Bacteria and yeasts as potential biocontrol agents for the management of blue mould and green mould diseases of 'Valencia' oranges caused by *Penicillium italicum* and *Penicillium digitatum*

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DISSERTATION SUMMARY

Citrus fruits can be infected by microorganisms that affect the quality of fruit. *Penicillium* moulds caused by *Penicillium digitatum* (green mould) and *Penicillium italicum* (blue moulds) are two of the most problematic plant pathogens that affect the Rutaceae. *Penicillium digitatum* is a mesophilic fungus that can produce a potential mycotoxin called citrinin that has the potential to be carcinogenic to animals and humans. Management strategies in place to control these pathogens such as synthetic fungicides provide control, however they have impacts on the environment which does not appeal to consumers. This study aimed to assess the efficacy of bacterial and yeast strains isolated from leaves and peels of lemon (*Citrus lemon*), oranges (*Citrus sinensis*) and limes (*Citrus aurantiifolia*) in controlling *P. digitatum* and *P. italicum* in laboratory and fruit storage trials.

In the *in vitro* studies, 102 isolates were screened against *P. digitatum* and *P. italicum* using the dual culture assay on petri dishes. The best seven isolates were selected for secondary *in vitro* screening against both fungi. The best two isolates from the secondary screening against *P. italicum* were UK37 (96.89%) and SCO13 (94.23%) and for *P. digitatum*, UK37 (98.83%) and SCO13 (95.29%). Isolates UK37 and SCO13 were sent to Inqaba Biotech Industries (Pty) Ltd for molecular characterization and identification. Both isolates were identified as *Bacillus amyloliquefaciens*. *Bacillus amyloliquefaciens* strain SCO13 and *B. amyloliquefaciens* strain B UK37 were used in *in vivo* experiments to assess their efficacy in controlling *P. digitatum* and *P. italicum* on oranges 'Valencia' stored at 25 ℃ for ten days. *Bacillus amyloliquefaciens* strain SCO13 showed potential as a biological control agent against both *P. digitatum* and *P. italicum*, with a low disease incidence of 19.54% and 35.87% respectively.

Two yeast strains of *Meyerozyma guilliermondii* that were previously isolated, were used in *in vivo* experiments to assess their efficacy in controlling *P. digitatum* and *P. italicum* on oranges 'Valencia' stored at 25 ℃ for ten days. Yeast treatments provided the least control of the *Penicillium* moulds on oranges. The SEM images of the mycelial growth of *P. digitatum Meyerozyma* spp. displayed antagonism by growing on *P. digitatum* hyphae and colonizing orange fruit surfaces. micrographs showed the bent hyphae and reduced number of conidia *in vitro* against *B. amyloliquefaciens* spp., with *B. amyloliquefaciens* B13 completely colonizing and growing over *P. digitatum in vitro* for seven days, incubated at 25℃.

In conclusion, *B. amyloliquefaciens* demonstrated potential in controlling *P. digitatum* and *P. italicum* infection on oranges. These *Bacillus* spp. have the potential in the integrated pest management of *Penicillium* spp.

DECLARATION

I, **Onosizo Asimdumise Zondi** declare that:

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

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

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

IV. This thesis does not contain other persons‟ work unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

   a. Where their exact words have been used, their writing has been placed inside quotation marks and referenced.

   b. Their words have been re-written, but the general information attributed to them has been referenced.

V. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

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Finally, I would like to thank God for seeing me through my research study.

DEDICATION

I dedicate my work to my mother Mrs B.G. Zondi and my father Mr V.M Zondi

Table of Contents

DISSERTATION INTRODUCTION

The Crop

Citrus fruits are one of the most traded horticultural crops in the world. They are the most consumed fruit crops after mangoes, tomato and bananas (Arouma *et al*., 2012; Turner and Burri, 2013). Sweet oranges, tangerines, clementines, sour oranges, lemons, limes, grapefruit and mandarins are all different kinds of citrus fruits (Ortiz, 2002). They are a good source of vitamin C, which is an essential nutrient that plays a role in iron metabolism, acts as an antioxidant, stimulates white blood cell function and inhibits blood clots (Wintergerst *et al*., 2006; Arouma *et al.*, 2012). Citrus fruits are a source of minerals and vitamins and contain active phytochemicals and phytophenolics that have biological properties such as anti-inflammatory, anti-tumor and antimicrobial activities (Arouma *et al*., 2012). Turner and Burri (2013) undertook a study which revealed that undeveloped countries in Sub-Saharan Africa and Southeast Asia experience high levels of malnutrition and consume the least citrus. In global citrus exports, South Africa ranks 13th in citrus production, 3rd in terms of value and 2nd in volume (Moore and Manrakhan, 2022). The citrus industry is indirectly responsible for about one million jobs, making it a major contributor to employment nationally (Dlikilili and van Rooyen, 2018). Citrus exports from South Africa create approximately 140 000 jobs directly, and bring in R30 billions of export revenue every year (Dlikilili and van Rooyen, 2018).

