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Preharvest management of *Botrytis cinerea* on *Leucospermum* 'Ayoba Sun' flowers using bacterial isolates

By

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PREFACE

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

DECLARATION

I, **NOKWANDA MBEDU (219044475)**, declare that,

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I would like to thank my sisters -Sinikiwe Khumalo, Andiswa Mbedu and my family for their support and encouragement from the beginning to the end of my project. I cannot thank you enough.

DEDICATION

I dedicate my work to God, my father A.V. Mbedu, my late mother B.B. Mbedu, my sister A. Mbedu and my brother Mzokhona Mbedu.

DISSERTATION SUMMARY

Ornamental plants and fresh-cut flowers play a crucial role in the South African economy, with over 80% of fynbos exported to the USA, Europe, England, Japan, China, and other countries. However, ornamental plants are prone to leaf spots, poor budding and small-sized flower development at preharvest. This is a result of *Botrytis* blight disease caused by *B. cinerea* which presents an enormous challenge for the fresh-cut flower industry. One of the ornamental plants, *Leucospermum* 'Ayoba Sun' flower, a new fynbos cultivar recently bred for disease resistance, growth vigor and its bright attractive color is prone to *Botrytis* blight disease at preharvest affecting the consumer acceptability and market value at postharvest. Preharvest treatments such as fungicides are used to manage *B. cinerea*. However, fungicides such as benzimidazole have been effective to manage *B. cinerea* with some limitations that include fungicides resistance, residues, environmental effects, and health concerns in humans. Furthermore, biological control agents such as *Bacillus brevis, Pseudomonas* sp. and *Bacillus subtilis* have been reported to be effective against *B. cinerea*. Hence, there is a need to evaluate other control strategies since few chemical and biological control methods have been effective against *B. cinerea* on ornamentals. Therefore, the aim of this research was to evaluate the effect of bacterial biological control agents to manage *B. cinerea* on *Leucospermum* 'Ayoba Sun' at preharvest. A total of 96 bacterial isolates were isolated from leaves of *Tetradenia riparia*, *Vernonia amygdalina*, *Plantago lanceolata* L., *Ipomoea batatas* (L.) Poir, *Cymbopogon marginatus,* anthers of maize (*Zea mays* L.), flowers of *Plantago lanceolata* L. and heads of wheat *Triticum aestivum* L., and soil samples collected at different locations in KwaZulu-Natal where macadamia nut trees (*Macadamia integrifolia*, *C. marginatus*) were planted. During *in vitro* screening, the six best isolates were selected *Triticum* as the most promising antagonists against *B. cinerea* based on their inhibition zones (65% to 82%) and used in the *in vitro* secondary screening. Furthermore, these bacterial isolates suppressed the mycelial growth *of B. cinerea* by shrinking, deformation, cutting, and bending of the pathogen hyphae when observed under the scanning electron microscope (SEM). Moreover, all the isolates were identified as *Bacillus amyloliquefaciens* strains when their sequences were subjected to Blast analyses.

The potential of the commercial product (CP) Double Nickel 55, a preventative bacterial inoculant with *B. amyloliquefaciens* (D747) and *B. amyloliquefaciens* strains A5 and SWL were evaluated on *Leucospermum* 'Ayoba Sun' leaves and buds under greenhouse conditions. The concentration of *B. amyloliquefaciens* SWL and *B. amyloliquefaciens* A5 suspension was adjusted to 1×10^8 cells/ml and the concentration of *B. cinerea* was adjusted to 1×10^5 conidia/ml using a hemocytometer. Double Nickel 55 was applied at 5×10^{10} CFU per gram. The leaves treated with *B. amyloliquefaciens* A5 had the lowest disease severity (13.2%), followed by *B. amyloliquefaciens* SWL (19.8%) and Double Nickel 55 CP (22.67%) compared to the control inoculated with the pathogen-only (76%). Double Nickel 55 (CP) treated leaves had a percentage disease incidence of 25.18%, *B. amyloliquefaciens* A5 (10.44%), *B. amyloliquefaciens* SWL (21.57%) and control (69.39%). The effect Double Nickel 55 (CP), *B. amyloliquefaciens* A5 and *B. amyloliquefaciens* SWL on *Leucospermum* 'Ayoba Sun' plant quality was scored 6, 3 and 5, respectively, compared to the control with a score of 0. Moreover, Double Nickel 55 (CP) treated buds had the lowest disease severity (3,3%) followed by *B. amyloliquefaciens* A5 (21,3%), *B. amyloliquefaciens* SWL (50%) and control (70%). Hence, *B. amyloliquefaciens* A5 treatment had the lowest disease severity and percentage disease incidence on the leaves and the least effect on plant phytotoxicity while Double Nickel 55 resulted in the lowest disease severity on the buds.

Leucospermum 'Ayoba Sun' leaves treated with Double Nickel 55, *B. amyloliquefaciens* A5, *B. amyloliquefaciens* SWL and water (control) were collected at 7, 14, and 35 days post-inoculation to determine the effect of *B. cinerea* on peroxidase activity (POD), total phenolic content (TPC) and total flavonoids (TFC). On day 35, the effect of Double Nickel 55 (CP) on TFC of *Leucospermum* 'Ayoba Sun' was 28.56 GAE/g dry matter, *B. amyloliquefaciens* A5 (13.56 GAE/g dry matter), *B. amyloliquefaciens* SWL (24.87 GAE/g dry matter) and control (26.63 GAE/g dry matter). The effect of Double Nickel 55 (CP) on TPC in *Leucospermum* 'Ayoba Sun' was 407 GAE/g dry matter, *B. amyloliquefaciens* A5 (410 GAE/g dry matter), *B. amyloliquefaciens* SWL (395.3 GAE/g dry matter) and control (441 GAE/g dry matter). The effect of Double Nickel 55 (CP) on POD in *Leucospermum* 'Ayoba Sun' was 0.2 U/min/g dry matter, *B. amyloliquefaciens* A5 (0.23 U/min/g dry matter), *B. amyloliquefaciens* SWL (0.26 U/min/g dry matter) and control (0.38 U/min/g dry

matter). Therefore, at 35 days post-inoculation the control and Double Nickel 55 treatments exhibited the highest TFC of 26,03 GAE/g dry matter and 28,56 GAE/g dry matter, respectively. The control (441 GAE/g dry matter) and *B. amyloliquefaciens* A5 (410 GAE/g dry matter) had the highest TPC. Moreover, the control (0,38 U/min/g dry matter) and *B. amyloliquefaciens* SWL (0,26 U/min/g dry matter) had the highest POD enzyme activity on day 35 compared to other treatments. Therefore, *B. amyloliquefaciens* A5 resulted in the lowest disease severity and disease incidence, high TPC, low TFC and POD at 35 days post-inoculation. While *B. amyloliquefaciens*, SWL resulted in lower disease severity and disease incidence but high POD, low TPC and TPC. Hence, the disease incidence and disease severity are indirectly proportional to all plant enzyme and antioxidant activity. *B. amyloliquefaciens* A5 and *B. amyloliquefaciens* SWL enhanced TPC and POD in *Leucospermum* 'Ayoba Sun' leaves, respectively. Moreover, an increase in TPC and POD content in *Leucospermum* 'Ayoba Sun' plants reduces the spread of *B. cinerea*. The findings suggest that *B. amyloliquefaciens* A5 has the potential to be used as a biological control agent against *B. cinerea* infecting the leaves and the buds of *Leucospermum* 'Ayoba Sun'.

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Chapter 1
Dissertation introduction

1.1 Background

Leucospermum 'Ayoba Sun' is an evergreen flower with broad leathery leaves and a bright orange-yellow pincushion flower head. It is a recently bred fynbos cultivar that was importantly bred for disease resistance, growth vigour and a bright attractive color. The *Leucospermum* species are cultivated in Australia, Hawaii, New Zealand, Portugal, and most species are native to South Africa, particularly KwaZulu-Natal and Western Cape (Leonhardt et al., 2000; Leonhardt et al., 2006). Fresh cut-flowers are of economic importance in South Africa and 80% of fynbos are exported to the USA, Europe, England, Japan, China, and other countries (Hughes et al., 2013). The cuttings of 'Ayoba Sun' are used for indoor adornment and the plant can be used to develop lovely gardens (Johnson et al., 2014).

In cut flower production, diseases have a significant impact on crop quality and cause blemishes, which affect the visual appeal of the product. According to Domingues and Ornelas (2022), diseases of the Proteaceae family are a result of *Colletotrichum sp*., *Mycosphaerella sp*., *Drechslera sp*., *Botryosphaeria sp*., *Alternaria sp*., *Elsinoe sp*., *Rosellinia necatrix* and *Armillaria mellea.* Other fungal diseases caused by *Phytophthora, Fusarium, Alternaria, Botrytis*, and *Cladosporium* pathogens have been reported and have been managed using biological control agents such *Trichoderma sp. (Trichoderma harzianum* and *Trichoderma virens*) and benomyl fungicides (Schiappacasse and Sandoval, 2008).

Botrytis cinerea, the causal agent of grey mould is one of the most important pathogens infecting fresh-cut flowers, reducing production and postharvest quality (McPartland et al., 2000; Williamson et al., 2007; Bika et al., 2021). The pathogen causes *Botrytis* blight on the leaves, branches, stems and flower heads in most ornamental plants (Bika et al., 2021; Ha et al., 2021). It is a threat to the floriculture industry causing huge losses and fewer exports (Chen et al., 2019). Severe grey mould infection is prevalent in greenhouses, field, storage, transportation, and handling (Bika et al., 2021). *Bacillus brevis* has been reported to be effective in managing *B. cinerea* in Chinese cabbage by antibiosis as it produces gramicidin S

antibiotic which is fungicidal (Edwards and Seddon, 2001). *Pseudomonas* sp. and *B. subtilis* have also been reported to be effective against *B. cinerea* in ornamental crops (Nakkeeran et al., 2020). Fungicides such as benzimidazole have been effective to manage *B. cinerea* with some limitations. *Botrytis cinerea* develops resistant strains and can be harmful to the environment and humans (Fillinger and Walker, 2016). Therefore, more research should be done to manage the disease since few chemical and biological control methods have been effective.

1.2 Problem statement

The cut flowers and potted flowers are a huge part of the floriculture industry that is dependent on the longevity, shape, form, size and colour of the flowers for marketing (Bika et al., 2021). However, *Botrytis* blight disease results in poor budding and small-sized flowers. *Leucospermum* 'Ayoba Sun' plants are prone to leaf spots and poor flower head development caused by *B. cinerea*. The pathogen *B. cinerea* causes blight of young growing shoots of Protea spp. in South Africa, resulting in tremendous losses (Serfontein and Knox-Davies, 1990). The local brown lesion infection becomes necrotic and spreads systemically in the plant (Bi et al., 2022). Furthermore, there is a failure of current control strategies to completely manage the disease in the field resulting in negative economic effects on the fresh-cut flower industry.

In commercial production, the disease is controlled by the application of synthetic fungicides such as benzimidazoles (e.g., benomyl), dicarboximides and phenylpyrroles (Hanh, 2014). However, continuous use of fungicides results in the development of resistant pathogen strains, phytotoxicity, residues, environmental effects, and health concerns (Baibakova et al., 2019). Approximately 8% of global fungicides are used to manage *B. cinerea* (Hua et al., 2018). Sanitation methods are ineffective as the pathogen can survive in the plant debris and spread in the next growing season. Therefore, other control strategies such as the use of biological control agents are alternatives to manage *B. cinerea* affecting the commercial production of *Leucospermum* 'Ayoba Sun' must be explored.

1.3 Research aim and objectives

The aim of this study was to evaluate the effect of bacterial isolates against *B. cinerea* of *Leucospermum* 'Ayoba Sun' flowers. The research objectives were to:

- Isolate and screen the bacterial isolates against *B*. *cinerea in vitro.*
- Evaluate the potential of the best bacterial isolates against *B. cinerea* on *Leucospermum* 'Ayoba Sun' and the effect on flower quality in a greenhouse trial.
- Evaluate the effect of *Bacillus amyloliquefaciens* strains on plant defense-related enzymes and antioxidants of *Leucospermum* 'Ayoba Sun' flowers.

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Chapter 2

Preharvest management of *Botrytis cinerea* on ornamental plants: A review

Abstract

Ornamentals are rich in secondary metabolites called anthocyanins that contribute to the diverse beautiful flower and leaf colours. The flower market flourish relies heavily on various factors such as pigmentation, size and form which are novelty markers in consumer choices. However, there is a significant threat to ornamental production in the field and greenhouses from phytopathogenic fungi, including *B. cinerea*. This pathogen affects up to 200 plant species globally. Fungicides such as cyprodinil and fludioxonil have not been highly effective in managing *B. cinerea*, as the pathogen develops resistance and has high adaptability. Additionally, other techniques applied to manage *B. cinerea* at preharvest include the application of cultural practices, relative humidity and temperature management, breeding methods for resistance, biological control, chemical control, and plant nutrition to reduce the occurrence of grey mould. However, some of these methods have been criticized due to their change in effectiveness and negative effects on the environment. Therefore, it is important to explore novel methods and consider natural strategies such as the use of biologicals to manage *B. cinerea*. Biological controls have been considered a promising strategy for sustainable agriculture as they are effective, environmentally friendly, and can improve disease control at a lower cost. Therefore, this review assesses the preharvest management strategies to manage *B. cinerea* on ornamentals at preharvest. Moreover, this review gives insight into the current control strategies to manage *B. cinerea* and makes future suggestions contributing to future research to manage *B. cinerea* on ornaments.

Keywords: Biological control, cultural practices, *B. cinerea*, breeding, chemical control

2.1 Introduction

Floriculture is a branch of horticulture that specializes in growing ornamental and flowering plants for use in gardens and floristry. This sector is driven by innovation and encompasses the marketing and production of various types of flowers and related plants, including cut flowers, foliage, potted plants, garden plants, nursery stock

(trees), flowering leafy plants, flower bulbs, and tubers (Proietti et al., 2022). These plants are commonly used for home decorations, as gifts, and to express condolences at funerals. Ornamentals are rich in secondary metabolites called anthocyanins that contribute to beautiful flower and leaf colors (Swami et al., 2020).

The fresh-cut flower market relies heavily on various factors such as pigmentation, scent, nectar, texture, size, longevity, and form which are novelty markers in consumer choices (Maleka et al., 2013; Datta, 2018; Bika et al., 2021). In Sub-Saharan Africa, floriculture is a significant source of income, through exporting fresh-cut flowers. However, there is a significant threat to ornamental production in the field and greenhouses from phytopathogenic fungi, including *B. cinerea*. This pathogen affects up to 200 plant species globally and causes losses of up to 100 million US dollars (Mbengue et al., 2016; Bennet et al., 2020). A recent study by Kim et al. (2020) found that the pathogen caused a disease incidence of 50-60% in peonies (*Paeonia lactiflora* Pall. 'Sarah Bernhardt') under greenhouse conditions. In La Pama, *B. cinerea* is the second most important pathogen, with a 1.94% disease incidence in proteas (Huertas et al., 2022). There were almost 26 million rand in revenue losses in 2002 by *Botrytis* affecting 15 to 20% of roses and gerbera bunches (Abbey et al., 2019).

Various methods have been attempted to control the spread of *B. cinerea* before harvest, but they have not been highly effective. This is a result of the pathogen adaptation to different environments, a wide range of hosts, different modes of infection and survival in both favorable and unfavorable conditions through sexual and asexual stages (Hua et al., 2018). Moreover, fungicides have not been able to effectively manage *B. cinerea*, as the pathogen develops resistance and has high adaptability (Hapon et al., 2018). Therefore, it is important to explore novel methods and consider natural strategies such as the use of biologicals. Hence, this review aims to assess the preharvest management strategies for *Botrytis cinerea* on ornamental plants.

2.2. Grey mould

2.2.1 Taxonomy

The term "botryose" refers to the clusters of macroconidia on the conidiophores that resemble grapes (Figure 2.1; Day, 2020). *B. cinerea* is a type of ascomycete fungus

that has two mating-type genes, MATI-1 or MATI-2, and multinucleate macroconidia, as well as uninucleate microconidia (sperm cells) (Budhika, 2017). The pathogen is known as *Botryotinia fuckeliana* which is its teleomorph name.

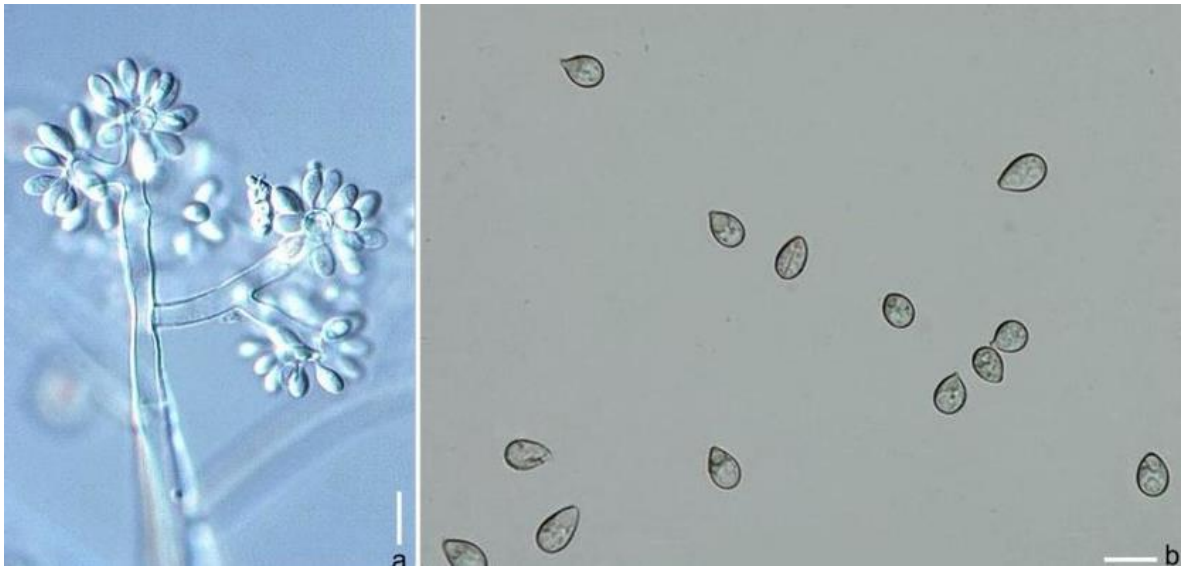

Figure 2.1: *Botrytis cinerea* (a) conidiophores and (b) conidia under the light microscope (Jayawardena et al., 2018).

Botrytis cinerea (anamorph) is classified under the Kingdom Fungi, phylum Ascomycota, Subphylum Pezizomycotina, Class Leotiomycetes, order Helotiales, family Sclerotiniaceae, genus *Botrytis* and species *cinerea*. The binomial name is *Botryotinia cinerea* (De Bay) (Williamson et al., 2007).

2.2.2 Disease cycle

Sclerotia, colored black or brown due to melanin pigments, are globular structures that form from mycelial branches (Romanazzi and Feiziani, 2014). They are overwintering structures in plant debris and soil, the melanin pigments provide a protective layer against UV radiation, pest attack, and desiccation (Carisse, 2016; Manasra and Jerusalem, 2020). Under favorable conditions, sclerotia germinates and release conidiophores, which are crucial in the *Botrytis* germination process (Figure 2.2). Asexually, these conidiophores release conidia, which can spread by wind and water. At a temperature ranging between 15-25°C and relative humidity of 93%, these spores germinate, leading to the production of an appressorium that penetrates the host by

secreting hydrogen peroxide and enzymes such as cutinases and lipase to damage the cuticle of a susceptible host plant (De Miccolis Angelini et al., 2015; Auda, 2021).

Sexually, through carpogenic germination, the microconidia that develop from the macroconidia germ tubes, appressoria and sclerotium are capable of functioning as spermatia and mate with the sclerotium to form a tiny mushroom-like structure called apothecium on the sclerotium (Romanazzi and Feliziani, 2014). The apothecium with asci releases ascospores that germinate and form hyphae (Schumacher and Tudzynski, 2012). However, the pathogen also produces thick-walled overwintering spores called chlamydospores by transforming vegetative mycelium parts to survive short-term under unfavorable conditions on the plant's surface and germinate under moist conditions to form macroconidia (Emmanuel, 2016). *Botrytis* microspores can form aggregates and survive up to six months due to their protective layer (Hulle) (Boine, 2012). Overall*, Botrytis* is a polycyclic disease that causes necrosis and apoptosis for successful infection (Veloso and van Kan, 2018).