Post-Harvest Pathogens of Citrus

The packing, transportation and cold storage of fruit are all vital processes for exportation because fruits must remain in a good condition until they reach their destination and are put on shelves for customers. However, a quarter of harvested fruit is attacked by fungal pathogens in developed countries, with almost half of total production of fruit being damaged in developing countries (Leelasuphakul *et al.*, 2008), including citrus. Research into the development of control measures for the sustainable management of post-harvest diseases is a priority for the citrus industry in South Africa. Citrus fruits are affected by several pests and diseases that cause economic losses. Green mould and blue mould caused by *Penicillium digitatum* (Pers: Fr) Sacc, *Penicillium italicum* Wehmer respectively, are amongst the most important post-harvest diseases impacting the citrus industry and are the three pathogens investigated in this study (Ahmed *et al*., 2022). *Penicillium* spp. are wound pathogens that thrive in warm temperatures from 22 to 30°C (Li *et al*., 2022). *Penicillium. digitatum* and *P. italicum* are wound pathogens that produce airborne conidia that can cause fruit decay even at temperatures as low as 4 °C

(Palou, 2009). The spores make contact with a wound and colonize the tissue rapidly; initial symptoms are water-soaked soft spots that are visible within 48h (Droby *et al*., 2008). Citrus fruit are often wounded in the harvesting, packing, transport and sale pipeline, creating sites for infection by the airborne conidia of these fungi. Fruits may be covered in green and blue conidia in a matter of 3 to 5 days, creating a new source of inoculum, which spreads rapidly onto other fruit (Bhatta, 2022). Due to the short life cycle of these pathogens, it is imperative to provide protection of citrus fruits from infection (Yang *et al*., 2019). This pathogen can complete its life cycle at low temperatures low as 10°C, which the temperature at which citrus fruit is typically stored (El-Ghaouth *et al*., 2002).

Control of Post-Harvest Pathogens of Citrus

The citrus industry has relied heavily on fungicides such as prochloraz, thiabendazole and imazalil to ensure disease free fruits for export and purchase by customers (Njombolwana *et al*., 2013). However, consumers have become increasingly inquisitive about what goes into fruit production and are demanding chemical free food (Yang *et al*., 2019). There has been immense pressure to move away from total fungicide use due to the health concern of fungicide residues, with constantly changing regulations in Europe concerning fungicide maximum residue levels (MRL). With Europe being the main importers of citrus from South Africa, it is crucial to adhere to the regulations to ensure economic stability (Wisniewski, *et al*., 2016). A second issue is that of fungicide resistance, which the industry is facing, as pathogens adapt and become resistant to fungicides due to their constant exposure to the same fungicides (Kinay *et al*., 2007). Therefore, a major task has been to find an efficient, nonchemical control measure that produces the same results and consistency as fungicides (Papoutsis *et al*., 2019).

Biological Control

Yeasts, bacteria and some fungi are microorganisms that have been studied and used as biological control agents of plant diseases. Biocontrol products such as Shemer® (based on *Metschnikowia fructicola* Kurtzman and Droby and Candifruit® (based on *Candida sake* Saito and Ota) have been formulated and registered to protect various fruits from postharvest diseases (Sparado and Droby, 2016).

Bacteria such as *Bacillus* spp. have been extensively researched and studied for their biocontrol properties (Guo *et al*., 2021). *Bacillus amyloliquefaciens* is an aerobic, spore-forming bacterium that is often found around plant roots (Wang *et al*., 2020; Liu *et al*., 2023). Various strains of *B. amyloliquefaciens* have been used as biocontrol agents and have proven to inhibit

disease decay and extend the shelf life of fruit (Guo *et al*., 2021). This species has been regarded as having potential biocontrol agents because of its antifungal capabilities and induction of systemic resistance (Dhumal *et al*., 2021). Bacterial biocontrol agents such as *B. subtilis*, *Burkholderia gladioli* pv. *agaricicola* and *Streptomyces* spp. are currently used for managing citrus diseases (Chen *et al*., 2020). *Bacillus amyloliquefaciens* is a generally regarded as a safe bacterium, as defined by European Food Safety Authority (EFSA) (Dhumal *et al.*, 2021). Chen *et al* (2018) also reported that *B. amyloliquefaciens* DH-4 significantly inhibited the growth of *P. digitatum* and *P. italicum* pathogens on citrus. This study focused on the isolation of yeast and bacteria strains from citrus fruit and leaves, followed by screening *in vitro* and *in vivo* for biocontrol activity against the two major citrus post-harvest fungi.

Research aims and objectives

The aim of this study was to find microbes that provide biocontrol of *Penicillium* species in postharvest citrus. This study focused on the specific objectives:

i. To investigate the efficacy of yeasts and bacteria for the biological control of *Penicillium* moulds in postharvest citrus by isolating novel yeast and bacterial strains from citrus fruit and leaves, and to screen them for efficacy against *P. digitatum.*
ii. To identify and optimize the best yeast or bacterial strains as biological control agents to prevent subsequent postharvest infections
iii. To evaluate the best yeasts and bacteria versus Yeast B13 *in vivo* versus both *Penicillium* species of interest;
iv. To identify the best strains; To use microscopy and biochemistry to determine their mode of action.

Dissertation structure

Chapter 1 is the Introduction of the dissertation and its goals; Chapter 2 is a literature review, focusing on citrus, its production and information on postharvest diseases of citrus; Chapter 3 focuses on the isolation of antagonistic yeasts and bacteria from citrus fruit and leaves, and then tested them for any inhibitory effects against *P. digitatum in vitro* and *in vivo*; Chapter 4 evaluates the efficacy of the antagonistic yeasts and bacteria in comparison with a previously isolated yeast, Strain B13; Chapter 5 is a summary of the major findings of the study and their implications.

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Chapter 1

Literature review

1.1.Introduction

1.1.1. The Crop: Citrus

Citrus fruit (genus *Citrus*; family *Rutaceae*) is grown in more than 100 countries (Ismail and Zhang, 2004). Citrus fruit such as oranges, grapefruit, lime, lemons and tangerines/mandarin, are not only delicious but are a hub of bioactive substances such as vitamin C, flavonoids, and hydroxycinnamic acids (Strano *et al*., 2017). Grapefruits and oranges contain flavonoids that regulate blood circulation which improves heart health (Ladaniya, 2008). Citrus is grown in mild temperature and frost-free winters as the trees are subtropical and the fruit is climacteric (DOA, 2003). In comparison to other tropical fruits, citrus have a long shelf life which is critical as they are exported from countries all over the continent. Oranges have the highest production of citrus fruits, with the global orange production for 2021/22 estimated up to 48.8 million tons (Talibi *et al*., 2014; USDA, 2022)

South Africa is a major player in the export market, with citrus accounting for 27% of total agricultural exports and ranking at number 12 in total global citrus production (Symington *et al*., 2004; FAO, 2020). Citrus was introduced to South Africa by Jan van Riebeek in 1652 and exports began in 1902 (Stanbury, 1996). In South Africa, citrus is grown in several provinces namely, Limpopo, Eastern Cape, Western Cape, Mpumalanga, KwaZulu Natal, Northern Cape, and North-West provinces (GAIN, 2020). Limpopo is the largest citrus-producing province in South Africa attributing for 42% of the area planted (GAIN, 2020). The most popular citrus fruit produced in SA is oranges, attributing to half of the total citrus area planted (GAIN, 2020). South Africa has four citrus types namely, grapefruit, oranges, mandarins/tangerines, and lemons/lime with different varieties that have different harvest periods. South Africa exports soft citrus to numerous countries, mainly to Europe (32%), Asia/Southeast Asia (23%), Middle East (21%) (Weare, 2020). The EU receives high quality produce due to the restrictions laid by the European Commission as exporters must comply with the food safety protocols and requirements (Potelwa, 2017). EU citrus exports are very important to South Africa's economy and reliability (Ndou, 2012). It is therefore imperative to have disease management solutions that are not only effective in control of diseases, but also acceptable to the EU commission.

1.1.2. Postharvest Diseases of Citrus

Citrus is vulnerable to many diseases caused by wound and latent pathogens due to their high nutrient composition and high-water content (Tripathi and Dubey, 2003). Underdeveloped countries experience greater loss of fresh fruit, where half of the loss is due to post-harvest diseases (Wisiniewksi, 1989; Ladaniya, 2008). Postharvest bacterial pathogens are not as significant as fungi due the high acidity of citrus fruit which is not favourable for the growth of bacteria (Talibi *et al*., 2014). Latent pathogens of economic importance include Anthracnose rot (*Colletotrichum gloeosporioides* (Penz) Penz and Sacc), Alternaria rot (*Alternaria citri* Ellis and Pierce), Aspergillus rot (*Aspergillus niger* van Tieghem), Diplodia stem-end rot (*Botryodiplodia theobromae* Patouillard) and Rhizopus rot (*Rhizopus oryzae* Went et Prinsen Geerligs) (Ladaniya, 2008; Talibi *et al*., 2014). The most devasting wound pathogens are *Penicillium digitatum* (Pers) Sacc, *Penicillium italicum* Wehm., and *Geotrichum citri-aurantii* (Ferraris) and Trichoderma brown rot (*Trichoderma* spp.) (Regnier *et al.*, 2014).

1.2.Penicillium Moulds

1.2.1. Green mould disease

1.2.1.1. Classification and taxonomy

Penicillium is a large genus of microscopic fungi and is important with approximately 400 species that are distributed worldwide (Ladaniya, 2008). Ninety percent of the total loss in postharvest citrus is due to green mould disease (Zhu *et al*., 2017). *Penicillium digitatum* (Pers) Sacc is a species classified as Fungi, division Ascomycota, subdivision Pezizomycotina, class Eurotiomycetes, subclass Eurotiomycetidae, order Eurotiales, family Trichocomaceae, and genus *Penicillium*. *Penicillium digitatum* causes the most devastation for citrus postharvest, infecting all citrus fruits in all citrus-producing areas in the world (Frisvad and Samson, 2004). Colonies appear olive green and become dull brown as they mature on growing media (Palou, 2014). *Penicillium digitatum* reproduces asexually by producing conidia, which are smooth and cylindrical and vary in size between 3.5-8.0 × 3.0 – 4.0 µm (Palou, 2014).