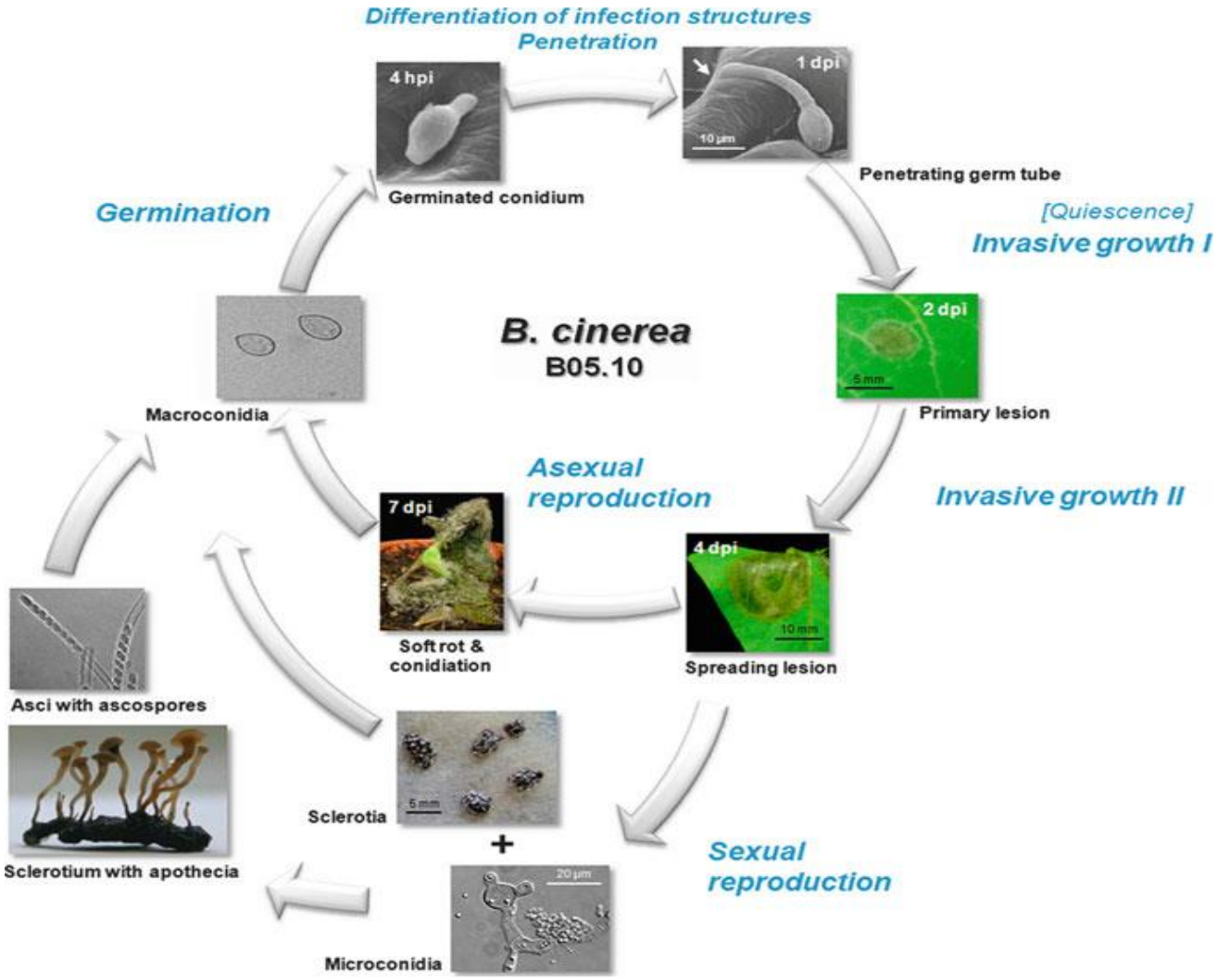

Figure 2.2: Sexual and asexual stages of *Botrytis cinerea* life cycle (Schumacher and Tudzynski, 2012).

2.2.3 Epidemiology

The pathogen remains quiescent in the soil as sclerotia, a saprophyte, or in an alternative host or infected debris and spreads in the upcoming growing season (Jaspers et al., 2016). *Botrytis* spores are very hydrophobic and are dispersed by water (rain), wind, physical or mechanical activity and flying insects such as thrips to other plants (Hawar, 2021). Upon contact with the leaf surface/ foliage, the spores enter the plant through wounds or natural openings, grow in the sub-tissue, and colonize the whole plant, causing necrotic lesions (Figure 2.2). At the beginning of spring under humid and wet conditions, the pathogen can grow and become necrotrophic or spread by planting infected cuttings (Schumacher, 2022). The

pathogen also spreads during the transportation, storage, and handling of materials (Bu et al., 2021).

2.2.4 Symptoms

B. cinerea has been reported to cause damping off and flower blight of *Protea* and *Leucadendron* in South Africa (Knoxx-Davis et al., 1987). The soft decay of tissues and fuzzy grey-brown mould, as well as velvety mats of sporulating tissues (Figure 2.3B), are all caused by the pathogen. It leads to pockmarks on flower petals (Figure 2.3A), blight on infected blossoms (Figure 2.3D), stem canker, and root rotting (Carisse, 2016). Moreover, it prevents flowers from opening and results in necrotic areas on the leaves (Figure 2.3C).

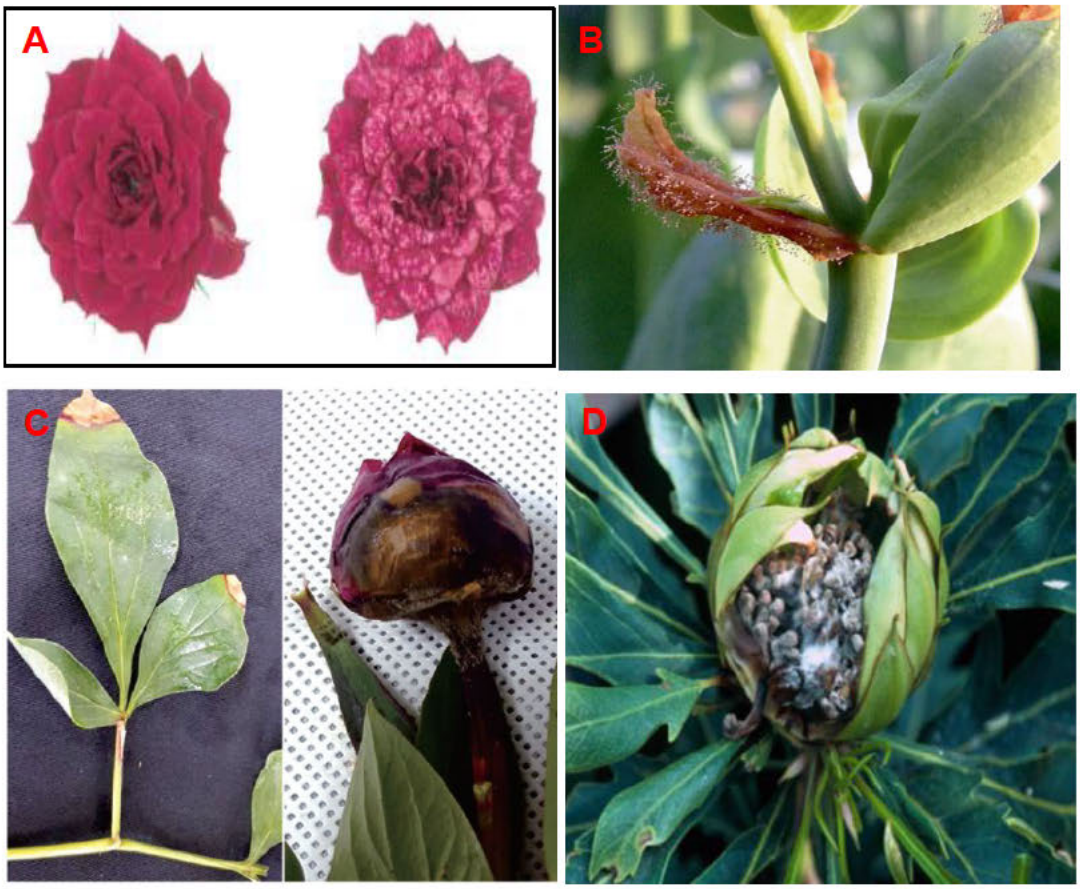

Figure 2.3: A) Pock symptoms on the flower petals (Williamson et al., 2007). B) Symptoms of Fuzzy grey-brown mould of *Botrytis cinerea* on Lisianthus cotyledon (Elad et al., 2016). C) Leaf necrosis and bud rot symptoms on peonies (Kim et al., 2020) and D) *Botrytis* blight symptoms on *Telopea sp*. (Proteaceae) flower head (Sumerell, 2017).

2.2.5 Distribution and host range

Botryotiana originated in the Northern hemisphere and was disseminated by the transportation of plant material, mammals or insects to the Southern regions of the world and further worldwide (Boine, 2012). Geographically, the species are found wherever there are host plants including the tropical and subtropical regions, and even in deserts where agriculture is practiced (Sharma and Kapoor, 2017). According to (Elad et al., 2016), *Botrytis sp.* has been reported to infect 596 genera with more than 1400 species. The pathogen infects various hosts such as cucumbers, strawberries, French beans, apples, tomatoes, pear, kiwi, red raspberries and citrus (Auda, 2021; Felizziani and Romanazzi, 2013). It infects seedlings and flowers of ornamental plants such as roses and gerbera flowers (Dik and Wubben, 2007; Williamson et al., 2007), freesia flowers (*Freesia hybridia* L.), petunia (Oliva et al., 2020), orchids, lisianthus, African violet, cyclamen (Bika et al., 2021). Other flowers including begonia (Piermann et al., 2022), poinsettia (*Euphorbia pulcherrima*) (Mahmoud et al., 2018), marigold flowers, peony (Tian et al., 2019), Lillium (Núñez de Cáceres González et al., 2015), geranium (Elmhirst et al., 2011), chrysanthemum (Kumar et al., 2020), zinnia (Szopińska, 2013) and Proteaceae (Summerell, 2017, Figure 2.4). Other ornamental plants that are susceptible to *B. cinerea* include anemone, calendula, hawthorn, heather, hydrangea, snapdragon, sunflower, dogwood, fuchsia, pansy, periwinkle, sweet pea and violet (Auda, 2021).

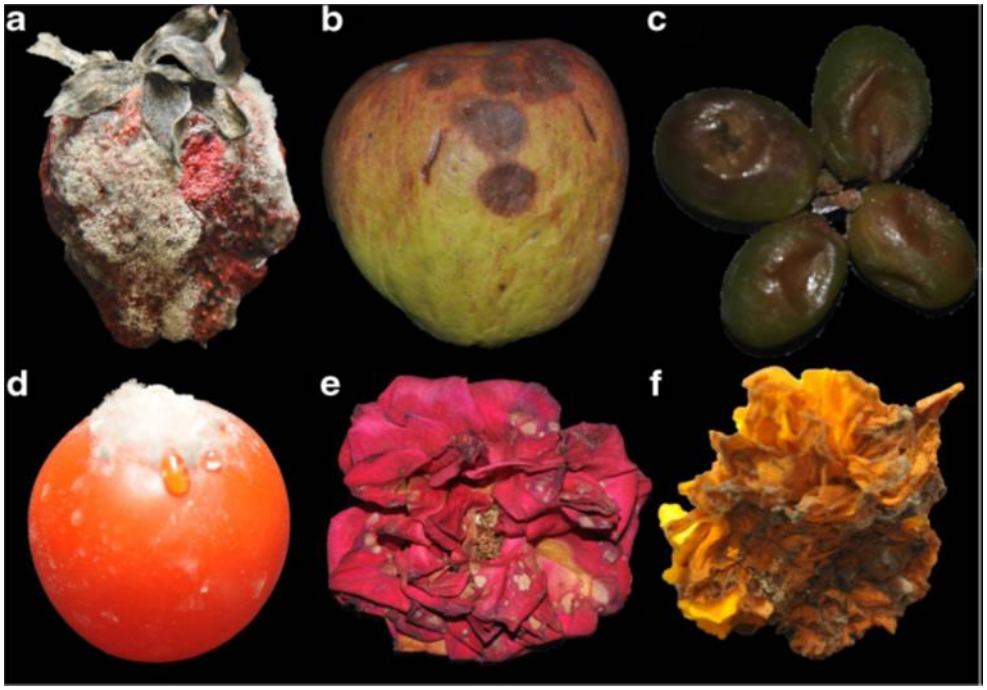

Figure 2.4: *Botrytis cinerea* infection on (a) strawberry, (b) apple, (c) grape berries,(d) tomato, (e) roses and (f) marigold flowers (Sharma and Kapoor, 2017).

2.3 Preharvest management strategies for *B. cinerea*

There are high economic losses on ornamental plants as a result of *B. cinerea* infection at preharvest. Various strategies have been investigated and implemented to reduce the occurrence of grey mould. These strategies include the use of cultural practices, relative humidity and temperature management, breeding for resistance, biological control, chemical control and plant nutrition.

2.3.1 Cultural practices

Cultural practices are traditional methods that enhance plant production by improving soil and crop health and reducing water waste (Fernández et al., 2020). These methods comprise of crop rotation, cover cropping, intercropping, sanitation, flooding, soil solarization, organic composting, irrigation to reduce weeds, pests and diseases (Katan, 2004; Hamadani et al., 2021). Certain cultural practices are essential in ornamental production to prevent the spread of *Botrytis* infection before harvest. These practices include removing dead and infected tissue, controlled irrigation, spacing plants appropriately, and maintaining cleanliness (Elad, 2016). *Botrytis cinerea* overwinters as sclerotia and dormant mycelia on plant debris and weeds germinates under higher humidity, wet weather and cool temperatures. According to

Bika et al. (2021), the plant debris from summer was the source of *Botrytis* blight infection in the greenhouse the following season. In New Zealand grape production, the debris served as the source of spores leading to the development of latent infections on berries during conditions favourable for *Botrytis* infection (Mundy et al., 2022).

Removing plant debris can prevent the pathogen from overwintering in the field and reduce the likelihood of disease occurrence. According to Putsa et al. (2024), in a field experiment in India, pruning reduced dieback severity in nuts orchards and increased yield. However, with such precautions, pathogen infection may still occur until harvest. Moreover, adequate plant spacing is crucial, as an infected plant can spread the pathogen throughout the field. The effect of plant spacing on the severity and incidence of stem rot (*Sclerotium rolfsii*) on peanuts (*Arachis hypogaea* L.) from 2000-2002 was evaluated and it was shown that severity and incidence of stem rot increased with an increase in plant spacing (Sconyers et al., 2005).

These cultural practices can reduce the need for costly and potentially harmful fungicides (Beckerman et al., 2015). However, they may not eliminate the pathogen, and additional control strategies such as fungicides may be necessary. Therefore, integrated management is key to effectively manage *B. cinerea*. Future research should focus on implementing more than one cultural practice integrated with fungicides, biological control agents or physical practices.

2.3.2 Relative humidity and temperature management

Weather conditions such as relative humidity and temperature favour pathogen spores to germinate resulting in epidemics of plant diseases (Zhao et al., 2022). Relative humidity of approximately ≤84% had a huge role in increasing conidia germination, disease incidence and severity of powdery mildew in grape and dropped sharply at (RH) above 87% (Carroll and Wilcox, 2003). However, plants have natural barriers such as a thick cuticle made of cutin polyester and waxes which limit the water and nutrient availability on the plant surface and prevents conidial germination (Arya et al., 2021). Cell walls are barriers that prevent further penetration of pathogen appressoria due to the formation callose, lignin and tannins at the site of penetration for cell wall reinforcement (Qin, 2020). Moreover, Ahmed et al. (2020) reported that the relative

humidity between 70-80% promoted vegetative growth of lettuce plants and reduced water stress when plants were exposed to artificial lighting. However, the yields of maize and soy were reduced in hot seasons where there was low precipitation and evapotranspiration and plants responded by closing the stomata (Lesk et al., 2021).

Relative humidity of more than 93% and temperatures between 10-20°C are optimum for *B. cinerea* infection (Ullah et al., 2024). This is usually common in greenhouses with susceptible hosts if such conditions are present. Growing plants very close to each other increases the relative humidity in the greenhouse and leads to disease incidence because it's directly related to transpiration (Shamshiri et al., 2018). Host foliage wetness results in an infection; hence, the duration of water resting on a host should be minimized (Cantoral et al., 2011). Organic mulch reduces evaporation, conserves soil moisture and enhances organic matter which minimizes disease infection (Demo and Asefa, 2024). The use of polyethylene mulch reduces the relative humidity and dew duration thus reducing infection (Shtienberg et al., 2010).

To prevent the spread of *Botrytis* infection, it is important to have an open canopy and ensure proper ventilation, as high humidity can increase the possibility of conidial germination and penetration (Baptista et al., 2012). However, managing heating, aeration, ventilation and avoiding overhead irrigation consumes high energy. If plants are wounded, it is also a disadvantage as wounds provide sufficient moisture and lead to symptom development. To prevent such infections fungicides are usually applied in an injured area (Jackson et al., 2012). Furthermore, maintaining relative humidity and temperature reduces the severity but does not eliminate the infection. Even using fan temperature regulator may transfer the spores of the pathogen to other plants due to the airflow. *Trichoderma* spp., *Aspergillus* spp., *Penicillium* spp., *Cladosporum* spp. and *Botrytis spp.* are the top five airborne fungi in the greenhouse and were identified to have between (36 426 to 49 729 spores/m^3) present where begonia, easter lily, geranium and other ornamental plants were propagated (Li et al., 2010).

2.3.3 Breeding

Breeding is the science of changing the genetic traits of plants in order to create plants that are resistant to various environmental stresses (Brummer et al., 2011). Disease resistant cultivars are selected based on several techniques. Field, greenhouse,

controlled climatic conditions, molecular techniques including real-time polymerase chain reaction (RT-PCR) and marker-assisted selection (MAS) should be utilised to screen genetic material to obtain germplasm for pathogen resistance breeding projects (Ismail et al., 2020; Aydogan, 2024). They reduce the use of fungicides and bactericides without causing any phytotoxicity to the plant. This improves ornamental crop resistance, quality and tolerance to environmental stresses and increases resistance to pests and pathogens. However, methods such as conventional breeding are effective but are resource consuming and time consuming from making crosses, screening large germplasms to selecting desired characteristics (Gaikwad et al., 2020). The availability of diverse gene pool to intergrate novel characteristics is reduced since this type of breeding is continuous. This leads to a need to obtain resistance genes from other plants and it is difficult to accomplish through conventional breeding as it can take up to 10 years and identifying disease resistant clones is a challenging exercise (Wanga et al., 2021). Thus, there is a need to explore other methods in order to develop resistant cultivars. A recent study by Xiang et al. (2022) reported that Tobacco rattle virus based virus-induced gene silencing (TRV-VIGS) method was a suitable and efficient method for screening *B. cinerea* resistant genes, with petal discs of lilies.

Other breeding methods such as genetic engineering have been implemented to produce transgenic plants and solve many problems associated with conventional breeding by introducing disease resistant genes into the plant without affecting the existing genotypes (Parmar et al., 2017). Lillium transgenic plants were developed using *Agrobacterium*-mediated transformation which had an over expression of Rice chitinase 10 gene (RCH10) which conferred resistance against *B. cinerea* and led to reduction in sporulation *in vitro* (Nunez et al., 2015). A study conducted by Liu et al. (2018) confirmed the role of brassinosteroids in rose (*Rosa hybrida*) petals defence against *B. cinerea* using RNA-seq analysis.

Even though the development of transgenic plants is a costly method, it is a promising strategy to manage *Botrytis* infection. More research should be done under field conditions since these results are usually based on greenhouse trials. Breeding for resistance is insufficient to manage *B. cinerea* since the pathogen has high genetic

plasticity and adaptability (Bika et al., 2021). Therefore, intergration of control measures can be highly effective to manage *B. cinerea*.

2.3.4 Chemical control

The use of pesticides is very common in modern agricultural production. In recent years, various fungicides with different modes of action have been introduced, including site-specific (systemic) and multisite inhibitors. For many years the two main groups of synthetic fungicides namely, benzimidazoles and dicarboximides have been applied to manage gray mould disease (Sofianos, 2020). The synthetic fungicides affect the osmoregulation, respiration, sterol biosynthesis and microtubule assembly of *B. cinerea* to manage gray mould infection (Dwivedi et al., 2020). Despite the benefits of fungicides, the development of resistance has become a concern with *B. cinerea* due to its polycyclic nature and high genetic variability (Cai et al., 2015). However, it is important to note that while fungicides are useful in disease management, their negative impact on the environment and their tendency to develop resistance in plants should not be overlooked.

The resistance of fungi to fungicides depends on various factors such as the chemistry and mode of action of the fungicidal compound, mutations of the pathogen, the biology and the frequency of fungicide use. Various fungicides have been reported to be effective in managing *B. cinerea* in different plants hosts but some plants develop resistance to these fungicides (Table 2.1). A study by Rupp et al. (2017) demonstrated that ornamental flowers exhibited an average of 20% resistance to fludioxonil and 75% resistance to trifloxystrobin. Moreover, a study by Dowling et al. (2021) discovered that the high resistance of *B. cinerea* to fludioxonil on calibrachoa flowers in China was due to the pathogen's high osmolarity glycerol mitogen-activated protein kinase pathway. Hence, it is imperative to avoid repeating use of chemicals. However, the combination of biological control and chemicals has been found to be effective to manage diseases (Ons et al., 2020). A study conducted by Hatem et al. (2023) investigated the management of *B. cinerea* by integrating *P. chrysogenum* VKM F-4876D (DMP) with tebuconazole, fludioxonil or difenoconazole *in vitro* for 14 days. The DPM (2.5 g/L) + difenoconazole (1.0 mg/L) combination was highly effective in reducing *B. cinerea* growth by 85.6% and after 14 days the inhibition level was 51.3%.

Table 2.1 Fungicides to manage *Botrytis cinerea* diseases in different plant hosts.

Fungicide/ fungicide class	**Effect**	**Active ingredient**	**References**
Cabrio top	At a concentration of 300ppm, the mycelial growth of *Botrytis cinerea* was reduced to 31mm *in vitro* compared to neem extract which reduced it to 23.33mm.	Pyraclostrobin	Gavilan et al., 2022
Tebuconazole 250 EW	A concentration of 5ppm was effective in reducing the pathogen's infection *in vitro* and *in vivo* under controlled conditions. It was able to reduce blossom blight incidence.	Tebuconazole	Vinodkumar and Nakkeeran, 2017
Phenylpyrrole	The mycelial growth of *B. cinerea* was reduced by 93.7% *in vitro* at a concentration of 0.1µg/ml.	Fludioxonil	Kim et al., 2016
Difen super, 55% wp and Skor 250 g/l EC	The mycelial growth of *B. cinerea* was reduced and no fungicide resistance occurred.	Difenoconazole	Zuparov et al., 2020

2.3.5 Plant nutrition

Proper nutrient management is also critical in plant health and overall disease management. High calcium and low nitrogen levels are effective in managing *Botrytis* in ornamental plants (Munoz, 2018). The use of a calcium spray is a commonly used chemical control strategy for managing grey mould in petunia (Bennett, 2019). Calcium ions regulate metabolic processes in the cytosol or organelles, strengthen the cell walls of hosts, reduce the ability of *B. cinerea* to digest the pectate, and play a role in normal cellular functions (Pirayesh et al., 2021). Calcium compound including calcium sulphate ($CaSO_4$), calcium chloride ($CaCl_2$) and calcium nitrate ($CaNO_3$) have been found promising to control *B. cinerea*. Reduced transpiration due to high humidity may cause less calcium ions transportation, which may result in less calcium in young and developing tissues (Gonzalez-Fontes et al., 2017). A research study by Bennett et al. (2020) reported that spraying $CaCl_2$ on petunia 'Pretty Grand Red' for 2 weeks reduced *Botrytis* blight by 96%. In ornamental production under greenhouse environments, $Ca(NO_3)^2$ and $CaSO_4.2H_2O$ are useful and inexpensive to supply calcium and reduce *Botrytis* blight incidence (Bika et al., 2021).

2.3.6 Biological control

The use of natural enemies for eradicating pathogens is an effective control method. Biological control agents (BCAs) function in a variety of ways by limiting space, hyperparasitism, antibiosis, cross-protection, competing for nutrients, or inducing host resistance (Heydari and Pessarakli, 2010). Biological control agents (BCAs) can release lipopeptides, bacteriocins, antibiotics, biosurfactants and cell-wall-degrading enzymes or microbial volatile compounds that will affect the phytopathogen metabolism and reduce pathogen infection (Raymaekers et al., 2020). The fungus *Gliocladium catenulatum* was one of the most successful biological control agents in managing *Botrytis* blight of geranium flowers (Elhimist et al., 2011). According to Ha et al. (2021), yeast and *Bacillus subtilis* secondary metabolites are highly recommended antagonists that are non-toxic and safe for use. Moreover, other BCAs have been reported to be effective against *B. cinerea* infection in various hosts (Table 2.2).

Understanding the mode of action of BCAs is crucial as some may pose risks to human and environmental health, or lead to the development of pathogen resistance. While the efficacy of BCAs may vary between laboratory and field conditions, certain

microorganisms may produce essential compounds or enzymes only at low concentrations *in situ* (Kohl et al., 2019). Although BCAs are cost-effective, reduce plant disease resistance, and play a significant role in integrated pest management, their performance cannot be controlled in ambient conditions, making them less reliable and efficient than chemical methods (Collinge et al., 2022). However, farmers can use BCA's with other control methods as an Intergrated pest management (IPM) system for *Botrytis* to achieve the desired results. Despite this, biologicals are still a promising strategy for sustainable agriculture as they are effective, environmentally friendly, and can improve disease control at a lower cost.

Table 2.2: Microbial biological control agensts used to manage *Botrytis cinerea* diseases in different plant hosts.

Microorganism	Host	Effect	References
Gliocladium roseum FR136 *Trichoderma inhamatum*	Rose (*Rosa hybrida*)	Reduced sporulation of B. cinerea by >90% on leaf residues	Tatagiba et al., 1998
Fluopimomide (50 g ha^{-1}) + *B. methylotrophicus* TA-1 at 10^8 cfu ml^{-1} (Treatment 1) Fluopimomide (100 g ha^{-1}) + *B. methylotrophicus* TA-10^8 cfu ml^{-1} (Treatment 2)	Tomato	Treatment 1 and treatment 2 reduced tomato gray mould caused by *B. cinerea* by 70.16 and 69.32%, respectively.	Ji et al., 2019
Bacillus amyloliquefaciens (VB7)	*Lillium* leaves	*B. amyloliquefaciens* reduced *B. cinerea* by 46% *in vitro*. Foliar application prevented conidial germination of *B. cinerea* and resulted in hyphae shrinkage.	Nakkeeran et al., 2020
Pseudomonas protegens AP54, *Pseudomonas chlororaphis* 14B11 *Pseudomonas fluorescens* 89F1	*Petunia* × *hybrida* 'Carpet Red Bright petunia	The three *Pseudomonas* strains were sprayed and drenched and reduced the *B. cinerea* disease severity by 26-39% indicating less disease occurrence.	South et al., 2020
B. velezensis strains (LBUM279, FZB42, LBUM1082) *B. subtilis* LBUM979	*Cannabis* leaves	Foliar application resulted to disease severity index (DSI) of 42%,35% and 58%, respectively. Gray mould severity was significantly reduced and DSI (52%).	Balthazar et al., 2022

2.3.7 Conclusion and future prospects

The production of ornamentals is highly affected by *B. cinerea*, which may result in revenue losses. The use of chemicals in managing diseases, including *B. cinerea*, is well-researched and widely documented. However, the potential of biological control methods has also received interest from researchers. There is still a need to evaluate more biological control agents and understand their mode of action. Currently, there are very few commercialised strains that are used as biological control agents against *B. cinerea*. Therefore, there is an urgent need to develop new preharvest control strategies that are eco-friendly, inexpensive, and extremely effective.

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Chapter 3

Isolation and *in vitro* screening of bacterial isolates against *Botrytis cinerea* of *Leucospermum* 'Ayoba Sun'

Abstract

Botrytis cinerea is a necrotrophic opportunist pathogen that infects over 200 plant species and causes more than 30% loss of plants produced globally. The pathogen releases cell wall degrading enzymes and toxins to colonize the host. It reduces the market competitiveness of plants as it affects the leaves and the blossom of ornamentals resulting in low quality and yield. A total of 96 bacterial isolates were isolated from leaves of *Tetradenia riparia*, *Vernonia amygdalina*, *Plantago lanceolata* L., *Ipomoea batatas* (L.) Poir, *Cymbopogon marginatus,* anthers of maize (*Zea mayz* L.), flowers of *Plantago lanceolata* L. and heads of *Triticum aestivum* L. The soil samples were collected where *C. marginatus* and from different locations in KwaZulu-Natal where macadamia nut trees (*Macadamia integrifolia*) were planted. During the *in vitro* primary screening trial, only 1.04% of the isolates resulted in high mycelial growth inhibition of *B. cinerea* with zones of inhibition ranging from 76-100% and were categorized as group 4. Notably, 84% of isolates had 51-75% inhibition. Six best isolates were selected as the most promising antagonists against *B. cinerea* based on their zones of inhibition and used in the secondary screening. The selected bacterial isolates effectiveness decreased and the inhibition levels ranged from 26-50% belonging to group 2 (P= 0.001). The A5, A3, PL3, A2, SM3 and SWL bacterial biological control agents suppressed *B. cinerea* growth by ripping, deforming, cutting and bending pathogen hyphae when observed under the scanning electron microscope (SEM). Moreover, the rod-shaped bacterial cells were observed on the hyphae where it was broken or bent. The pathogenicity of *B. cinerea* was evaluated on the leaves and buds of *Leucospermum* 'Ayoba Sun' at 7- and 14-days post-inoculation, respectively. The buds were highly susceptible to grey mould and were fully colonized by the pathogen within 7 days. The phylogenetic tree revealed that the bacterial isolates A2, A3, A5, SWL, PL3 and SM3 are closely related to each other and to *B. amyloliquefaciens* strain BCRC 11601 and *B. amyloliquefaciens* strain NBRC 1553. Moreover, the six bacterial isolates were further amplified using internal transcriber sequencing ITS1 and ITS4 primers and the polymerase chain reaction

(PCR) products were sequenced. BLAST prediction identified the A5, A3, PL3, A2, SM3 and SWL isolates as *Bacillus amyloliquefaciens* (NR117946.1). The results suggest that the isolated *Bacillus* isolates have the potential to be used as a biological control agent against *B. cinerea*.

Keywords: *Bacillus amyloliquefaciens*, primers, PCR, *Leucospermum* 'Ayoba Sun', *Botrytis cinerea*, buds and leaves.

3.1 Introduction

South African plant species such as *Disa*, *Leucospermum*, *Leucandron*, *Pelargonium*, *Strelitzia Eucomics* are of great international interest to breeders to gain commercial success (Reinten et al., 2011; Darras, 2021). These species are popular in Kirstenbosch Botanical Gardens which is one of the main tourist attractions with diverse and unique flora (Reinten et al., 2011). These species are prominent among breeders worldwide as the source of genetic material for cut flowers that have been hybridized and registered (Reinten et al., 2011). 'Ayoba Sun' is a newly bred future fynbos cultivar that is mainly commercialized as cut flower bouquets to be used for decorations and exports. It is beneficial to the environment as it feeds honeybees and attracts birds. *Freesias, Gerbera, Gladioli, Nerine, Protea, Leucospermum, Leucadendron, Zantedeschia, Agapanthus, Lachenalia, Ericas, Strelitzias* and *Ornithogalum* contribute to the South African indigenous flora and account 10% of the world's plants (Reinten and Van, 2017). However, diseases pose a huge threat to the production of these ornamental species.

These ornamental plants are susceptible to diseases caused by fungi, bacteria, viruses, and phytoplasmas. This reduces the market competitiveness as it affects the leaves and the blossom resulting in low quality and yield (Singh et al., 2011). Fungal plant diseases are considered the most important in agriculture as they account for huge annual losses and export reductions (Heydari and Pessarakli, 2010). The genus *Botrytis* has more than 30 species and *Botrytis cinerea* is the second most destructive pathogen worldwide causing grey mould disease in ornamental plants, vegetables, and fruits both at pre- and postharvest (Aktaruzzaman et al., 2017; Yuan et al., 2024). *Botrytis cinerea* is a necrotrophic opportunist pathogen that infects over 200 plant species and releases cell wall degrading enzymes and toxins to colonize the host

(Amselem et al., 2011). It is highly virulent fungus as it has long terminal repeat retrotransposon called Boty and a DNA transportation called Flipper that aids in successful infection (Yuan et al., 2024). The disease is more severe in greenhouses, nurseries, and fields and during transportation under humid conditions (RH> 95%) and temperatures between 20- 25°C causing more than 30% plant losses and deterioration (Dik and Wubben, 2007; Bika et al., 2021).

Several control strategies have been implemented to manage *Botrytis* including chemical and cultural methods. Cultural practices are not efficient in disease control and chemicals release toxic residues leading to soil quality degradation (Wang et al., 2018). Moreover, pathogen resistance development reduces the effectiveness of fungicides since *B. cinerea* has genetic variability and is resistant to most fungicides (Haider et al., 2017). *Botrytis cinerea* perecentage resistance was reported for fungicides thiophanate-methyl (94%), pyraclostrobin (80%), boscalid (67%), iprodione (65%), fenhexamid (38%), cyprodinil (38%), fludioxonil (21%), and fluopyram (13%) applied on infected petunia, geranium, and poinsettia in greenhouse (Lusasko and Hausbek, 2024).

Biological control agents (BCA) are a promising and environmental-friendly alternative to fungicides for controlling plant diseases (Tariq et al., 2020). Biological control agents (BCAs) use various modes of action such as hyperparasitism, predation, antibiosis, cross-protection, competition for nutrients, and induced plant resistance (Kohl et al., 2019). Various species of bacteria, fungi, and yeasts have been studied and *Bacillus subtilis*, *Trichoderma harzianum* and the strains under the genus *Pseudomonas* and *Paenibacillus* are proven to have the highest antifungal activity (Roca-Couso et al., 2021). The genus *Bacillus* is the most effective genera against pathogens as it produces antibiotic substances such as peptides and bacteriocins and can adapt to high heat and drought conditions (Raaijmakers et al., 2011). Thus, the objective of this study was to isolate and screen the bacterial isolates against *B. cinerea in vitro*.

3.2 Materials and methods

3.2.1 Pathogen isolation

Diseased grapes showing symptoms of grey mould were purchased from a local supermarket (Pietermaritzburg, South Africa) and used to isolate *B. cinerea.* A sterile

needle was used to harvest the mycelia from the infected grapes and transferred into Petri dishes with potato dextrose agar (PDA) media. The Petri dishes were incubated at 22°C for 3 days, thereafter a pure culture of the pathogen with grey mycelia was cut using a cork borer and inoculated at the centre of fresh PDA plates and incubated at 22°C.

3.2.2 Molecular identification of the pathogen

The pathogen was identified using method according to White et al. (1990). Genomic DNA was extracted from the pure cultures using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The ITS target region of DNA was amplified using NEB OneTaq 2X MasterMix with Standard Buffer (Catalogue No. M0482S, Thermo Fisher Scientific) and the ITS specific primers (Table 3.1) in a PCR (Weisburg et al., 1991; Turner et al., 1999). The PCR parameters used were NEB OneTaq 2X, MasterMix with Standard Buffer (Catalogue No. M0482S, Thermo Fisher Scientific), Genomic DNA (10-30ng/µl), Forward primer (10µM), Reverse primer (10µM) and Nuclease free water (Catalogue No. E476). The PCR protocol was denaturation at 94°C for 5 min, annealing at 94°C for 30 sec, elongation at 50°C for 30 sec, 68°C for 1 min and 68°C for 10 min. There were 35 cycles performed and a hold at 4°C.

For agarose gel analysis, the integrity of the PCR amplicons was visualized on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye. The NEB Fast Ladder was used on all gels (N3238) as a size standard. The PCR Amplicon fragments were enzymically purified using the ExoSAP procedure (NEB M0293L; NEB M0371). The amplicons were then purified for sequencing using DNA kit (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050), and sequenced in the forward and reverse direction (Nimagen, BrilliantDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) using the ABI 3730xl Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific). FinchTV (https://finchtv.software.informer.com/1.4/) was used to view the raw chromatogram files (.abi). The CLC Bio Main Workbench was used to assemble the forward and reverse sequencing reads to form a consensus sequence for each sample. BLASTn analysis with default parameters (Altschul et al., 1997) was performed on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine if a sequence in the

database matches the query sequence above a certain threshold (99% query coverage; 99% identity).

Table 3.1: ITS primer sequences used in the molecular identification of *Botrytis cinerea*.

Name of Primer	Target	Sequence (5' to 3')
ITS1	Internal Transcribed Spacer Region	TCCGTAGGTGAACCTGCGG
ITS4	Internal Transcribed Spacer Region	TCCTCCGCTTATTGATATGC

3.2.3 Isolation of bacterial isolates

The bacteria were isolated from the leaves of *Tetradenia riparia*, *Vernonia amygdalina*, *Plantago lanceolata* L., *Ipomoea batatas* (L.) Poir and *Cymbopogon marginatus*. They were also isolated from the anthers of maize (*Zea mayz* L.), flowers of *Plantago lanceolata* L. and heads of *Triticum aestivum* L. and also from soils collected at Ukulinga Research Farm (Pietermaritzburg) where macadamia nut trees *M. integrifolia* were planted.

Approximately 10g of leaves were weighed on the Sartorius AY212 M-Prove weighing scale (H&C weighing systems, USA). The leaves were rinsed with distilled water to remove soil particles. They were then crushed and ground in the mortar using pestle with the gradual addition of 20ml distilled water to facilitate the grinding. The solution was transferred into a 500ml beaker, sealed with foil and heated in the water bath at 80°C for 1hr for isolation of bacteria. The 20g of soil was also weighed in a weighing boat on a Sartorius M-prove weighing scale (H & C weighing systems, USA) and 20ml of distilled water was added. The 10g of anthers and 20g of flowers were also weighed, ground, transferred into beakers and placed in a water bath (Labotech water bath, Thermo Fisher Scientific, United states) at 80°C for 1hr. They were then transferred in a 500ml beaker, sealed with foil and transferred in water bath for 1 hour to ensure only bacteria survives. The stock solutions were left for 1 hour at room temperature to cool. Serial dilutions from 1×10^0 to 1×10^5 were prepared by transferring 1ml of the stock solution into sterilized McCartney bottles containing 9ml of sterilized distilled water.

Potato dextrose agar (PDA) (NutriSelect® Plus, Germany) media was prepared to grow the bacterial isolates and poured into 90mm Petri dishes. Once the PDA was cooled down and the media solidified, 20 µL of the solution of each McCartney bottle with a different concentration was plated in PDA plates and stored at 28°C for 3 days. For each concentration, there were 3 replicates of 1 petri dish per replicate. After 3 days post-inoculation, the plates with morphologically different colonies were subcultured onto fresh PDA to obtain pure cultures. The pure cultures were stored in 70% glycerol (v/v) at -80°C for further use.

3.2.4 *In vitro* screening of bacterial isolates

In vitro primary screening of 96 bacterial isolates against *B. cinerea* was conducted in 3 replicates of 1 Petri dish per replicate in a completely randomized design (CRD). Potato dextrose agar (PDA) media was prepared and poured into 90mm Petri dishes and left to solidify. Two perpendicular lines of the bacterial isolates were streaked using a sterile inoculating loop at the bottom of each Petri dish. A cork borer was used to cut the mycelial plugs of *B. cinerea* and this was placed upside down at the center of the Petri dish. Plates were incubated at 25℃ for 7 days. After 7 days of incubation, the diameter pathogen mycelial growth (mm) was measured using vernier caliper (Merck, South Africa). The % inhibition was calculated for all the isolates using the formula:

$$\%\ Inhibition = \frac{C - T}{C} x\ 100$$

Where:
C = Diameter of *B. cinerea* of the control plate
T = Diameter of *B. cinerea* of the dual plate

Six of the best isolates from primary screening were selected for secondary screening using a similar method described above. There were 3 replicates per treatment, 1 Petri dish per replicate and the Petri dishes were stored at 22℃ for 7 days. Mycelial growth of *B. cinerea* was measured on day 3,5 and 7 post-inoculation using a vernier caliper.

3.2.5 Molecular identification of bacterial isolates

Pure cultures of biological control agents were prepared by picking single colonies, transferring to fresh PDA and incubated at 22°C for 3-7 days. Genomic DNA was extracted from the pure cultures using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The ITS target region was amplified using the primers presented in Table 3.1. The bacterial isolates sequences obtained were aligned from the database of National Center of Biotechnology Information (NCBI) using Basic Local Alignment Search Tools (BLAST). The phylogenetic tree was established using MEGA 12 program Tamura-Nei model, Maximum-likelihood method and the bootstrap was generated using 1000 replications.