1.2.1.2. Disease Cycle and Epidemiology

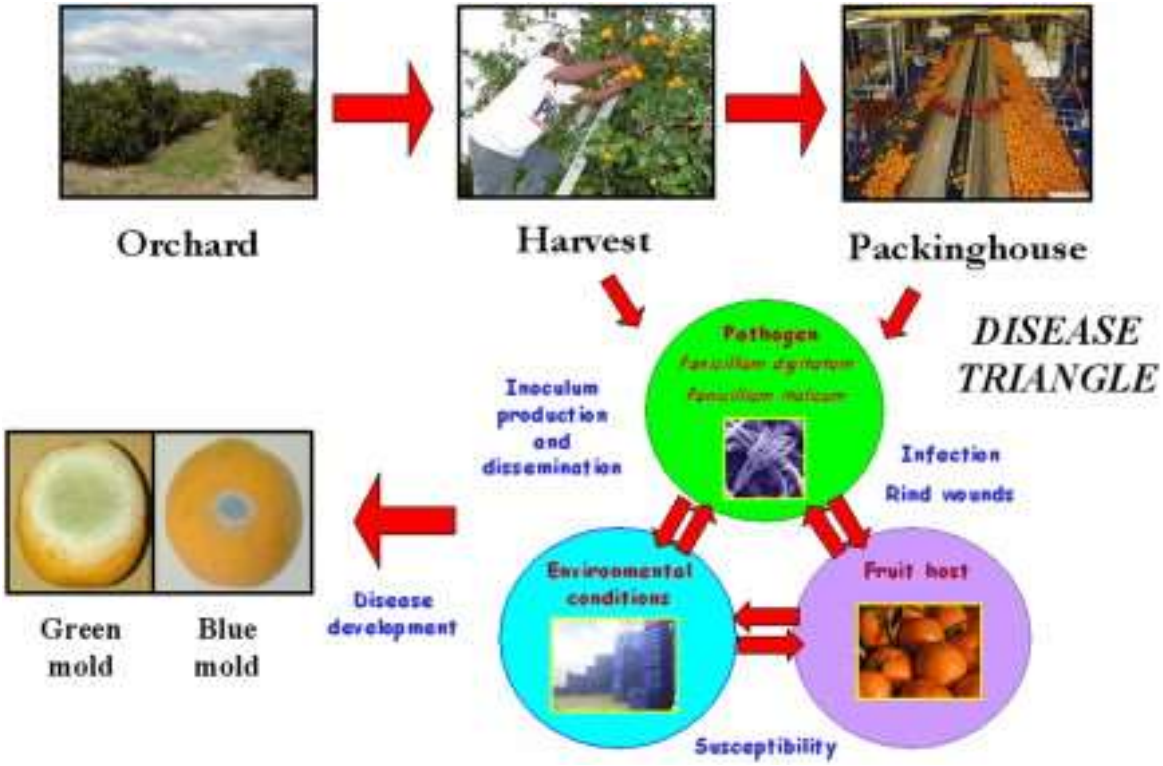

Figure 1.1: Disease cycle of *Penicillium* moulds (Palou, 2014).

Penicillium digitatum is a fungus that sporulates on wounds that result from injury during harvesting or caused by insects (Eckert and Eaks 1989). This fungus has a short disease cycle, about three to five days, approximately two billion conidia production which are transported through wind currents to healthy fruits (Holmes and Eckert 1999). Figure 1.1 indicates the occurrence of wounds during harvesting, packing, transportation, and damage from insects. Fungal spores are carried by the wind from diseased fruit on the orchard floor to contaminate healthy fruit (Soma, 2020). Sporulation starts after three to five days if temperatures are between 15 and 28 ℃, after seven to eight days the centre of the lesions become olive green and are surrounded by a thick layer of non-sporulating white mycelium (Palou, 2014).

1.2.1.3. Host Range and Symptomology

Green mould disease affects numerous fruit namely, papaya, maize, beet, melon, apple, and goldenberry (Farr and Rossman, 2013). *Penicillium digitatum* causes green mould which results in softening of tissue and fruit decay (Chen *et al*., 2016). Citrus fruit are susceptible to *P. digitatum*, which appears as a mass of green conidia that appears on the surface of infected fruit as displayed in Figure 1.2 (Abraham *et al.*, 2010; Chen *et al*., 2016).