3.2.6 Scanning electron microscopy (SEM)

Petri dishes from the *in vitro* secondary screening experiment which demonstrated the inhibition of the mycelial growth of *B. cinerea* by the best 6 isolates were viewed under SEM microscope. Mycelial plugs (5mm × 5mm) of were cut where the bacteria and *B. cinerea* meet from 10-day old cultures that were incubated under ultraviolet light (UV light) to sporulate. The mycelial plugs were fixed for 1-2 hours in 3% glutaraldehyde buffered with sodium cacodylate. They were then washed with sodium cacodylate buffer twice for 5 minutes each. After this, they were dehydrated with a series of ethanol starting from 10%, 30%, 50%, 70%, 90% and 100% each for 10 min. At 100% the samples were dehydrated three times. After dehydration, they were transferred into critical point baskets and dried with a Quorum K850 critical point dryer machine. They were then mounted onto stubs using carbon double-sided tape and coated with gold to make them conductive using Quorum 150R ES machine. The sample morphologies were then viewed under the ZEISS EVO LS 15 SEM.

3.2.7 Pathogenicity of *B. cinerea* on *Leucospermum* 'Ayoba Sun'

Leucospermum 'Ayoba Sun' plants were purchased from Click-n-Plant nursery (Cape Town, South Africa). The leaves and buds were detached from *Leucospermum* 'Ayoba Sun' plants and stored in sterile plastic bags. They were then washed with tap water and sprayed with 70% ethanol (v/v) for sterilization. They were then left to air dry in a laminar flow for 1 hour. The leaves were wounded with a sterile needle at an equatorial site, and 13 wounds were made at each site. The buds were not wounded. Water agar (WA) was prepared and poured into 90mm Petri dishes and allowed to solidify. The

buds and the leaves were then placed in WA water agar plates to provide moisture. A volume of 20µL of *B. cinerea* suspension ($1x10^5$ conidia/ml) was inoculated on the leaves and sprayed on the buds until run-off. The Petri plates were then incubated at 22°C, and the disease incidence was recorded on days 7, 10, and 14 post-inoculation. There were 3 replicates per *B. cinerea* treatment and uninocontrol with 3 leaves and 3 buds per treatment in a completely randomized design (CRD). The experiment was repeated twice. Moreover, the number of leaves and buds infected were counted and recorded at day 7, 10 and 14 for the leaves and day 3, 5 and 7 for the buds over the total and multiplied by 100 to measure percentage disease incidence.

3.2.8 Statistical analysis

The data collected was subjected to analysis of variance (ANOVA) using Genstat® 23rd Edition. Duncan's multiple range test was performed to determine the significance of the mean differences obtained among the treatments at the 5% significance level.

3.3 Results

3.3.1 Pathogen identification

The fungal pathogen isolated was identified as *Botrytis cinerea* or *Botrytis fabae* with 100% identity confirmation (Table 3.3).

Table 3.3: Molecular identification of the pathogen using amplified ITS target region and percentage identity.

Isolate	Identified Pathogen	Sequence length (bp)	Accession number	% Identity
BC	*Botrytis cinerea or Botrytis fabae*	497	MN891765.1/ MN589852.1	100

3.3.2 *In vitro* primary screening

A total of 96 bacterial isolates were used during the primary screening test (Appendix 1). The groups were created according to the ranges of average percentage inhibition and the majority of the bacterial isolates fell under group 3 with percentage inhibition

between 51 and 75% (Table 3.4). Only 1.04% had the highest percentage inhibition between 76-100% under group 4. The control plates without bacterial isolates were completely colonised by the pathogen.

Table 3.4: Bacterial isolates grouped according to their average percentage inhibition of *B. cinerea* during primary *in vitro* screening after 7 days at 22℃.

Groups	**Ranges of average percentage Inhibition in each group**	**Percentage of bacteria isolates**
Group 1	0-25	6,3
Group 2	26-50	8,3
Group 3	51-75	84,0
Group 4	76-100	1,0

3.3.3 *In vitro* secondary screening

Six isolates were selected based on their ability to inhibit *B. cinerea* mycelial growth during the primary screening test (Appendix 1). All the bacterial isolates significantly ($P < 0.001$) reduced the pathogen mycelial growth compared to the control. The bacterial isolates had inhibition ranging from 40.6% to 51.3% (Table 3.5). The highest level of inhibition was exhibited by bacterial isolates SWL and A5 with 51.3% and 49.4%, respectively. Five of the bacterial isolates were categorised under group 2 and one under group 3. A change in appearance of the mycelia from white to grey with pin-like structures (conidiophores with conidia) was observed in some of the plates after day 7 (Figure 3.1).

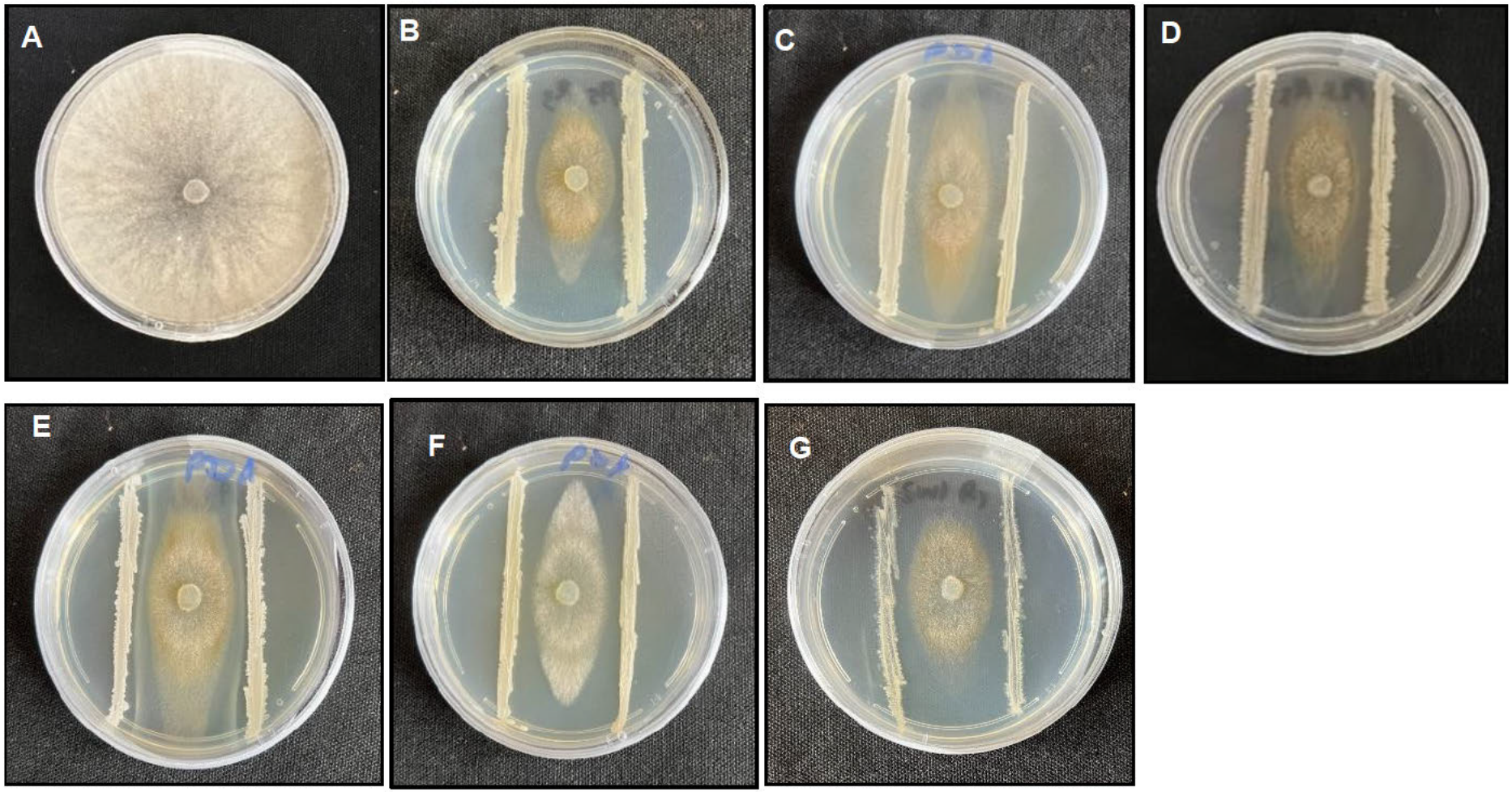

Figure 3.1 *In vitro* antagonistic effect of the isolated bacterial isolates on the mycelial growth of *Botrytis cinerea* after 7 days at 22°C. Untreated mycelia for *B. cinerea* control (A). Mycelial growth inhibition of *B. cinerea* treated with bacterial isolates A5

(B), A3 (C), PL3 (D), A2 (E), SM3 (F) and SWL (G).

Table 3.5: The mycelial growth and percentage inhibition of the 6 bacterial isolates against *Botrytis cinerea* after 7 days at 22°C during *in vitro* secondary screening.

Treatments	Mycelial growth (mm)	Inhibition (%)	Group
SWL	38.6 a	51.3	3
A3	47.8 b	39.8	2
A2	42.9 ab	46.0	2
A5	40.2 ab	49.4	2
SM3	46.1 ab	41.9	2
PL3	47.1 ab	40.6	2
Control	79.3 c		
P-value	<0.001		
LSD	5.2		
CV%	6.00		
SED	2.4		

Each value is an average of 3 replicates, mycelial growth (mm) followed by the same letters are not significantly different based on Duncan's Multiple Range Test at 5% significance level.

3.3.5 Molecular identification of bacterial isolates

The phylogenetic analysis (Figure 3.2) revealed that the bacterial isolates A2, A3, A5, SWL, PL3 and SM3 are closely genetically to each other, the *B. amyloliquefaciens* strain NBRC 15535 and the *B. amyloliquefaciens* strain BCRC 11601. The isolated bacteria are distantly similar to other *Bacillus* sp. including *Bacillus subtilis* and *Bacillus velezensis*. Furthermore, the isolates used for secondary screening were identified as different strains of the same pathogen, *Bacillus amyloliquefaciens*. The percentage similarity ranged between 99.16% to 99.86%. Three isolates A2, SWL and SM3 showed 99.86% similarity.

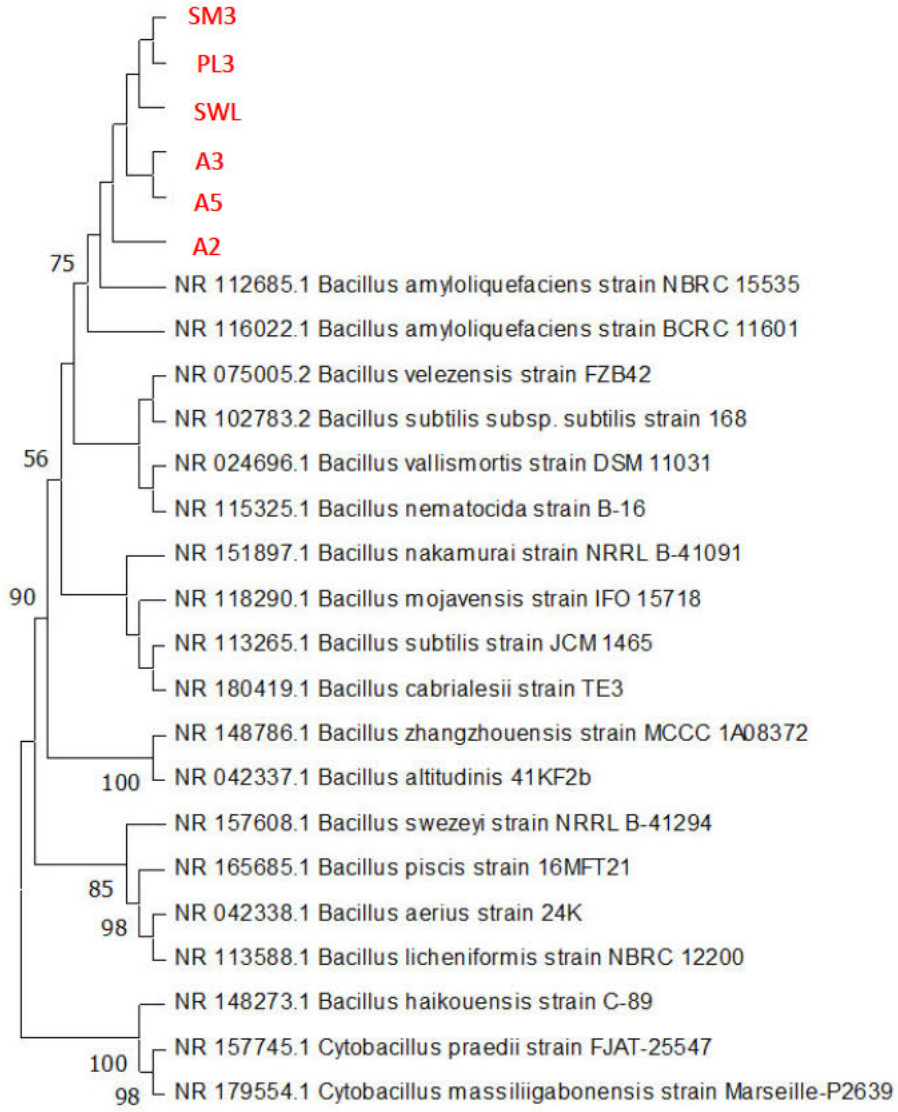

Figure 3.2: Likelihood phylogenetic tree using Kimura 2-parameter model depicting the phylogenetic relationship between bacterial isolates *(*A2, A3, A5, SWL, PL3 and SM3) to other pathogen species. The phylogenetic tree represents the identification of isolates A2, A3, A5, SWL, PL3 and SM3. Values for frequencies less than 50% are not given. The numbers above the nodes are percentages of the trees from bootstrap analyses.

3.3.6 SEM analysis

Smooth hyphae and oval-shaped spores were observed in *B. cinerea* control treatment (Figure 3.3A). The bacterial isolates suppressed *B. cinerea* growth by

shrinking, deforming, cutting and bending pathogen hyphae (Figure 3.3B-G). Smooth hyphae and oval-shaped spores were observed in *B. cinerea* control treatment (A). The hyphae of *B. cinerea* treated with bacterial isolates A3 and PL3 were deformed and bent respectively. Rod shaped bacterial cells were observed on the broken or bent hyphae treated with A2 and PL3, respectively. In addition, the bacterial isolates caused deformation and bending of *B. cinerea* hyphae.

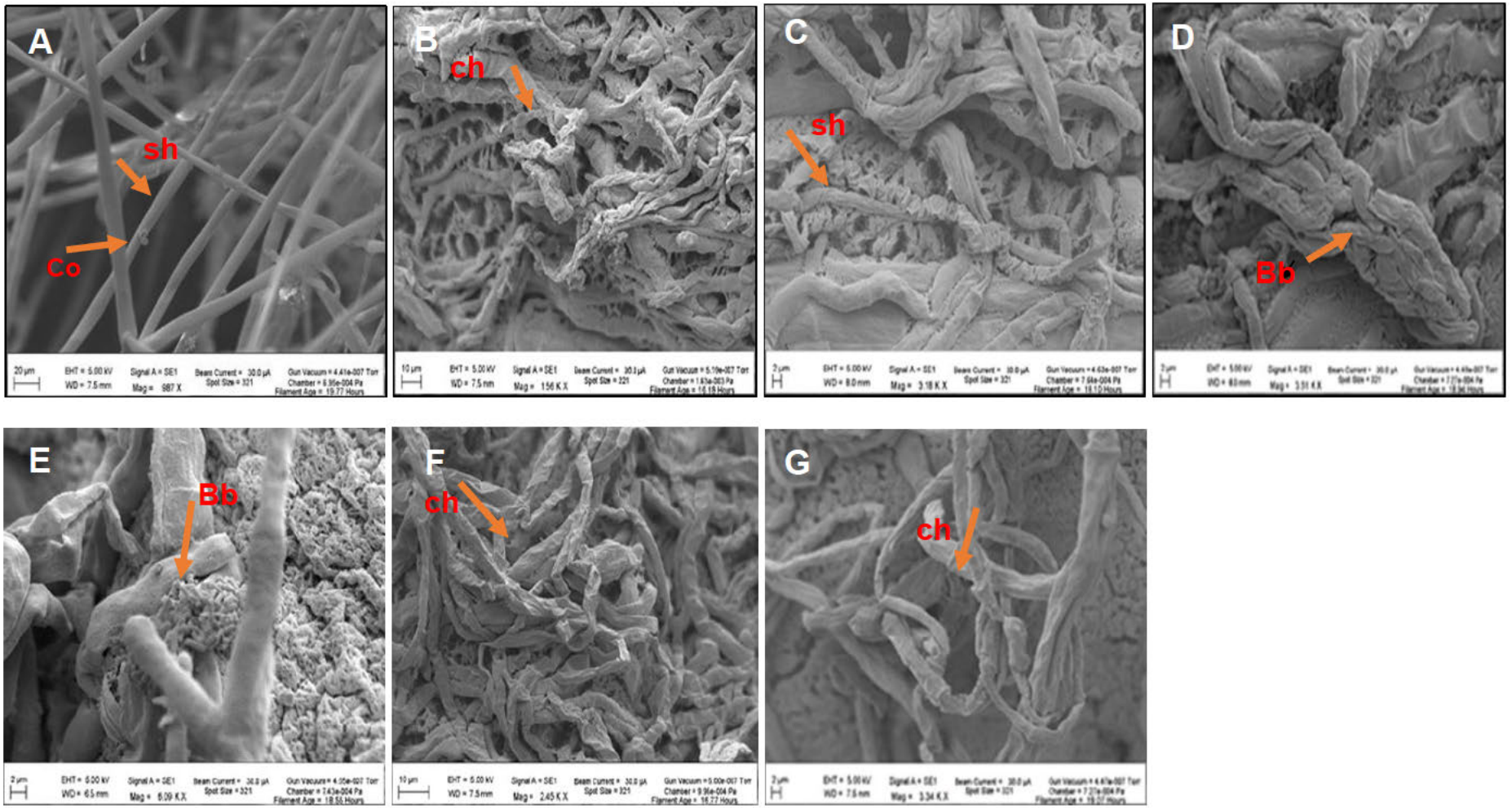

Figure 3.3: Scanning electron micrograph of *B. cinerea* hyphae after 7 days at 22°C. Untreated *B. cinerea* control (A) showing smooth hyphae (Sh) with no damage and oval shaped conidia (Co); Morphological changes on the hyphae treated with bacterial isolates A5 (B), A3 (C) PL3 (D), A2 (E), SM3 (F) and SWL (G), such as collapsed hyphae (Ch), bent hyphae colonized by bacterial cells (Bb); shrinked hyphae (Sh) and broken hyphae (Bh).

3.3.7 Pathogenicity test of *B. cinerea* on the leaves and buds of *Leucospermum* 'Ayoba Sun'

The leaf infection of *B. cinerea* increased with time. At day 7 there was slight visible infection of *B. cinerea* (Figure 3.4 A), at day 10 the pathogen sporulated demonstrating pin-like structures and conidiophores from the mycelia (Figure 3.4 B). At day 14 of incubation, there was high disease severity and the leaves changed colour to brown and black (Figure 3.4C) and there was 0% infection on the control (Figure 3.4 D). The percentage disease incidence of *B. cinerea* in the leaves increased from day 7 to day 14 from 9.7-53.7% (Figure 3.5).