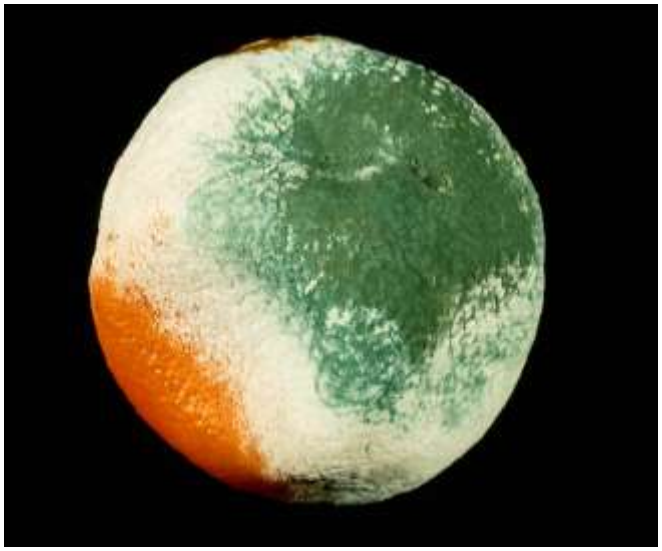

Figure 1.2: Orange displaying green mould disease symptoms

Credit: Nigel Cattlin- Nature Picture Library

1.2.2. Blue Mould Disease

1.2.2.1. Classification and Morphology

Penicillium italicum Wehm., is a fungus that causes blue mould disease which is under-investigated compared to green mould disease (Wehmer, 1894; Kanashiro *et al*., 2020). The classification of *P. italicum* is identical to that of *P. digitatum*. Conidia produces by this fungus are that are cylindrical/ovate in a string-like chain form (Eckert, 1987; Kanashiro *et al*., 2020). The conidia are about 4.0-5.0 × 2.5-3.5 µm in size, smooth, and bluish-greenish (Palou, 2014).

1.2.2.2. Disease Cycle and Epidemiology

The pathogens disease cycle is as displayed in Figure 1.1 although *P. italicum* is less aggressive and develops slower compared to *P. digitatum*. However, it is more resistant to cold temperatures and low water availability (Plaza *et al*., 2003; Kanshiro *et al*., 2020). Infection occurs only if there is a presence of wounds, which is essential for germination to occur (Talibi *et al*., 2014). Temperatures between 20-25℃ are suitable for optimum disease development for this pathogen and symptoms can be observed visually on the fruit (Papoutsis *et al*., 2019).

1.2.2.3. Host Range and Symptomology

Hosts range for this fungus includes avocado (*Persia americana*), sweet potato (*Ipomoea batatas*), melon (*Cucumis melo*), persimmon (*Diospyros kaki*), and mango (*Mangifera indica*) (Farr and Rossman, 2013). It has been concluded that citrus fruit is the primary host for *P. italicum* as it does not cause any economically important diseases in other host plants (Frisvad and Samson, 2004). Infected fruit becomes tender and water-soaked in the infected area due to

the enzymes produced by the fungus such as polygalacturonase and glucosidase that are accountable for the maceration of tissue (Barkai-Golan and Karadavid, 1991; Louw and Korsten, 2015). Initially, the fruit surface is covered with a sheet of white mycelium which grows deeper into the fruit, which sporulates into blue conidia as displayed in Figure 1.3 (Kanashiro *et al*., 2020).

Figure 1.3: Lemon fruit cv. showing powdery blue symptoms of blue mould (*Penicillium italicum*)

Credit: Gerald Holms, Strawberry Center, Cal Poly San Luis Obispo.

1.3. Control Strategies

1.3.1. Sanitation Practices

Infected fruit carries a large number of conidia which are transported by air currents onto neighbouring fruit trees (Abraham *et al*., 2010). Packhouses use sterilizers such as chlorine to sterilize the fruit surface (Rouissi *et al*., 2009). During harvesting and transportation to packhouses, it is vital to ensure that the flavedo of the fruit is not wounded as this will most likely result in infection of mould diseases (Abraham *et al*, 2010). It is important to sterilize equipment, tools, and surfaces to prevent infection of healthy fruit as fungal inoculum is constantly present during the season and after harvest (Kanetis *et al*., 2007).

1.3.2. Physical Control

Physical treatments provide control of postharvest decay and leave no residues or harmful substances that are harmful to the environment or pose a threat to human health (Palou *et al*., 2008).

1.3.2.1. Heat Treatments

Heat is an effective physical treatment that can protect the fruit from postharvest diseases by inducing physiological changes to accomplish a significant level of control (Ladaniya, 2008). However, heat treatments alone are impractical in large packhouses and are often combined with other methods of disease management (Montesinos-Herrero and Palou, 2010). Treating fruit with heat can result in fruit damage, hence the time fruit is exposed to heat is relatively short (Montesinos-Herrero and Palou, 2010). Fresh fruit is treated after harvesting by hot water dipping, hot water brushing, rinsing, and hot vapour (Terry and Joyce, 2004; Palou *et al*., 2008). The direct mode of action of heat on *P. italicum* and *P. digitatum* is the inhibition of mycelial growth and spore germination (Palou *et al*., 2008). Exposure to high temperatures interrupts spore growth of fungi up to 48 hrs (Ladaniya, 2008). The indirect mode of action of heat treatment is the induction of resistance that results from heat shock which triggers lignin formation, production of phytoalexins and the production of reactive oxygen species (ROS) against *P. digitatum* (Ben-Yehoshua *et al*., 1998; Schirra *et al*., 2000; Ladaniya, 2008). Hot water dips of fruits for 150 s at temperatures 50 to 55 ℃ reduced the disease severity of oranges and mandarins inoculated with *P. digitatum* (Palou, 2013).

Fruits are thermally cured by being exposed to temperatures higher than 30℃ for 2-5 days and relative humidity higher than 90% which has been reported to control green mould on citrus species (Hopkins and Loucks, 1948; Palou *et al*., 2008; Palou, 2013). Phenolic compounds and

lignin develop as a reaction of curing, which increases the plants defence system as these compounds promote self-healing of injuries by the fruit (Ismail and Zhang, 2004).