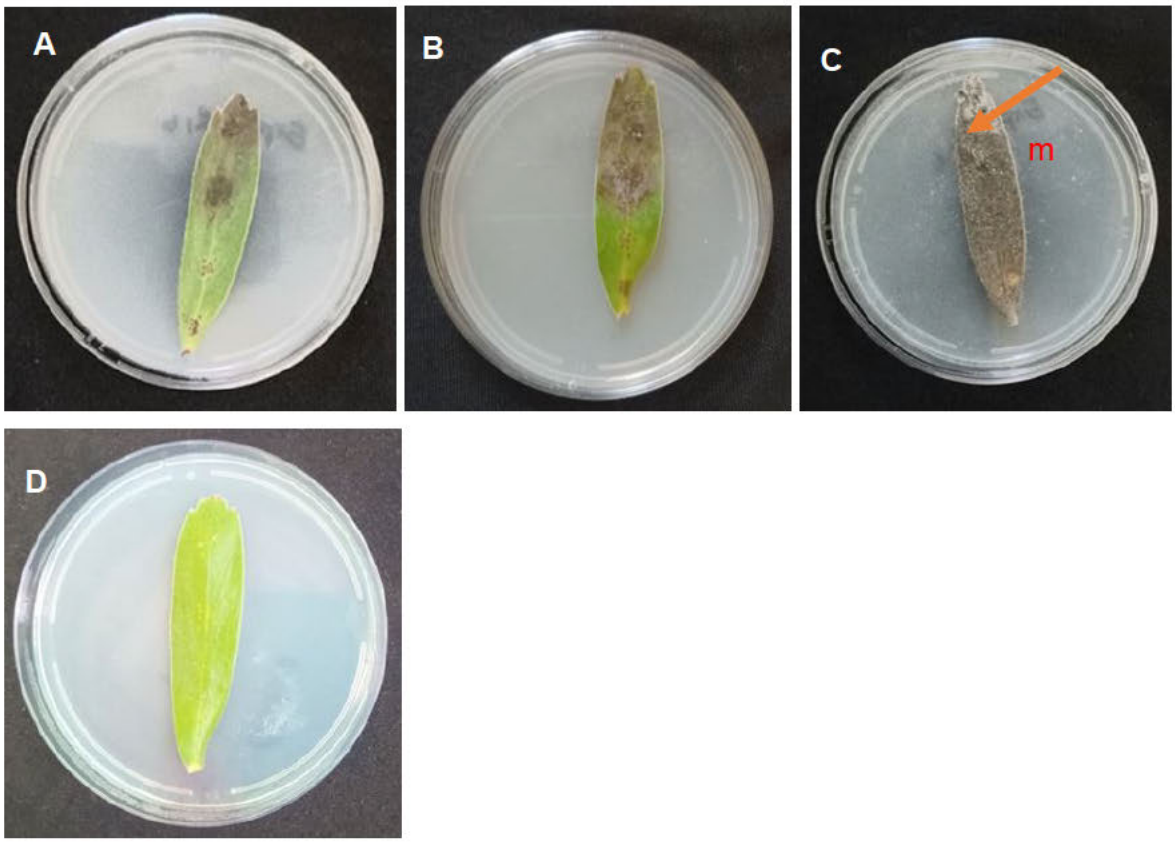

Figure 3.4: Disease incidence of *Botrytis cinerea* on *Leucospermum* 'Ayoba Sun' leaves after (A) 7, (B) 10 and (C) 14 days post-inoculation at 25°C. (A-C) The increase in the infection of *B. cinerea* colonised the leaves, changing their appearance from green to dark brown and forming grey mycelia (m) on top of the leaves. (D) The uninoculated control was symptomless and remained green.

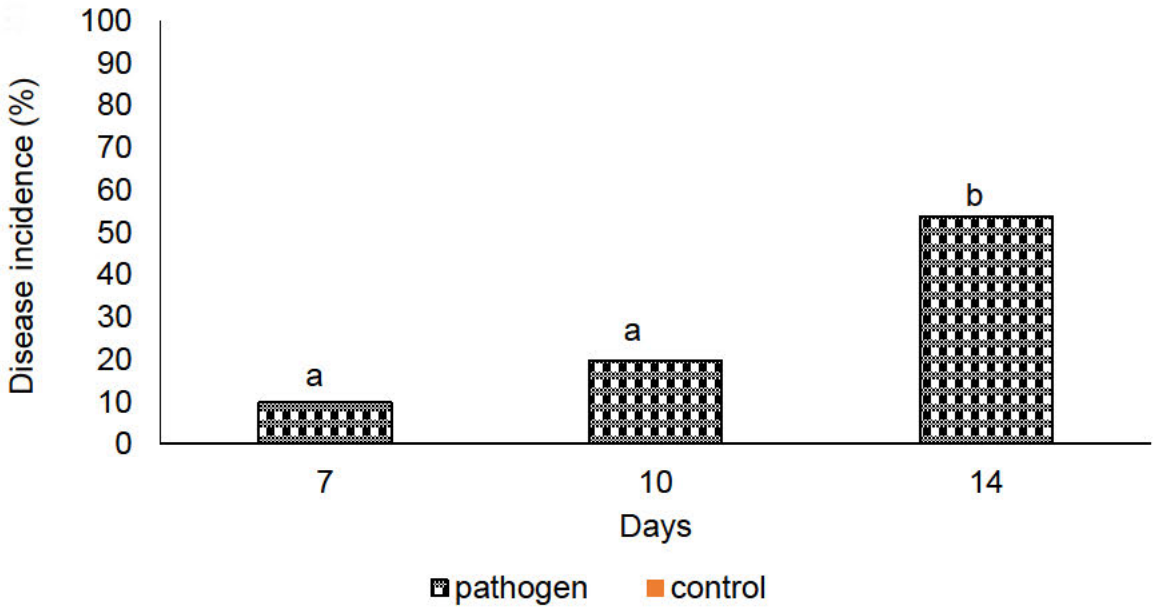

Figure 3.5: Gray mould incidence caused by B*otrytis cinerea* on *Leucospermum* 'Ayoba Sun' leaves after 14 days post-inoculation ($p<0.001$).

For buds, at 3 days post-inoculation there was a slight infection, which increased with time. The control (Figure 3.6A) was not infected showing no symptoms until day 7. Buds replicates were slightly infected showing signs of resistance to the pathogen infection at day 5 (Figure 3.6B). Most of buds replicates were fully colonised with white mycelia on the surface at day 7 (Figure 3.6C). Moreover, at day 7 there was high percentage disease incidence of 66,7% for all the replicates (Figure 3.7).

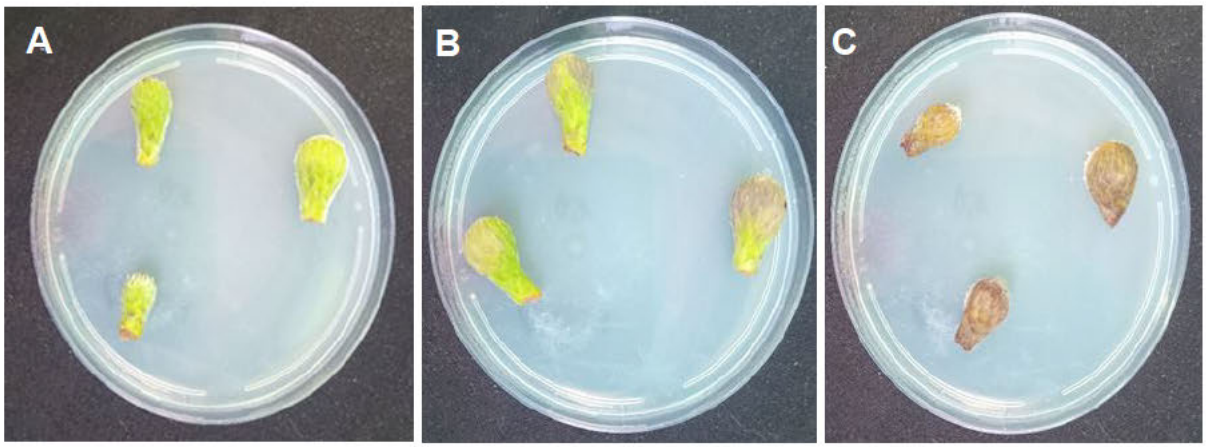

Figure 3.6: Pathogenicity of *Botrytis cinerea* infection on the *Leucospermum* 'Ayoba Sun' buds after 7 days post-inoculation at 25°C. The control showing no symptoms

(Figure 3.6A), buds replicates were slightly infected at day 5 (Figure 3.6B) and buds replicates were fully colonised by *B. cinerea* at day 7 (Figure 3.6C).

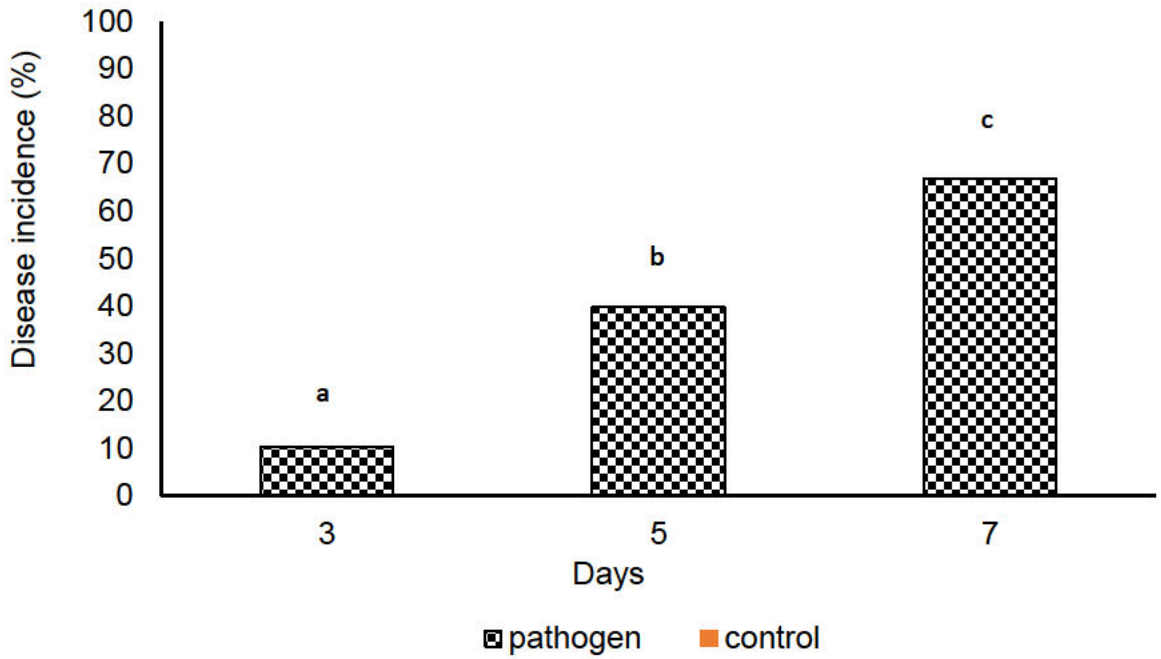

Figure 3.7: The percentage disease incidence of *Botrytis cinerea* on the *Leucospermum* 'Ayoba Sun' buds after 7 days post-inoculation ($p<0.001$) at 25°C.

3.4 Discussion

During the *in vitro* primary screening, 84% of bacterial isolates were categorized under group 3 with 51-75% inhibition of *Botrytis cinerea*. This illustrates that most biological controls are effective in reducing botrytis infection *in vitro* and can manage *Botrytis* blight disease. The selected bacterial isolates which had the highest percentage inhibition against *B. cinerea* during primary screening with 51-100% inhibition was less effective under secondary screening with inhibition between 26-50% when compared to the control. This can be a result of the change in environmental conditions mainly temperature which highly affects bacteria sporulation thus reducing the effectiveness of the bacterial isolates (Friere et al., 2024). The isolated pathogen was identified as *B. cinerea* or *B. fabae* with 100% identity under molecular studies using ITS 1 and ITS 4 primers. According to Bellemain et al. (2010), the use of ITS primers is the accurate method for the identification of fungi. However, identification methods like TUB (β-tubulin) and translation elongation factor (TEF1-α) genes are superlative to further distinguish between two closely related pathogens (Shu et al., 2020).

Pure culture of *B. cinerea* had smooth hyphae and oval-shaped spores under a scanning electron microscope. According to Ma et al. (2018), *Botrytis cinerea* has smooth hyphae and colourless oval-shaped conidia. A change in the appearance of the mycelia from white to grey with pin-like structures and conidiophores was observed. According to Zhou et al. (2018), *B. cinerea* has greyish mycelia and conidia. The bacterial isolates damaged the hyphae causing deformation, shrinking and bending to inhibit growth under SEM. The bacterial isolates effectiveness to inhibit *B. cinerea* mycelial growth under *in vitro* and *in situ* conditions is a result of the production of signalling compounds, enzymes such as chitinase and interfering metabolites (Kohl et al., 2019). *Botrytis cinerea* treated with bacterial isolate SM3 showed modifications such as rod-shaped bacterial cells on the hyphae causing bending and collapsing of the hyphae which prevented further infection. According to Wang et al. (2021), β-1,3-1,4-Glucanase released by *Bacillus amyloliquefaciens* resulted in bent, swollen and deformed hyphae as a form of protection against *B. cinerea* of ginseng (*Panax ginseng*) under *in vitro* and *in vivo* trials.

All six bacterial isolates used for secondary screening were identified as *Bacillus amyloliquefaciens* with percentage similarity ranging between 99,16% to 99,86% to the actual pathogen. *Bacillus amyloliquefaciens* is reported to be the most dominant bacteria producing secondary metabolites and antibiotics that suppress various diseases (Arguelles-Arias et al., 2009; Chen et al., 2009; Lagerlof et al., 2015; Dimopoulou et al., 2021). According to Samaras et al. (2021), *B. amyloliquefaciens* significantly reduced the disease severity and succinate dehydrogenase inhibitors (SDHI) resistance of *B. cinerea* on beans in the greenhouse. The six *B. amyloliquefaciens* are related to *B. amyloliquefaciens* strain NBRC 15535 and *B. amyloliquefaciens* strain BCRC 11601. The isolated bacteria are genetically similar to *Bacillus subtilis* and *Bacillus velezensis*. However, according to Chun et al. (2019), the *B*. *amyloliquefaciens*, *B*. *siamensis*, and *B*. *velezensis* genome sequences and metabolic features are similar. Moreover, *B*. *velezensis* has more genes in its core-genome which are involved in the biosynthesis of antimicrobial compounds and has a potential to be effective in plant disease management.

Infection by *B. cinerea* was faster and higher on the buds than on the leaves reaching 66% disease incidence. It took 7 days to fully colonize most of the buds and 14 days for most of the leaves. Hence the leaves are slightly resistant to infection than the buds. The pathogen *B. cinerea* is economically important and is reported to be the causal agent of bud rots worldwide (Jerushalmi et al., 2020; Garfinkel, 2021; Punja and Ni, 2021). This poses a threat to the floriculture industry, particularly affecting the production of *Leucospermum* 'Ayoba Sun' cut flowers. Therefore, this will reduce production and exports due to its significant impact on quality.

3.5 Conclusion

Buds were significantly more susceptible to *B. cinerea* than the leaves. Various strains of *B. amyloliquefaciens* have been identified for effective control *B. cinerea in vitro*. *B. amyloliquefaciens* mode of action against B. cinerea is deformation, shrinking and bending of hyphae. Therefore, they should be evaluated for their potential to manage the pathogen in *planta* under greenhouse environment. Other closely related *Bacillus sp*. such as *B. amyloliquefaciens* strain NBRC 15535, *Bacillus nematocida* and *B. subtilis* should be evaluated against *B. cinerea in vitro* as they related to the isolated *B. amyloliquefaciens* and have the potential to be highly effective. Moreover, the identification of volatile antifungal compounds from the *in vitro* screening in the bacterial secretome should be evaluated using a gas chromatography-mass spectrometry (Nakkeetan et al., 2020).

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Chapter 4

Evaluating the effect of *Bacillus amyloliquefaciens* strains against *B. cinerea* of *Leucospermum* 'Ayoba Sun' under greenhouse conditions

Abstract

Botrytis cinerea is a destructive necrotrophic pathogen known for causing devasting losses on ornamental plants affecting the leaves, stems and blossom. Cultural and chemical methods against *B. cinerea* have been less effective since the pathogen has high genetic plasticity and adaptability. Hence, the pathogen develops resistance to fungicides resulting in less pesticide use. In this study, the potential of the commercial product (CP) Double Nickel 55, a preventative bacterial inoculant with *Bacillus amyloliquefaciens* (D747) and two isolated *Bacillus amyloliquefaciens* strains (A5 and SWL) were evaluated on *Leucospermum* 'Ayoba Sun' leaves and buds under greenhouse conditions. *Leucospermum* 'Ayoba Sun' seedlings during their flowering stage with blossoms were sourced from the Western Cape KZN nursery. *Bacillus amyloliquefaciens* SWL and *B. amyloliquefaciens* A5 spore suspension concentrations were prepared at 1×10^8 cells/ml and *B. cinerea* was prepared at 1×10^5 spores/ml. The *B. amyloliquefaciens* commercial product (Double Nickel 55) had 5×10^{10} CFU per gram. The treatments significantly affected the disease severity and Area Under Disease Progression Curve (AUDPC), reducing *B. cinerea* on the leaves and buds ($P<0.001$). The plants treated with *B. amyloliquefaciens* A5 had the lowest disease severity (13.2%) on the leaves followed by *B. amyloliquefaciens* SWL (19,8%) and the *B. amyloliquefaciens* commercial product (22.7%), compared to the untreated control with 76% infection. Moreover, *B. amyloliquefaciens* A5 had the lowest disease incidence (10.44%) and the lowest effect on plant quality with few leaf lesions. The commercial product (CP) was highly effective in managing *B. cinerea* on the buds with 3.3% disease severity while *B. amyloliquefaciens* A5 also showed promising results. The findings suggest that *B. amyloliquefaciens* A5 can be used as a biological control agent against *B. cinerea* infecting the leaves and the buds of *Leucospermum* 'Ayoba Sun'. However, it must be sprayed on newly growing leaves since the pathogen moves systemically.

Keywords: *Botrytis cinerea, B. amyloliquefaciens* A5, *Leucospermum* 'Ayoba Sun', quality and disease severity

4.1 Introduction

Botrytis cinerea results in huge losses in many ornamental plants, affecting the leaves, stems and blossom which results in poor quality and rejection of cut flowers by consumers (Munoz et al., 2019). Currently, the ornamental horticulture industry is highly dependent on multi-site and site-specific fungicides for suppressing *B. cinerea*. Carbendazim, procymidone, and diethofencarb fungicides have been proven effective against *B. cinerea* by inhibiting mycelial growth and conidia production on peonies (Tian et al., 2019). However, *B. cinerea* develops resistance to fungicides, resulting in less pesticide use since the pathogen has high genetic plasticity and adaptability (Hahn et al., 2014). For instance, He et al. (2020) reported *B. cinerea* resistance against carbendazim (multi-site) application due to pathogen mutation and the fungicide has lost its effectiveness in managing gray mould. Moreover, the use of chemicals leads to pollution and more research has been done to develop biological control and biofungicides for disease management.

Biological control is an effective alternative to fungicides as it is safe for the environment, humans and relatively easy to apply (Lahlali et al., 2022). The use of chemicals such as fungicides, fertilizers and pesticides in crop protection tremendously affects the environment, causing soil and aquifer pollution, soil degradation and salinization, and loss of biodiversity (Valenzuela et al., 2020). Plant-growth promoting rhizobacteria (PGPR) including *Bacillus* sp. mostly inhabit the soil and secrete metabolites that are antibiotic and reduce the growth of other microorganisms (Khan et al., 2021). The genus *Bacillus* is the dominant source of biological control agents due to their high abundance, diversity in ecosystems, production of desiccation-resistant spores, production of antibiotics and antifungal compounds that allow them to survive adverse conditions making them versatile in biocontrol application (Dammie, 2017; Villarreal-Delgado et al., 2018). The genus can promote plant growth and naturally form endospores, enabling bacteria to survive higher temperatures and decrease plant disease incidence (Egamberdieva, 2016). According to Salvatierra-Martinez et al. (2018), *B. amyloliquefaciens* was effective against *B. cinerea* on tomatoes by secreting lipopeptide-like compounds to

inhibit mycelial growth *in vitro*. Blossom blight on roses caused by *B. cinerea* was reduced by 64% compared to the control when *B. amyloliquefaciens* was sprayed four times weekly (Nakkeeran et al., 2020). However, no published reports have investigated the effects of *B. amyloliquefaciens* on inhibiting the growth of gray mould in *Leucospermum* 'Ayoba Sun' leaves and buds. Therefore, this study evaluated the potential of the *B. amyloliquefaciens* antagonists for controlling *B. cinerea* under greenhouse conditions on *Leucospermum* 'Ayoba Sun'.

4.2 Methods and materials

4.2.1 *In vivo* screening

Leucospermum 'Ayoba Sun' was grown in 15cm diameter plastic pots in a rich and well-drained growing medium with coir and slow-release fertilizer applied on top. Drip irrigation was installed and applied 3 times a day for 5 min each cycle. The plants were kept in a glasshouse under uncontrolled ambient conditions. A preventative spraying regime was conducted to evaluate the efficacy of *B. amyloliquefaciens* A5, *B. amyloliquefaciens* SWL and Double Nickel 55 (active ingredient *B. amyloliquefaciens*) in managing *B. cinerea* on the leaves and buds. Cultures of *B. cinerea* was grown at 22℃ and placed under ultraviolet light for at least 10 days to allow sporulation. The spores were harvested by washing the pathogen culture with sterilised distilled water and using a glass rod to dislodge them. The spores were counted using a hemocytometer, and the concentration was adjusted to 1×10^5 cells/ml. The suspensions of *B. amyloliquefaciens* SWL and *B. amyloliquefaciens* A5 were prepared by washing the bacterial cultures incubated at 28℃ for 2 days with sterile distilled water. The spores were counted using a hemocytometer and the concentration was adjusted to 1×10^8 cells/ml. The concentration of Double Nickel 55 was prepared according to the manufacturer's instructions and adjusted to 5×10^{10} CFU per gram. The leaves were sprayed until run-off with *B. amyloliquefaciens* SWL and *B. amyloliquefaciens* A5 strains. The negative and positive controls were sprayed with sterilized distilled water and *B. cinerea* spore suspension (1×10^5 cells/ml) until run-off, respectively. Black polyethylene bags were placed over each plant after treatment, and after 24 hours the bags were removed and sprayed with the pathogen suspension until run-off. Clear polyethylene bags were placed back over each plant inoculated

with the pathogen to create humidity and prevent contamination and were removed after 24 hours. The experiment was laid out in a completely randomized design (CRD) and repeated twice with three replicates per treatment. The disease severity and quality ratings were recorded weekly; 8 times post inoculation. Moreover, the same experimental procedure was done during bud development from July-August and the disease severity ratings were evaluated weekly 6 times post inoculation before full blossom development.

4.2.2 Disease assessment

The disease severity was visually scored on a scale of 0 to 10 where 0 = 100% of the leaves and buds were covered by brown *B. cinerea* lesions: 1 = 1–10%; 2 = 11–20%; 3 = 21–30%; 4 = 31–40%; 5 = 41–50%; 6 = 51–60%; 7 = 61–70%; 8 = 71–80%; 9 = 81–90%; and 10 = 91–100% of total leaf area (Shrestha and Hausbeck, 2023).The midpoint values were used to calculate the Area Under Disease Progression Curve AUDPC for all the treatments before statistical analysis. As previously described by Ge et al. (2020), the disease incidence was calculated using the following equation with amendments:

$$\%\ Disease\ incidence = \frac{number\ of\ symptomatic\ leaves}{total\ number\ of\ leaves} x\ 100$$

4.2.3 Phytotoxicity assessment

The quality of the plants was rated on a visual scale according to Elmhirst et al. (2011) using a phytotoxicity scale of 1-10 with amendments where 10= (91–100%) is severe necrosis, chlorosis, stunting, blossom spotting or leaf distortion, 9 = 81–90%, 8 = 71–80%; 7 = 61–70%; 6 = 51–60%; 5 = 41–50%; 4 = 31–40%; 3 = 21–30%; 2 = 11–20%;1 = 1–10%.

4.2.4 Data analysis

The AUDPC was calculated, and the final disease severity values were subjected to analyzed for variance using GenStat 23rd edition for analyses of variance. Treatment means were separated using Duncan multiple range test at the 5% probability level (p=0,05).

4.3 Results

4.3.1 Effect of selected bacterial isolates against *B. cinerea* on *Leucospermum* 'Ayoba Sun' leaves

The treatments had a significant effect on reducing final disease severity of *B. cinerea* on the leaves ($P<0.001$, Figure 4.1) and the AUDPC ($P<0.001$, Figure 4.2). Treatment with *B. amyloliquefaciens* A5 had the lowest disease severity of 13.2%, *B. amyloliquefaciens* SWL (19.8%) and Double Nickel 55 (22.67%) compared to the control inoculated with the pathogen. The control inoculated with sterilized distilled water had no infection while 76% of infection occurred on the pathogen inoculated control. There was no significant difference on the AUDPC among the *B. amyloliquefaciens* treatments, A5 and SWL and CP, Double Nickel 55 containing *Bacillus amyloliquefaciens* D747 (Figure 4.2). The AUDPC was 294.7, 476.2 and 649.3, respectively whereas the control had 2118.2. Overall, the treatments with *B. amyloliquefaciens* A5 resulted in the lowest disease severity (Figure 4.1) and low effect on plant phytotoxicity (Figure 4.4). Leaf blemishes started from the leaf apex (Figure 4.7), chlorosis and leaf abscission were observed on the treated plants. New developing leaves were instantly infected by *B. cinerea* compared to the leaves sprayed with the *B. amyloliquefaciens* A5, *B. amyloliquefaciens* SWL and Double Nickel 55 treatments on all treated the plants. The lesions of *B. cinerea* moved systemically to the unsprayed leaves. The treatments had a significant effect on *B. cinerea* percentage disease incidence on the leaves ($P<0.001$). *B. amyloliquefaciens* A5 resulted in the lowest percentage disease incidence of 10.44% compared to the control with 69.39%.

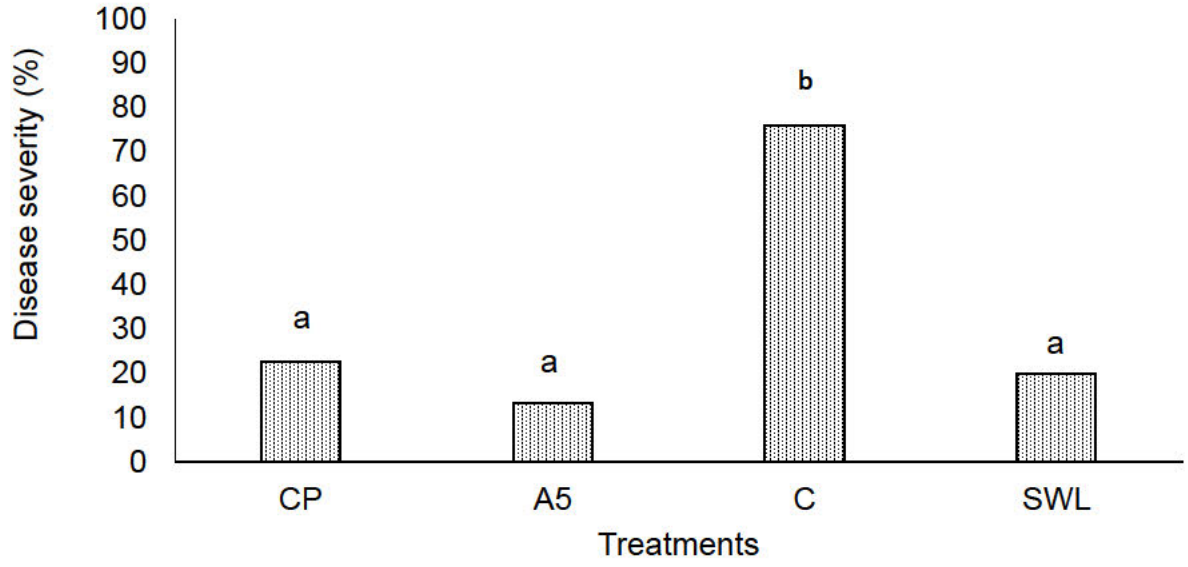

Figure 4.1: The effect of different *Bacillus amyloliquefaciens* and Double Nickel 55 on the disease severity of *Botrytis cinerea* on the *Leucospermum* 'Ayoba Sun' leaves under greenhouse conditions. CP (Double Nickel 55, commercial product containing *Bacillus amyloliquefaciens* (D747), (A5) *B. amyloliquefaciens* A5, (SWL) *B. amyloliquefaciens* SWL and C (control).

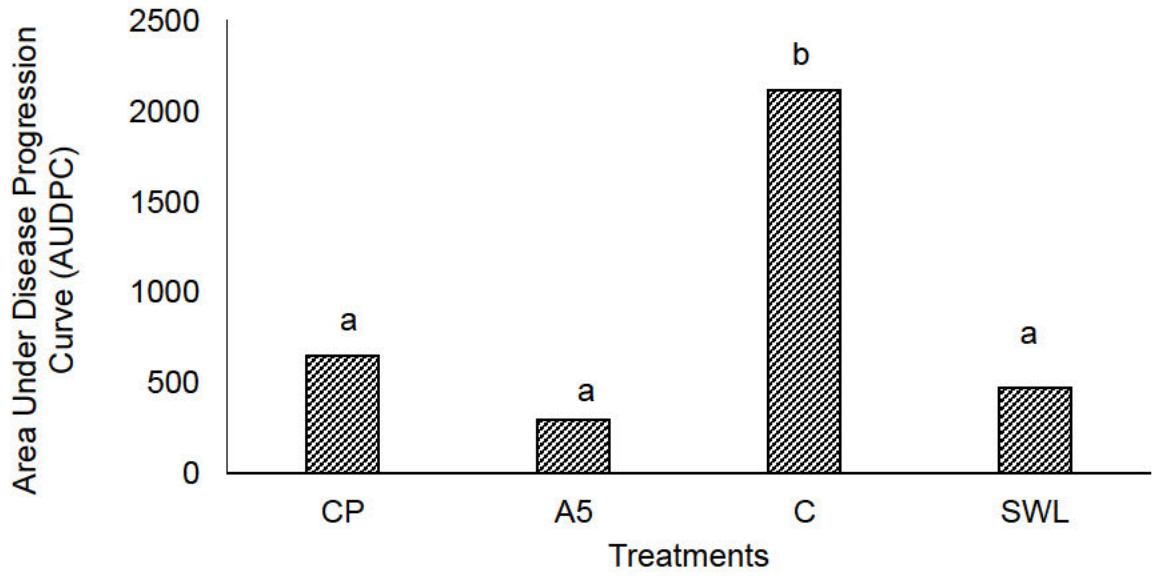

Figure 4.2: The AUDPC showing the resistance of the *Leucospermum* Ayoba Sun' leaves to *Botrytis cinerea* infection due to the effect of *Bacillus amyloliquefaciens* strains and Double Nickel (CP) treatments. CP (Double Nickel 55, commercial product

containing *Bacillus amyloliquefaciens* (D747), (A5) *B. amyloliquefaciens* A5, (SWL) *B. amyloliquefaciens* SWL and C (control).

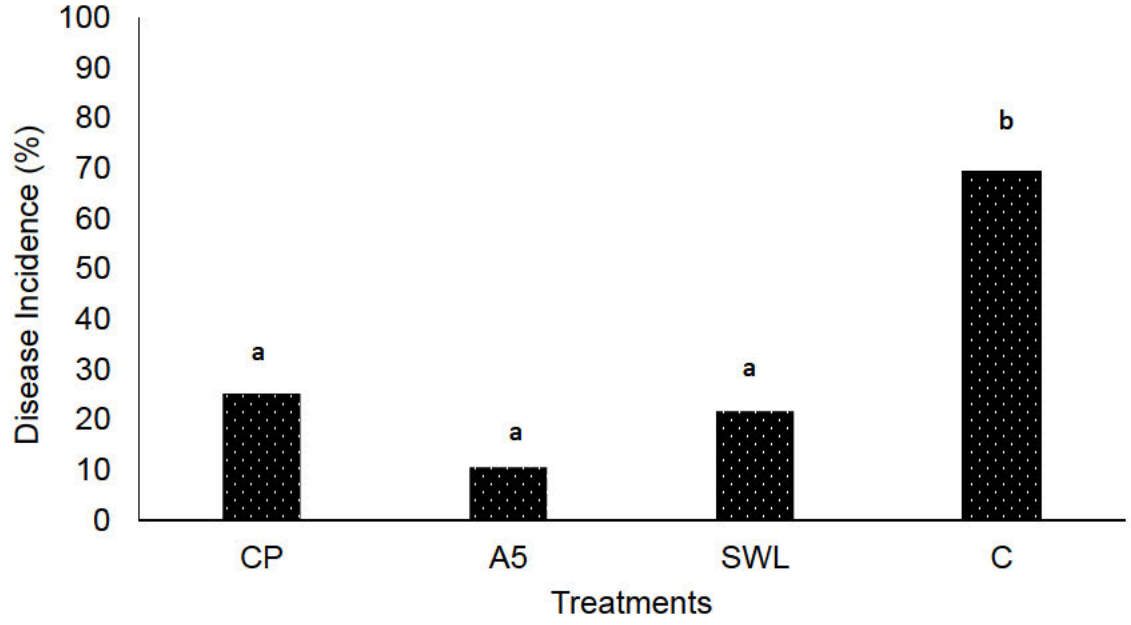

Figure 4.3: The effect of different *Bacillus amyloliquefaciens* strains and Double Nickel (CP) on the percentage disease incidence of *B. cinerea* on the *Leucospermum* 'Ayoba Sun' leaves. CP (Double Nickel 55, commercial product containing *Bacillus amyloliquefaciens* (D747), (A5) *B. amyloliquefaciens* A5, (SWL) *B. amyloliquefaciens* SWL and C (control).

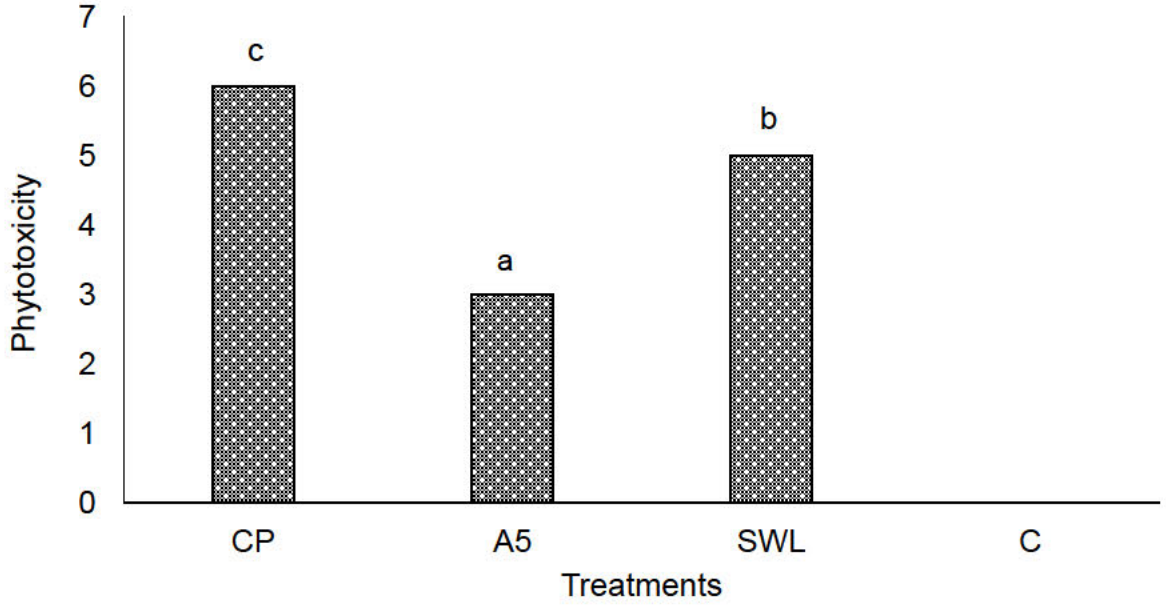

Figure 4.4: The effect of *Bacillus amyloliquefaciens* strains and Double nickel on *Leucospermum* 'Ayoba Sun' plant flower phytotoxicity. CP (Double Nickel 55, commercial product containing *Bacillus amyloliquefaciens* (D747), (A5) *B. amyloliquefaciens* A5, (SWL) *B. amyloliquefaciens* SWL and C (control).

4.3.2 Effect of selected bacterial isolates on *B. cinerea* of *Leucospermum* 'Ayoba' Sun buds

The disease severity on the buds was significantly reduced by the *Bacillus amyloliquefaciens* strains and the commercial product (Figure 4.5). Moreover, the AUDPC significantly differed among the treatments (P<0,001). Notably, Double Nickel 55 (CP) had the lowest disease severity (3,3%), *B. amyloliquefaciens* A5 (21,3%) and *B. amyloliquefaciens* SWL (50%). Overall, the commercial product was highly effective in managing *B. cinerea* on the *Leucospermum* 'Ayoba Sun' buds and *B. amyloliquefaciens* A5 was promising.

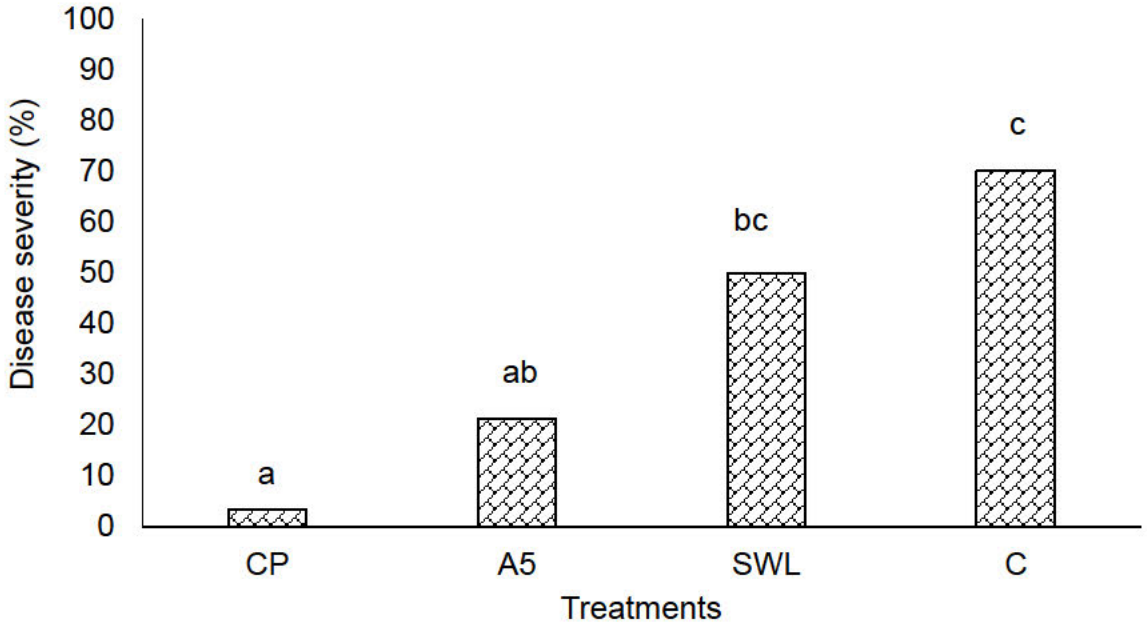

Figure 4.5: The effect of different *Bacillus amyloliquefaciens* strains on the disease severity of *Botrytis cinerea* on the *Leucospermum* 'Ayoba Sun' buds. CP (Double Nickel 55, commercial product containing *Bacillus amyloliquefaciens* (D747), (A5) *B. amyloliquefaciens* A5, (SWL) *B. amyloliquefaciens* SWL and C (control).

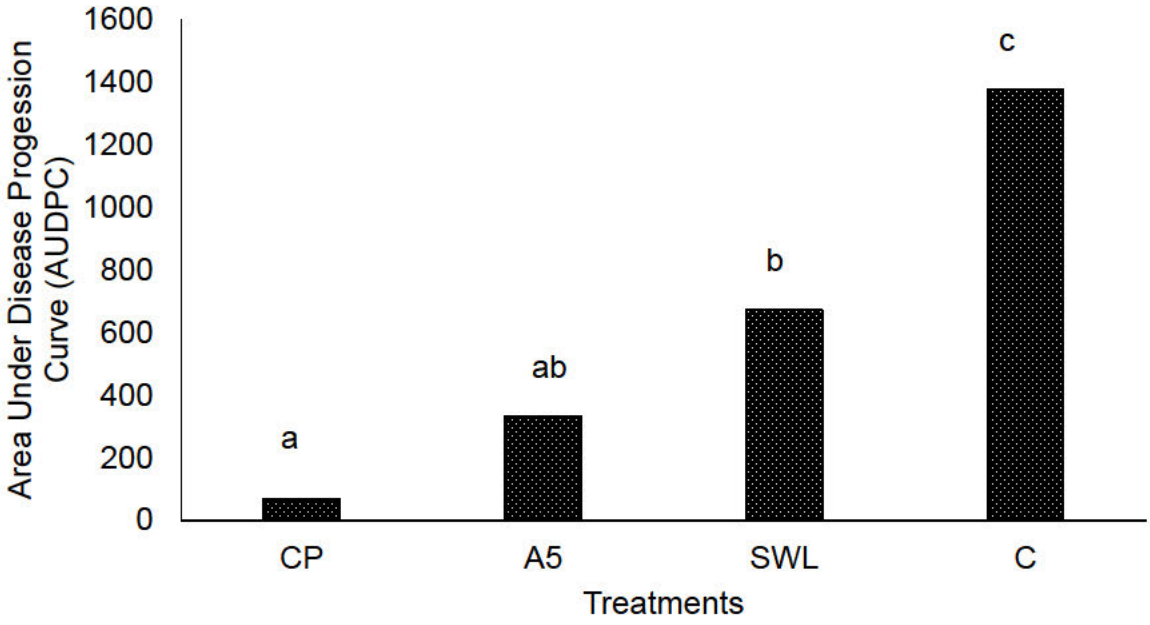

Figure 4.6: The effect of *Bacillus amyloliquefaciens* strains on the *Leucospermum* 'Ayoba Sun' plants disease resistance (AUDPC) when infected with *B. cinerea*.