1.3.2.2. Irradiation treatments

Irradiation treatments such as exposing the fruit to UV-C illumination (100-280 nm wavelength) or gamma radiation have proven to be an efficient method of postharvest treatment of citrus and has been used as early as 1978 (Ladaniya, 2008). Reports on UV-C illumination between 1.6 to 8 kj/m^2 on grapefruit picked from November to February, triggers the plant's defence system and induces resistance (Droby *et al*., 1993). Stevens *et al* (1997) undertook a study which revealed that the use of ultraviolet light at 254 nm-UVC combined with treatment of an antagonistic yeast, *Debaryomyces hansenii*, proved to control green mould disease.

1.3.2.3. Surface wax and coatings

Citrus fruits are transported for long periods and they reach consumers in good condition for purchase. Surface waxes and coatings are used to maintain the fruit quality and extend the shelf life of the fruit (Njobolwana *et al*., 2013). These waxes and coatings also protect the fruit peel from being wounded easily during transportation, and protect fruits against postharvest physiological disorders (Dou et al., 2001). Surface wax coatings can be synthetic, such as polyethylene coating or can be natural, such as carnauba wax coating which is a natural vegetable wax (Njombolwana *et al*., 2013). Polysaccharide-based coatings such as Chitosan, which is a de-acetlylated form of chitin, which is used on citrus and can increase volatile compounds which improve the taste of fruit (Arnon *et al*., 2015; Kassim *et al*., 2020).

1.3.3. Chemical control

Fruits are produced, transported to consumers in different continents, where consumers expect fresh produce. The use of fungicides has been the main method to control fungal pathogens due to their reliability and affordability. Chemicals are easy to apply whether fruits are sprayed, drenched, or dipped. Fungicides also provide preventative and curative action. Different countries have different restrictions regarding fungicides as they have been proven to be toxic to human health and the environment (Tripathi and Dubey, 2003; Palou *et al*., 2008). Table 1.1 shows the list of fungicides currently being used in citrus packhouses.

1.3.3.1. Current fungicides used in the South African citrus industry

Table 1.1: Fungicide active ingredients used in packhouses

Fungicide Active Ingredient	**References**
Sodium Ortho-Phenyl-Phenol (SOPP)	Harding (1962); Talibi *et al* (2014)
Thiabendazole	Harding (1972); Talibi *et al* (2014)
Guazatine (GZT)	Wild (1983); Brown (1988)
Imazalil	Bus *et al*. (1991); Kinay *et al*. (2007); Kanetis *et al*. (2008); Erasmus *et al*. (2015)
Pyrimenthanil (PYR)	Talibi *et al* (2014); Erasmus *et al*. (2015); Government Gazette (2020)
Propiconazole (PPZ)	Erasmus *et al*. (2015); Government Gazette (2020)
Fludioxonil (FLU)	Talibi *et al* (2014); Erasmus *et al*. (2015); Government Gazette (2020)

1.3.3.2. Resistance to Fungicides by *Penicillium* species.

The constant use of fungicide has resulted in resistant strains of *P. digitatum* and *P. italicum*, furthermore, fungicide residues can cause risks that affect human health. The public concern for residues on fruit has increased over the years, applying more pressure on the food production industry to develop efficient, practical, and affordable alternatives for disease control (Auret, 2007). Citrus packhouses in California experienced resistance to widely used fungicides namely, IMZ, TBZ, and SOPP (Eckert 1987, 1990). Resistance to conventional fungicides results in the use of alternative fungicides with different modes of action such as FLU and PYR, however, resistance to the alternative fungicides has also been reported in California packhouses (Kanetis *et al*., 2008). Alternative solutions such as integrating fungicides with bicarbonate of soda ($NaHCO_3$) and heating fungicide solutions have been used in an attempt to restore their efficacy (Smilanick *et al*., 2005; Kinay *et al*., 2007).

1.3.3.3. Fungicide Restrictions in South Africa (SA) and Globally

Fungicides must be registered and used according to the dosage regulations required by commissions and unions of each country. The European Union (EU) has been responsible for controlling pesticide residues since 1996 through the European Food Safety Authority and the Standing Committee on the Food Chain and Animal Health (Mutengwe *et al*., 2016). In 2011 the EU regulators current list of permitted agrochemicals approved Imazalil® for 10 years until 2021 with a maximum residue level (MRL) of 5 mg kg^{-1} (Palou, 2014). Propiconazole was discontinued by EU commission regulation (EU) 2018/1865. Prochloraz® and guazatine were withdrawn from the list of active ingredients allowed in pesticides in 2012 by the EU. SOPP is on an extension by EU regulators in 2009 (Palou, 2014). Citrus packhouses in California experienced resistance to widely used fungicides namely, IMZ, TBZ, and SOPP against *G. citri* and *P. digitatum* (Eckert 1987, 1990).