(Double Nickel 55, commercial product containing *Bacillus amyloliquefaciens* (D747), (A5) *B. amyloliquefaciens* A5, (SWL) *B. amyloliquefaciens* SWL and C (control).

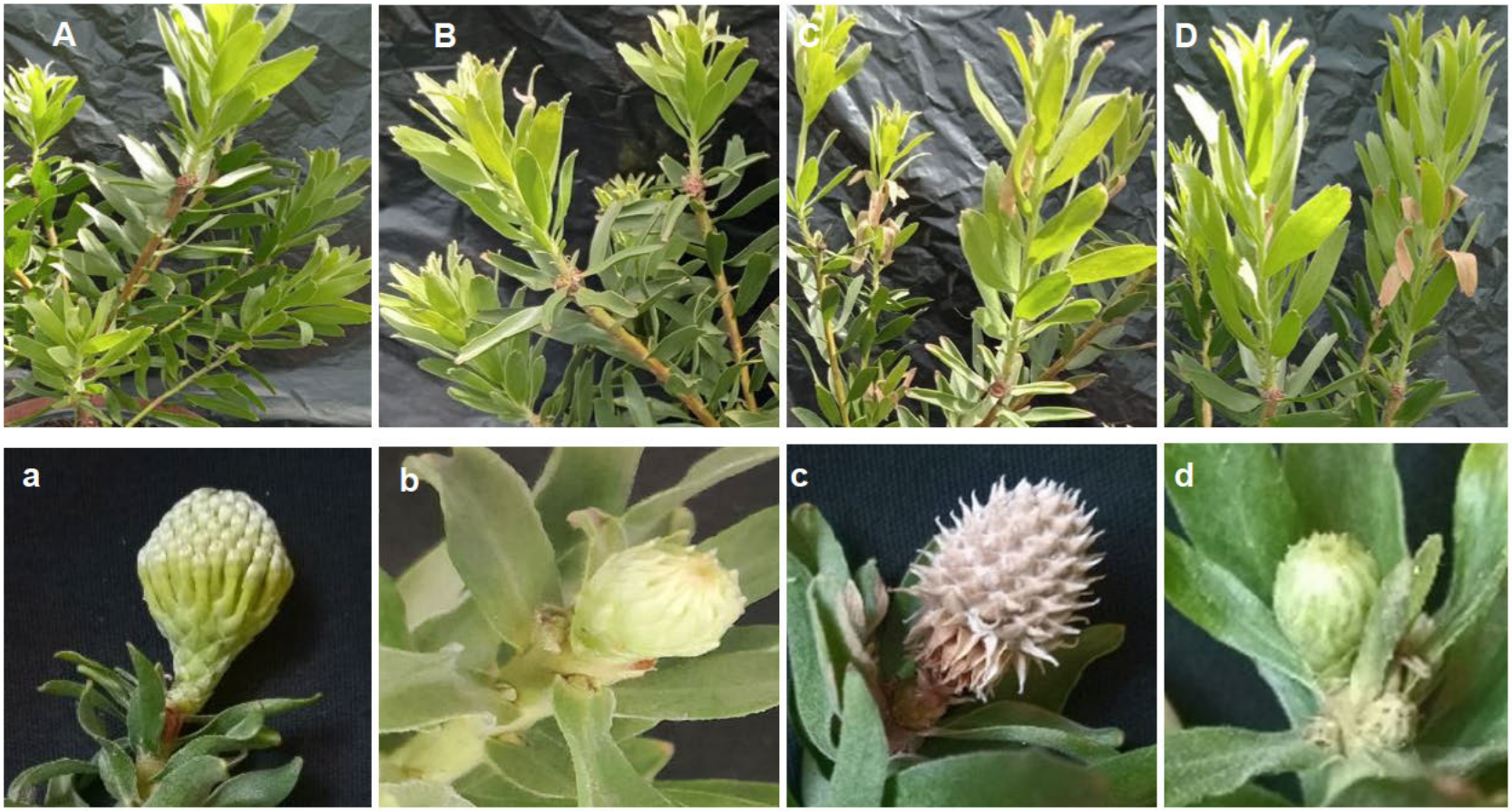

Figure 4.7: The *in vivo* antagonistic effect by different *Bacillus amyloliquefaciens* strains on the disease severity of *B. cinerea* and the effect on phytotoxicity. (A) Uninoculated control showing no necrosis on the leaves and (a) the buds are symptomless; (B) the effect of *B. amyloliquefaciens* A5 application resulted in less infection of *Botrytis cinerea* on the leaves which increases plant quality (b), *B. amyloliquefaciens* A5 managed to reduce *B. cinerea* infection on the buds and caused browning at the centre (C) *B. amyloliquefaciens* SWL was ineffective to manage *B. cinerea* infection on the leaves and resulted in low plant quality and many

brown leaves infected from the apex to the base and (c) was also highly ineffective to reduce *B. cinerea* infection on the buds which were fully colonized resulting in bud blight. (D) The commercial product CP resulted in poor inhibition of *B. cinerea* on the leaves resulting on the upper leaves browning but (d) was highly effective to manage *B. cinerea* infection on the buds resulting in healthy green buds.

4.4 Discussion

The results demonstrate that *B. amyloliquefaciens* is an effective biological agent against *B. cinerea* under greenhouse conditions. Moreover, *B. amyloliquefaciens* is effective to manage other diseases in various hosts. *B. amyloliquefaciens* has been reported to reduce Anthracnose disease when sprayed in chilli by 71% and also cause increased plant growth (Gowtham et al., 2018). Moreover, *B. amyloliquefaciens* SB14 significantly reduced seedling damping-off of sugar beet caused by *Rhizoctonia solani* AG-4 by 58% and 52.5% caused by *R. solani* AG2-2 when the seeds were soaked in the formulated bacteria CMC 1% solution (Karim et al., 2016).

The experiments under the greenhouse demonstrated a change in the effectiveness of the treatments to managing *B. cinerea* and their effect on flower plant quality. *B. amyloliquefaciens* A5 had the highest inhibition of *B. cinerea* on the leaves and the lowest efficacy on leaf quality. The change in the performance of the treatments could be due to environmental factors such as the mode of application of the biological controls and temperature changes in the glasshouse. According to Ahlem et al. (2012) biocontrol survival and adaptation can be affected by abiotic environmental conditions such as temperature, pH and relative humidity.

B. amyloliquefaciens strain A5 and SWL were effective to manage *B. cinerea* at different rates on the *Leucospermum* 'Ayoba Sun' leaves. According to Singh et al. (2016), the effectiveness of pathogen strains differs due to the types of fatty acids in the cell which regulate its virulence. The commercial product, Double Nickel 55 had the lowest percentage reduction of *B. cinerea* and a huge effect on plant quality in both experiments. According to Lengai and Muthomi (2018), *Bacillus spp., Pseudomonas spp., Burkholderia spp*. and *Xanthomonas spp*. are one of the most commercialised biopesticides and their effectiveness depends on their formulation and the used substrate. The Double Nickel 55, *B. amyloliquefaciens* A5 and *B. amyloliquefaciens* SWL treatments significantly reduced *B. cinerea* on the leaves. The effectiveness of the *B. amyloliquefaciens* strains could be due to their mode of action such as the production of volatiles and sub-lethal cyclic lipopeptides concentrations through induced systemic resistance (ISR) pathway (Chowdhury et al., 2015). New developing leaves were highly and instantly infected by *B. cinerea* compared to the leaves sprayed with the Double Nickel 55, *B. amyloliquefaciens* A5 and *B. amyloliquefaciens*

SWL treatments on all the plants treated. According to Liang et al. (2023), *B. cinerea* moves systemically to untreated parts of the plant.

The *B. amyloliquefaciens* strains and Double Nickel 55 used in this study significantly reduced *B. cinerea* disease severity on the buds. The reduction in the efficacy of the treatments can be due to temperature and humidity in the greenhouse that affects disease severity. The commercial product, Double Nickel 55 was highly effective with the highest inhibition against *B. cinerea* and *B. amyloliquefaciens* A5 was more effective compared to *B. amyloliquefaciens* SWL. Therefore, Double Nickel can be applied by farmers on the buds to manage *B. cinerea* infection.

4.5 Conclusion

Double Nickel 55 was effective in managing *B. cinerea* only on the *Leucospermum* 'Ayoba Sun buds and can be extensively tested under field and nursery conditions and then applied by farmers to manage *B. cinerea* infection on the buds. However, *B. amyloliquefaciens* A5 was proven to be effective in suppressing *B. cinerea* on the leaves and the buds. Further studies are needed to evaluate the use of this strain to manage *B. cinerea* of *Leucospermum* 'Ayoba Sun' plants in the field. However, it must always be sprayed on newly growing leaves for effective results since the pathogen moves systemically. The evaluation of the mode of action of *B. amyloliquefaciens* A5 to supress *B. cinerea* on *Leucospermum* 'Ayoba Sun' should be conducted. Moreover, studies to test the efficacy of the strain when the suspension cells concentration is increased should be conducted for better *B. cinerea* management.

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Chapter 5

The effect of *Bacillus amyloliquefaciens* strains on plant defense-related enzymes and antioxidants in *Leucospermum* 'Ayoba Sun' flowers

Abstract

Botrytis cinerea is a necrotrophic fungal pathogen with a broad host range of about 1400 plant species. Synthetic fungicides such as benzimidazoles and dicarboximides are known as the primary strategy to manage *B. cinerea.* However, these chemicals are harmful to humans as they have toxic residues and pathogens develop resistance. Hence, environmentally safe strategies such as biological control are an effective eco-friendly alternative to fungicides. Therefore, the aim of this research is to evaluate the potential of the best isolated *B. amyloliquefaciens* antagonists and Double Nickel 55 (*Bacillus amyloliquefaciens* D747) to enhance phenolics (TPC), flavonoids (TFC) and peroxidase (POD) in *Leucospermum* 'Ayoba Sun' leaves against *B. cinerea*. The *Leucospermum* 'Ayoba Sun' infected leaves during the *in vivo* screening were collected for each treatment with three replicates at three different days during the experiment. The samples were stored at -80℃, freeze-dried and ground into powder using a mortar and pestle. The peroxidase activity, total phenolic content (TPC) and total flavonoids (TFC) was determined on *Leucospermum* 'Ayoba Sun' leaves treated with *B. amyloliquefaciens* A5, *B. amyloliquefaciens* SWL and commercial product (Double Nickel). The control and CP treatments exhibited the highest TFC of 26.03 GAE/g dry matter and 28.56 GAE/g dry matter, respectively on day 35. Overall, the control (441GAE/g dry matter) and *B. amyloliquefaciens* A5 (410 GAE/g dry matter) had the highest TPC on day 35. The control (0.38 U/min/g dry matter) and *B. amyloliquefaciens* SWL (0.26 U/min/g dry matter) had the highest POD enzyme activity on day 35 compared to other treatments. Therefore, an increase in *B. cinerea* severity does not result in all plant enzyme activity enhancement against the infection. The effect of *B. amyloliquefaciens* on the other defense enzymes including phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) should be evaluated on *Leucospermum* 'Ayoba Sun' plants infected by *B. cinerea.* In conclusion, *B. amyloliquefaciens* does enhance plant defense enzymes in plants to manage *B. cinerea*.

Keywords: *B. cinerea*, Peroxidase activity, phenolics, flavonoids, *B. amyloliquefaciens* A5 and *B. amyloliquefaciens* SWL.

5.1 Introduction

Botrytis cinerea is a necrotrophic fungal pathogen with a broad host range of about 1400 plant species including on fruits, vegetables and ornamental plants, affecting the leaves, stems and blossom which results in poor quality of cut flowers (Ullah et al., 2024). Application of synthetic fungicides such as benzimidazoles and dicarboximides is the primary strategy to manage *B. cinerea*, however these chemicals are harmful to humans and plants develop resistance. Therefore, there is a need to explore environmentally safe strategies such as biological control which is an effective eco-friendly alternative to fungicides.

Bacillus amyloliquefaciens is known for its potential for plant growth promotion and plant disease growth inhibition. The pathogen produces α-amylase, protease, lipase, cellulase, xylanase, pectinase, aminotransferase, barnase, peroxidase, glucanases, and chitinase enzymes to induce resistance against pathogens (Zalila-Kolsi et al., 2023). According to Gowtham et al. (2018), *B. amyloliquefaciens* enhanced growth of chili and induced plant resistance against anthracnose by increasing defense enzymes phenylalanine ammonia lyase (PAL), peroxidase (POD), polyphenol oxidase (PPO) and β-1,3-glucanase. According to Prathuangwong and Buensanteai (2007), there was an increase in production of total phenol, phenylalanine ammonia lyase, peroxidases and 1,3-β-glucanases when the soybean seeds infected with *Xanthomonas axonopodis* pv. *Glycines* were treated with *Bacillus amyloliquefaciens* strain KPS46. Moreover *B. amyloliquefaciens* (FZB24) treatment on tomato plants infected by *F. oxysporum* f.sp. *lycopersici* induced defense enzymatic activities such as POD, PAL, CAT and SOD (Elanchezhiyan et al., 2018). According to Lu et al. (2021) study, *B. subtilis* KLBC BS6 enhanced disease related enzymes including chitinase, phenylalanine ammonia lyase, peroxidase and polyphenol oxidase on blueberry fruits with grey mould caused by *B. cinerea*. Zhou et al. (2020) showed that the activities of polyphenol oxidase, peroxidase, chitinase, and β-1,3-glucanase on ‘Red Globe’ grapes infected by *B. cinerea* were enhanced during

different storage periods at postharvest. Therefore, this study evaluated the potential of the best isolated *B. amyloliquefaciens* antagonists and the *B. amyloliquefaciens* commercial product to enhance phenolics, flavonoids and peroxidase (PAL) in *Leucospermum* 'Ayoba Sun' leaves infected with *B. cinerea*.

5.2 Methods and materials

5.2.1 Sample collection and preparation

The *Leucospermum* 'Ayoba Sun' infected leaves during the *in vivo* screening (Chapter 4, Figure 4.7 results) were collected for each treatment with three replicates at three different days during the experiment. The samples were stored at -20°C. They were then stored at -80°C for 24 hours and freeze-dried in a Virtis freeze drier, model #2KBTES-55 (Thermo Fisher Scientific, USA). They were ground into powder using a mortar and pestle and stored -20°C.

5.2.2 Determination of peroxidase activity (POD)

In triplicate, sample powder (1 g) was extracted with 10 mL sodium phosphate buffer (0.2 M, pH 7.0). The extract was centrifuged at 15,000 rpm for 15 minutes at 4°C. Enzyme extract (0.1 mL) was mixed with 2.7 mL sodium phosphate buffer (0.05 M), 1% p-phenylenediamine (0.2 mL), and 0.1 mL hydrogen peroxide (1.5% w/v). The absorbance was measured at 485 nm using the spectrophotometer SPECTRO UV-16 (Trilab support, MRC laboratories, Cambridge, United Kingdom). Enzyme activity was defined as an increase in absorbance at 485 nm per mg protein per minute.

5.2.3 Determination of total flavonoid content

In triplicate, 0.5 g sample powder was extracted with 80% methanol at 40°C for 60 minutes. Samples were cooled down and centrifuged for 15 minutes at 10,000 rpm. Plant extract (0.1 mL) was added to 0.3 mL deionized water, followed by 0.03 mL sodium nitrate 5%, and incubated at 25°C for five minutes. Aluminum chloride 10% (0.03 mL) was added; after five minutes,0.2 mL sodium hydroxide (1 mM) was added to the solution, and the reaction mixture was diluted to 1 mL using deionized water. The absorbance was measured at 510 nm using a spectrophotometer against

methanol as a blank. Results were expressed as mg gallic acid equivalent (GAE)/g plant dry matter.

5.2.4 Determination of total phenolics

The total phenolic content was determined using a method described by Singleton et al. (1999). The *Leucospermum* 'Ayoba Sun' leaves (1g) collected during the *in vivo* screening were homogenized with 5ml of 80% methanol. Thereafter, 2.6ml of distilled water and 200µl of Folin-Ciocalteu's phenol reagent was added to 200µl of methanolic extracts. After 6 min, 2ml of 7% (w/v) sodium carbonate was added to the reaction mixture. The absorbance was measured at 750nm after 90 min using a spectrophotometer. The total phenolic content was expressed as mg gallic acid equivalents GAE g^{-1} plant dry matter.

5.2.4 Data analyses

The POD, total flavonoid and total phenolics values were subjected to analyzed for variance using GenStat 23rd edition for analyses of variance. Treatment means were separated using Duncan multiple range test at the 5% probability level (p=0.05).

5.3 Results

5.3.1 Total phenolic and flavonoid content

There were significant differences among the treatments on the total flavonoid content (TFC) and total phenolic content (TPC). On day 35 the effect of Double Nickel 55 (CP) on TFC in *Leucospermum* 'Ayoba Sun' was 28.56 GAE/g dry matter, *B. amyloliquefaciens* A5 (13.56 GAE/g dry matter), *B. amyloliquefaciens* SWL (24.87 GAE/g dry matter) and control (26.63 GAE/g dry matter) (Figure 5.1). The highest TFC was observed on the commercial product (CP) (38.12 GAE/g dry matter) and *B. amyloliquefaciens* A5 (33.03 GAE/g dry matter) on day 14 and it decreased on day 35. The lowest TFC was observed on day 14 on *B. amyloliquefaciens* SWL (10.1 GAE/g dry matter). The *B. amyloliquefaciens* SWL and the control had the lowest TFC however, it decreased on day 14 and increased on day 35. The control and CP treatments exhibited the highest TFC of 26.03 GAE/g and 28.56 GAE/g, respectively, on day 35.

On day 35 the effect of Double Nickel 55 (CP) on TPC in *Leucospermum* 'Ayoba Sun' was 407 GAE/g dry matter, *B. amyloliquefaciens* A5 (410 GAE/g dry matter), *B. amyloliquefaciens* SWL (395.3 GAE/g dry matter) and control (441 GAE/g dry matter) (Figure 5.2). The highest TPC was observed on day 14 on the *B. amyloliquefaciens* SWL (434.9 GAE/g dry matter) treated plants. The TPC increased on *B. amyloliquefaciens* SWL and *B. amyloliquefaciens* A5 overtime. The lowest TPC was observed on the control (228.4 GAE/g dry matter) on day 14 and increased on day 35. Overall, the control (441 GAE/g dry matter) and *B. amyloliquefaciens* A5 (410 GAE/g dry matter) had the highest TPC on day 35.

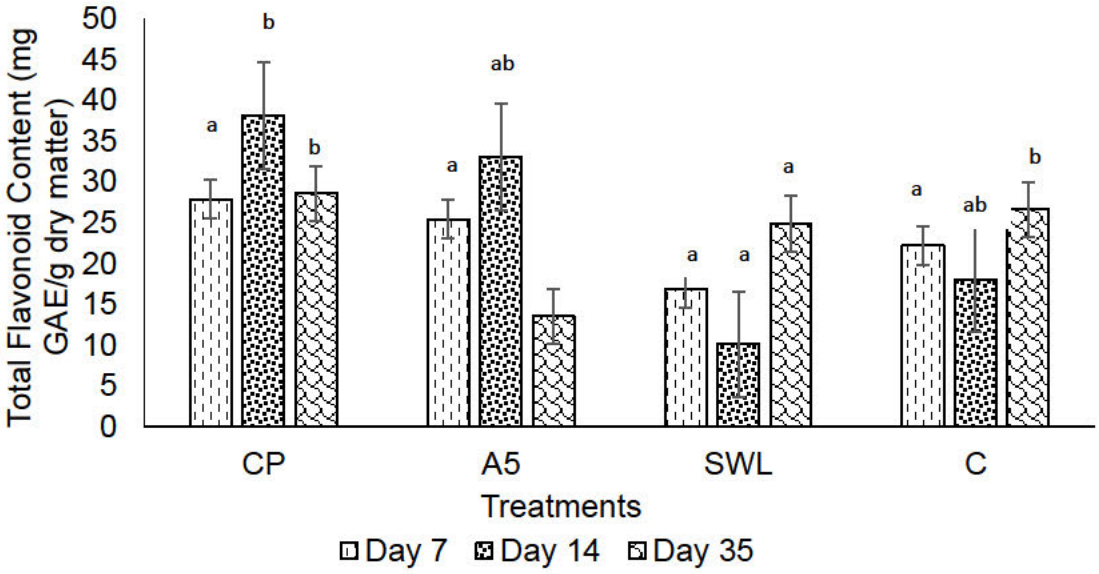

Figure 5.1: The effect of various treatments on the total flavonoids of *Leucospermum* 'Ayoba Sun' plants after *B. cinerea* inoculation. Means with different letters indicate significant differences according to Duncan's multiple range test ($p<0.05$). CP (Double Nickel 55), (A5) *B. amyloliquefaciens* A5, (SWL) *B. amyloliquefaciens* SWL and C (control).