In SA, the Perishable Product Export Control Board (PPECB) which is an outlet of the Department of Agriculture, Forestry and Fisheries (DAFF) is responsible for sampling for pesticide residue analysis (De Beer *et al*., 2003). Unfortunately, the DAFF has not developed a pesticide monitoring program as yet, and there are no annual reports on pesticide residue levels for individual food commodities (Mutengwe *et al*., 2016). In a recent study on fungicide residues in SA, conducted from 2009 to 2014, it was reported that Imazalil® is the most frequently found pesticide that exceeds the MRLs, which is expected as it is widely used for pre-and post-harvest treatment of fruit, vegetables, and ornamentals (Mutengwe *et al*., 2016).

1.3.4. Biological control

The development of a sustainable, affordable, and efficient alternative to replace toxic fungicides that are currently being used is the citrus industry is a necessity. It is important to find alternatives that are non-toxic so the fruit can be imported and exported more freely due to differences in fungicide regulations and restrictions. Bacteria, fungi, and viruses are microorganisms that can control plant diseases using their different modes of action (Kohl *et al*., 2019). Therefore, the use of naturally occurring microorganisms is regarded as the simplest and best option as an alternative for disease management (Pererya *et al*., 2021). Currently, only four bio-fungicides are commercially registered for the control of Penicillium moulds namely; Aspire® (*Candida oleophila* I-182), Shemer® (*Metschnikowia fructicola* Strain NRRL Y-27328), Pantovital® (*Pantoea agglomerans* Strain CPA-2) and Bio-Save10 LP® (*Pseudomonas syringae* Strain ESC-10) (Bazioli *et al*., 2019).

1.3.4.1.1. Current biological control agents.

Several studies have been conducted to investigate the effect of potential biological control agents as seen in Table 1.2.

Table 1.2: Biological control agents reported to control citrus postharvest diseases

Microorganism group	**Antagonist**	**Pathogen(s)**	**Reference(s)**
Yeast	*Aureobasidium pullulans*	*P. digitatum/P. italicum*	Chalutz and Wilson (1990)
	C. fermentati	*P. digitatum*	Abraha *et al*., (2010)
	C. guilliermondii	*P. digitatum*	McGuire (1994); Arras (1996)
	C. oleophila	*P. digitatum*	Droby *et al*., (2002)
	C. saitoana	*P. digitatum*	El-Ghaouth *et al*. (2000)
	C. sake	*P. digitatum/P.italicum*	Arras *et al*. (1998); Droby *et al*. (1999), Abadias *et al*. (2003)
	Clavispora lusitaniae.	*P. digitatum*	Perez *et al*. (2016); Pereyra *et al*. (2021)
	Cryptococcus laurentii	*P. digitatum*	Mekbib *et al*. (2011)
	Debaryomyces hansenii	*P. digitatum/P. italicum/G. citri-aurantii*	Chalutz and Wilson (1990); Cheah and Tran (1995)
	Kluyveromyces sp.	*P. digitatum*	Cheah and Tran (1995)
	Metschnikowia spp.	*Alternaria* spp., *Aspergillus* spp., *B. cinerea*, *Fusarium* spp. *Penicillium* spp.	Lui *et al*. (2019)

	Pichia guilliermondii	*P. digitatum/P. italicum*	Droby *et al.* (1997)
	Saccharomyces cerevisiae	*P. digitatum*	Cheah and Tran (1995)
	Rhodotorula minuta	*G. citri-aurantii*	Moraes-Bazioli *et al.* (2019)
Bacteria	*Bacillus amyloliquefaciens*	*P. digitatum, P. digitatum, G. citri-aurantii*	Parisa *et al* (2017), Dunlap (2019)
	B. pumilus	*P. digitatum*	Moraes-Bazioli *et al.* (2019)
	B. subtilis	*Alternaria citri, Geotrichum citri-aurantii, P. digitatum, Botrytis cinerea, Colletotrichum gloeosporioides, P. italicum*	Singh and Deverall (1984), Mohammadipour *et al.*, 2009)
	Streptomyces sp	*P. digitatum*	Moraes-Bazioli *et al.* (2019)
Fungi	*Myrothecium roridum*	*P. digitatum*	Appel *et al*. (1988)
	Debaryomyces hansenii	*P. digitatum*	Stevens *et al.* (1997)
	M. verrucaria	*P. digitatum*	Appel *et al*. (1988)
	Paecilomyces lilacinus	*P. digitatum, P. italicum*	Wang *et al*. (1996)
	Pseudomonas cepacia	*P. digitatum, P. italicum*	Smilanick and Denis-Arrue, (1982); Wilson and Chalutz (1989); Huang *et al*., (1991)
	P. corrugate	*P. digitatum*	Smilanick and Denis-Arrue, (1992)
	P. fluorescens	*P. digitatum*	Smilanick and Denis-Arrue, (1992); Wang *et al*. (2018)

	P. glathei	*P. digitatum*	Huang *et al*. (1995)
	P. syringae	*P. digitatum, P. italicum*	Auret (2007)
	Trichoderma viride	*G. citri-aurantii, P. digitatum*	De Matos (1984)

1.3.4.2.Yeasts as Biocontrol Agents

Bacterial and yeasts agents are both interesting microorganisms that are at the centre of biocontrol research. Yeasts are found in large populations in the rhizosphere, and they can grow well in adverse conditions (Liu *et al*., 2013; Zaic *et al*., 2020). The efficacy of a yeast BCA relies on the concentrations of the antagonist and the amount of inoculum of a pathogen (Thomé *et al*., 2020). The advantage of using yeasts over bacterial agents is that yeasts do not produce allergenic spores and are considered safe for humans (Zhimo *et al*., 2014). Abraha *et al* (2010) reported that in several screenings, yeast isolates had higher efficacy in the control of *P. digitatum* in comparison to *Bacillus* species.