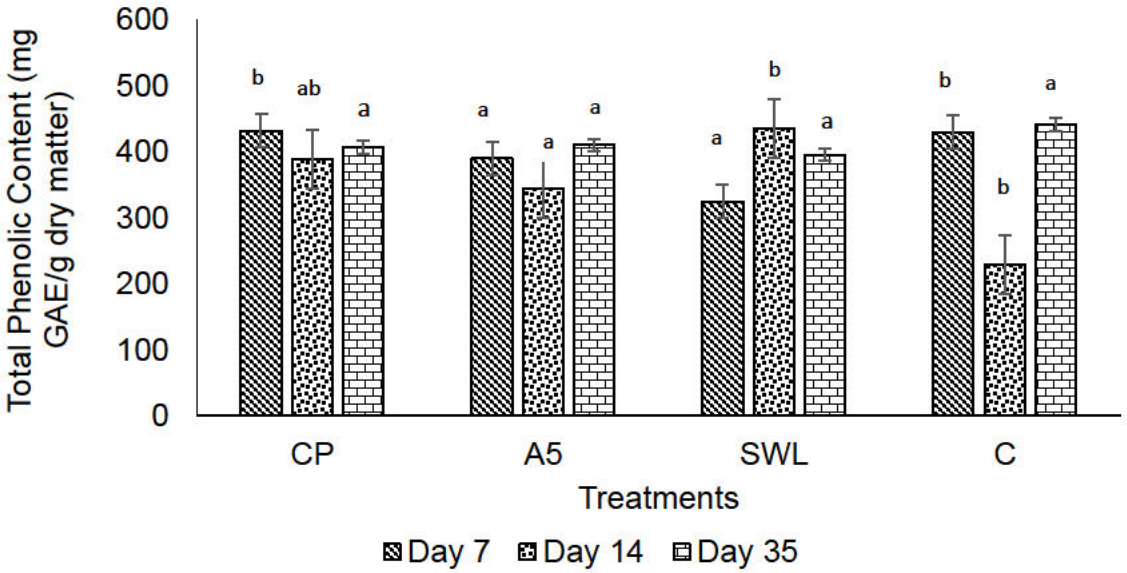

Figure 5.2: The effect of various treatments on the total phenolics in *Leucospermum* 'Ayoba Sun' plants after *B. cinerea* inoculation. Means with different letters indicate significant differences according to Duncan's multiple range test ($p<0.05$). CP (Double Nickel, (A5) *B. amyloliquefaciens* A5, (SWL) *B. amyloliquefaciens* SWL and C (control).

5.3.2 Defense-related enzyme activity

Double Nickel 55, *B. amyloliquefaciens* A5, *B. amyloliquefaciens* SWL and the control had no significant difference on the POD enzyme on *Leucospermum* 'Ayoba Sun' (Data not shown). On day 35 the effect of Double Nickel 55 (CP) on POD in *Leucospermum* 'Ayoba Sun' was 0.2 U/min/g dry matter, *B. amyloliquefaciens* A5 (0.23 U/min/g dry matter), *B. amyloliquefaciens* SWL (0.26 U/min/g dry matter) and control (0.38 U/min/g dry matter). The control (0.38 U/min/g dry matter) and *B. amyloliquefaciens* SWL (0.26 U/min/g dry matter) had the highest POD enzyme activity on day 35 compared to other treatments. The effect of *B. amyloliquefaciens* A5 and Double Nickel 55 on POD activity decreased from day 7 to day 35.

5.4 Discussion

The total flavonoid content increased in plants treated with *B. amyloliquefaciens* SWL and *B. amyloliquefaciens* A5 strains until day 14 and decreased afterwards. The findings of this study illustrate that among the *B. amyloliquefaciens* strains, *B. amyloliquefaciens* SWL strain had the highest average total phenolic content whereas *B. amyloliquefaciens* had the highest total phenolic content at day 7. Moreover, plants

treated with *B. amyloliquefaciens* A5 strain resulted in an increase in TFC on day 14 demonstrating that *B. amyloliquefaciens* does increase flavonoids in plants. Aldayel et al. (2024) reported that the tomato plants infected by *Alternaria solani* were treated with *B. amyloliquefaciens* in a greenhouse and had the highest flavonoid and phenolic content compared to fungicide mancozeb and Zinc nanoparticles treated plants. The flavonoid content on *B. amyloliquefaciens* A5 treated plants was observed increasing and decreasing towards the end of the experiment which could be attributed to the low accumulation of transcripts of the genes encoding for various pathogenesis-related (PR) proteins in leaves which are associated with host defense to limit pathogen progress (Abbey et al., 2023). Moreover, flavonoids decreased due to *B. cinerea* developing resistance. Flavonoids are secondary metabolites involved in plant protection against diseases, however, pathogens develop resistance against these compounds (Vargas et al., 2013). Double Nickel 55 had the highest flavonoid content compared to the BCAs and untreated control. The Double Nickel 55 product induced systemic acquired resistance which can involve high production of flavonoids to protect against diseases (Abd-Elsalam et al., 2023)**.** Overall, the inoculated control had the highest TFC and TPC on day 35 due to stimulation of natural plant defenses upon *B. cinerea* inoculation which can be due to plants being stressed and secreting more phenolics and flavonoids.

Among the treatments, the control and *B. amyloliquefaciens* SWL had the highest POD enzyme activity while it was decreasing on other treatments. According to Jiao et al. (2020), *B. amyloliquefaciens* YN201732 strain resulted in the decrease of POD and PAL enzyme secretion in tobacco plants against Powderly mildew. Therefore, an increase in *B. cinerea* severity does not result in all plant enzyme activity enhancement against the infection.

5.5 Conclusion

The *B. amyloliquefaciens* SWL and A5 antagonists and Double Nickel 55 (*B. amyloliquefaciens* d747) enhance phenolics, flavonoids and peroxidase (PAL) on *Leucospermum* ‘Ayoba Sun’ leaves against *B. cinerea* The control had the highest phenolics, flavonoids and POD compared to the other treatments at day 35. *B. amyloliquefaciens* A5 had the second highest TPC content while *B. amyloliquefaciens* SWL had the second highest POD enzyme activity at day 35. The effect of *B.*

amyloliquefaciens on the defense-related enzymes including PAL and PPO should be evaluated on *Leucospermum* ‘Ayoba Sun’ plants infected by *B. cinerea*.

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Chapter 6
Thesis overview

6.1 Introduction

The flower market relies on various factors such as pigmentation, scent, nectar, texture, size, longevity, and form which greatly influence consumer choices (Datta, 2018). However, the phytopathogenic fungus *B. cinerea* is a significant threat to ornamental production in the field and greenhouses. *Botrytis cinerea* is a necrotrophic opportunist pathogen that is a threat and infects over 200 plant species and causes more than 30% of plant losses globally (AbuQamar et al., 2016). It reduces the market competitiveness of plants as it affects the leaves and the blossoms of ornamentals resulting in low quality and yield. Moreover, *B. cinerea* infects a broad host range of about 1400 plant species including fruits, vegetables and ornamental plants. It affects the leaves, stems and blossoms which results in poor quality of cut flowers (Abbey et al., 2019). Several control strategies have been implemented to manage *Botrytis* including chemical and cultural methods but *B. cinerea* has genetic variability and is resistant to most fungicides (Shao et al., 2021). Hence, environmentally safe strategies such as biological control are an effective eco-friendly alternative to fungicides.

Bacillus amyloliquefaciens is known for its potential for plant growth promotion and plant disease growth inhibition (Asari et al., 2016). The pathogen produces α-amylase, protease, lipase, cellulase, xylanase, pectinase, aminotransferase, barnase, peroxidase, glucanases, and chitinase enzymes to induce resistance against pathogens (Zalila-Kolsi et al., 2023). However, no research has been conducted on ornamentals to manage *B. cinerea* on *Leucospermum* ‘Ayoba Sun’. Hence, the present study evaluates the effect of *B. amyloliquefaciens* on managing *B. cinerea* on *Leucospermum* ‘Ayoba Sun’ at preharvest.

6.2 Research objectives and major findings

The aim of this research study was to evaluate the effect of biological control agents against *Botrytis cinerea* of *Leucospermum* ‘Ayoba Sun’ flowers. The specific objectives of this research were to: (1) isolate and screen the bacterial biological

control agents against *Botrytis cinerea in vitro*, (2) Evaluate the potential of the best biological control agents in controlling *B. cinerea* on *Leucospermum* 'Ayoba Sun' and their effect on flower quality under greenhouse conditions and (3) evaluate the effect of *Bacillus amyloliquefaciens* strains on plant defense-related enzymes and antioxidants of *Leucospermum* 'Ayoba Sun' flowers. The major research findings are highlighted below.

Chapter 3: Isolation and *in vitro* screening of bacterial isolates against *Botrytis cinerea* of *Leucospermum* 'Ayoba Sun'

- The selected six bacterial isolates (A2, A3, A5, SWL, PL3 and SM3) inhibition levels and effectiveness decreased during the secondary screening.
- The phylogenetic tree revealed that the bacterial isolates A2, A3, A5, SWL, PL3 and SM3 are closely related to each other and to *B. amyloliquefaciens* strain BCRC 11601 and *B. amyloliquefaciens* strain NBRC 1553. Moreover, the bacterial isolates are distantly related to *B. subtills*, *B. velezensis* and other *Bacillus* species.
- The isolates used for secondary screening were identified to be the same bacteria, *Bacillus amyloliquefaciens*.
- *B. amyloliquefaciens* strains inhibited *B. cinerea* by shrinking, deformating, cutting and bending the pathogen hyphae when observed under the scanning electron microscope (SEM).
- Rod shaped bacterial cells were observed on the bent and broken pathogen under SEM.
- The buds were highly susceptible to infection than the leaves and most were fully colonized by *B. cinerea* within 7 days.

Chapter 4: Evaluating the effect of *Bacillus amyloliquefaciens* strains against *B. cinerea* of *Leucospermum* 'Ayoba Sun' under greenhouse conditions

- *B. amyloliquefaciens* A5 resulted in the lowest disease severity and percentage disease incidence on the leaves and low effect on plant quality of *Leucospermum* 'Ayoba Sun'.

- Chlorosis and leaf abscission were observed on *Leucospermum* 'Ayoba Sun' plants inoculated with *B. amyloliquefaciens* SWL and Double Nickel 55.
- New developing leaves were instantly infected by *B. cinerea* compared to the leaves sprayed by the treatments on all the plants treated with *B. amyloliquefaciens* A5, *B. amyloliquefaciens* SWL and Double Nickel 55. *B. cinerea* moved systemically to the unsprayed leaves.
- The commercial product, Double Nickel 55 was highly effective in managing *B. cinerea* on the *Leucospermum* 'Ayoba Sun' buds and *B. amyloliquefaciens* A5 was promising.

Chapter 5: The effect of *Bacillus amyloliquefaciens* strains on plant defense-related enzymes and antioxidants of *Leucospermum* 'Ayoba Sun' flowers

- The control and CP treatments exhibited the highest TFC on day 35.
- The TPC increased on *B. amyloliquefaciens* SWL and *B. amyloliquefaciens* A5 treated plants overtime. However, the control and *B. amyloliquefaciens* A5 had the highest TPC on day 35.
- The effect of *B. amyloliquefaciens* A5 and *B. amyloliquefaciens* CP on POD activity decreased from day 7 to day 35.
- The control and *B. amyloliquefaciens* SWL had the highest POD enzyme activity on day 35 compared to other treatments.
- An increase in *B. cinerea* severity does not result in all plant enzyme activity enhancement against the infection.

6.3 Conclusion and recommendations

Various strains of *Bacillus amyloliquefaciens* have been identified for effectively controlling *B. cinerea in vitro.* Buds of *Leucospermum* 'Ayoba Sun were more susceptible to *B. cinerea* infection than the leaves. Moreover, the *B. amyloliquefaciens* commercial product CP was effective to manage *B. cinerea* only on the *Leucospermum* 'Ayoba Sun buds and can be applied by farmers to manage *B. cinerea* infection on the buds. Additionally, *B. amyloliquefaciens* A5 has been proven to be effective in suppressing *B. cinerea* on the leaves and the buds. Therefore, it can be tested under field conditions before commercialised and then used against *B. cinerea*

of *Leucospermum* ‘Ayoba Sun’ plants in the field. However, it must always be sprayed on newly growing leaves for effective results since the pathogen moves systemically. Overall, the control had the highest phenolics, flavonoids and POD compared to the other treatments. *B. amyloliquefaciens* A5 had the highest TPC content at day 35 and *B. amyloliquefaciens* SWL had the highest POD enzyme activity. Therefore, the application of *B. amyloliquefaciens* strain against *B. cinerea* enhanced the production of some antioxidants or enzymes in *Leucospermum* ‘Ayoba Sun’. Hence, there is a need to explore other biological agents that can increase numerous enzymes and antioxidants in *Leucospermum* ‘Ayoba Sun’ against *B. cinerea*.

Future studies should focus on: 1) ‘Further evaluation of the mode of action of other *B. amyloliquefaciens* strains in suppressing *B. cinerea* on *Leucospermum* ‘Ayoba Sun’ in greenhouse conditions, along with their effects on plant growth promotion, should be conducted , 2) The efficacy of the *B. amyloliquefaciens* A5 strain should be tested at higher suspension cell concentrations for improved *B. cinerea* management.’, 3) other *Bacillus sp*. including *B. amyloliquefaciens* strain BCRC 11601, *B. amyloliquefaciens* strain NBRC 1553, *B. subtilis* and *B. velezensis* should be evaluated against *B. cinerea in vitro*, 4) identify volatile antifungal compounds from the *in vitro* screening in the bacterial secretome should be evaluated using gas chromatography-mass spectrometry, 5) determine the effect of *B. amyloliquefaciens* on the defense-related enzymes including PAL and PPO on *Leucospermum* ‘Ayoba Sun’ plants infected by *B. cinerea* and 6) investigate an integrated control strategy for *B. cinerea* using plant leaf extracts or edible coatings and biological agents.

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Appendices

Appendix 1: Primary screening of bacterial isolates against *B. cinerea in vitro* after 7 days at 25°C.

Isolate name	Source	Mycelial growth (mm)	Inhibition (%)	Group
Control	-	88.89		
PL2	*Plantago lanceolata* L.	86.86	2.51	1
WLF1	*Citrus limon*	86,.89	2.47	1
PL28	*Plantago lanceolata* L.	42.09	57.87	3
MF4	*Zea mayz* L.	38.69	62.06	3
PL14	*Plantago lanceolata* L.	37.87	63.08	3
MLC9	*Citrus limon*	41.16	59.01	3
PF8	*Plantago lanceolata* L	45.26	53.94	3
PL20	*Plantago lanceolata* L.	44.20	55.25	3
PL10	*Plantago lanceolata* L.	39.31	61.30	3
MF2	*Zea mayz* L.,	42.38	57.50	3
SPLB2	*Ipomoea batatas* (L.)	45.28	53.91	3
WLF5	*Citrus limon*	33.91	67.96	3
SBF1	*Citrus limon*	39.08	61.58	3
PL5	*Plantago lanceolata* L.	37.92	63.01	3
PL15	*Plantago lanceolata* L.	39.55	61.00	3
PF2	*Citrus limon*	40.56	59.73	3
MCL3	*Citrus limon*	39.08	61.58	3
PL3	*Plantago lanceolata* L.	35.65	65.82	3
SPLB1	*Ipomoea batatas* (L.)	86.86	2.51	1
WH1	*Triticum aestivum* L.	43.46	56.17	3
S37	*Citrus limon*	38.56	62.22	3
PL30	*Plantago lanceolata* L.	34.76	66.93	3
PL19	*Plantago lanceolata* L.	41.99	57.98	3
MLC12	*Citrus limon*	35.68	65.79	3
MF3	*Zea mayz* L.	36.96	64.21	3
SPLB5	*Ipomoea batatas* (L.)	40.38	59.97	3
PL26	*Plantago lanceolata* L.	34.17	67.63	3
WLF4	*Citrus limon*	45.34	53.83	3

PL9	*Plantago lanceolata* L.	58.08	38.09	2
PF3	*Citrus limon*	37.04	64.10	3
MLC1	*Citrus limon*	35.78	65.66	3
MF1	*Zea mayz* L.	44.47	54.92	3
MLC2	*Citrus limon*	39.00	61.67	3
CL2	*Citrus limon*	38.21	62.65	3
SM3	*Citrus limon*	30.06	72.73	3
Soil 2.5	*Citrus limon*	46.80	52.03	3
YP60	*Citrus limon*	32.11	70.20	3
BJO2	*Citrus limon*	37.34	63.73	3
SD2	*Citrus limon*	42.53	57.31	3
SD5	*Citrus limon*	83.64	6.50	1
GN1	*Citrus limon*	43.30	56.36	3
SO3	*Citrus limon*	34.98	66.64	3
LM6	*Citrus limon*	42.65	57.17	3
DLC	*Citrus limon*	42.27	57.64	3
LBB1	*Citrus limon*	43.36	56.29	3
YP25	*Citrus limon*	40.52	58.97	3
BJB14	*Citrus limon*	35.97	65.43	3
Lemla	*Citrus limon*	34.70	66.99	3
LLCO	*Citrus limon*	36.42	64.87	3
PL18	*Plantago lanceolata* L.	41.58	58.49	3
BJO3	*Citrus limon*	39.34	61.26	3
SWL	*Citrus limon*	28.19	75.04	3
BJB1	*Citrus limon*	36.50	64.77	3
PL27	*Plantago lanceolata* L.	43.92	55.6	3
PF16	*Citrus limon*	86.86	2.51	1
PL25	*Plantago lanceolata* L.	40.97	59.24	3
WH9	*Citrus limon*	46.99	51.80	3
WH3	*Citrus limon*	41.17	59.00	3
MLC5	*Citrus limon*	44.74	54.58	3
MF8	*Zea mayz* L.	39.99	60.45	3
MCS6	*Citrus limon*	46.45	52.47	3

PL16	*Plantago lanceolata* L.	32.90	69.22	3
WLC	*Citrus limon*	43.50	56.12	3
PL4	*Plantago lanceolata* L.	40.87	59.37	3
PL7	*Plantago lanceolata* L.	37.41	63.65	3
MLC15	*Citrus limon*	39.13	61.52	3
MLC8	*Citrus limon*	39.67	55.91	3
WLZ	*Citrus limon*	34.67	62.09	3
PL8	*Plantago lanceolata* L.	40.83	54.46	3
CL1	*Citrus limon*	54.50	37.57	2
PF6	*Citrus limon*	45.67	48.49	2
PL12	*Plantago lanceolata* L.	38.83	56.94	3
PL21	*Plantago lanceolata* L.	91.00	-7.55	1
PF4	*Citrus limon*	49.00	44.37	2
BNM	*Citrus limon*	36.00	60.44	3
WH2	*Citrus limon*	50.17	42.93	2
WLF6	*Citrus limon*	48.50	44.99	2
SPL13	*Citrus limon*	49.17	44.17	2
PL18	*Plantago lanceolata* L.	41.50	53.64	3
CM1	*Cymbompogon marginatus*	42.96	56.78	3
TR1	*Tetradenia riparia*	43.49	56.13	3
CM2	*Cymbompogon marginatus*	43.11	56.60	3
TR2	*Tetradenia riparia*	34.65	67.05	3
A2	Soil	22.82	81.68	4
Baci hons	*Leucospermum* ‘Ayoba Sun’	47.31	51.40	3
A6	Soil	38.65	62.12	3
A5	Soil	29.29	73.68	3
CMS3	*Cymbompogon marginatus*	53.37	43.92	3
CMS2	*Cymbompogon marginatus*	61.38	34.20	2

A1	Soil	38.30	62.54	3
B2	Soil	43.20	56.51	3
CMS4	*Cymbompogon marginatus*	45.10	54.14	3
B3	Soil	53.56	43.68	2
TR3	*Tetradenia riparia*	37.25	63.84	3
TR4	*Tetradenia riparia*	34.72	66.96	3
A3	Soil	29.43	73.51	3