1.3.4.2.1. Modes of Action

(i) Competition for Space and Nutrients

Fungal pathogens and antagonistic yeasts both need nutrients and to occupy space so they can colonize and grow. Antagonistic yeasts colonize the surface of wounded fruit and use up nutrients so that fungal spores are deprived of nutrients and do not germinate (Li *et al*., 2008; Zhang *et al*., 2020). Cunha *et al.* (2020) reported the efficacy of two Strains of *C. laurentii* (MeJtW 10-2 and TiC 4-2) and *C. sake* (TiL 4-3) in managing *P. digitatum* by colonizing the fruit surface and colonies, suggesting that competition for nutrients is one of the mechanisms of action used by the yeasts. The main nutrients necessary for the growth of microorganisms are carbon, nitrogen, and iron ions. The lack of nitrogen can cause inhibition of the fungal pathogen as fruit have limited amino acids (Zhang *et al.*, 2020). Iron competition is an important mode of action as iron plays an important role in the virulence and growth of fungal pathogens (Sparado and Droby, 2016; Dukare *et al.*, 2018). Some yeasts inhibit the germination of fungi by producing siderophores to compete for iron. *Metschnikowia* spp. antagonise numerous fungal species by consuming all the iron (Liu *et al*., 2019). Yeasts adhere to pathogen cell walls or host tissues, developing biofilms which are dense microbial clusters (Scherm *et al*., 2003; Corsta-Orlandi *et al.*, 2017). Competition for nutrients is the most assumed mode of action, although least proven, as it is often identified due to the lack of evidence of other mechanisms of action.

(ii) Mycoparasitism

Antagonistic yeasts parasitize other fungi by attaching to the hyphae of fungal pathogens and releasing cell degrading enzymes which lyse fungal structures. Enzymes such as chitinases, proteases, and glucanases are responsible for the antagonistic activity of yeasts. *Metschnikowia*

spp. has been reported to secrete lytic enzymes that parasitize *Penicillium* spp. (Liu *et al*., 2019).

(iii)Induction of host resistance

Plants, like all living organisms, have an immune system and therefore are alerted if infection occurs, where plants can identify and respond to invasive microorganisms (Liu et al., 2013). The application of yeasts to induce host defence mechanisms has been widely studied and reported. Antagonistic yeasts promote and enhance the defense-related genes of hosts which results in the enhancement of defence-related enzymes (Zhang *et al*., 2020). Defence related enzymes such as chitinase, glucanase, phenylalanine ammonia-lyase, and peroxidase can be stimulated by the application of yeasts.

(iv)Production of Killer Toxins and Volatile Organic Compounds

Antagonistic yeasts produce volatile organic compounds (VOCs), which are small and have a low molecular weight (<300 Da) (Contarino *et al*., 2009). The growth and infection of fungal pathogens such as *B. cinerea, Colletotrichum acutatum,* and *Penicillium* spp. are reported to be inhibited by two strains of *Aureobasidium pullulans in vitro* due to the production of killer toxins (Di Francesco *et al*., 2015; Zhang *et al*., 2020). Di Francesco *et al*. (2015) reported 100% inhibition of conidia germination of *Penicillium* spp. by VOCs produced by *A. pullulans* in laboratory conditions, and inhibited the pathogens activity by 96% *in vivo* on oranges.

1.3.4.2.2. Isolation

Antagonistic microorganisms can be isolated from leaf and fruit surfaces as they colonize surfaces rapidly (Liu *et al*., 2013). Yeasts are isolated selectively by using the water from washing harvested fruit and pipetting the solution onto yeast selective agar and allowing yeasts to grow and eventually streak onto potato dextrose agar and form colonies (Wilson *et al*, 1993; Abraham *et al*., 2010). Whole fruits or fruit peels can be dipped whole into sterile water and shaken in a water bath for an hour, the washing water can be pipetted onto nutrient yeast dextrose agar (NYDA), yeast extract peptone dextrose (YEPD) (Chalutz and Wilson, 1990; Abraham *et al*., 2010). Fruits displaying minimal or no symptoms are used to isolate colonies from wounds onto nutrient agar plates, where colonies can be differentiated as yeasts or bacteria (Chalutz and Wilson, 1993; Pereyra *et al*., 2021).

1.3.4.2.3. Formulation for application to fruit

The formulation and application of biological control agents (BCAs) play an important role in their success in the commercial market. BCAs must be compatible with other disease management protocols used in the packhouse. McGuire (1994) reported that mixing coatings that are based on derivatives of chitin, cellulose and protein with antagonists can be an effective method of application. Bio-fungicide products containing yeasts are often powder formulations that need to be mixed with water for application. Bio-fungicides can be applied using hand-held equipment, drench/dip tanks, or in-line/nonrecovery sprays. Nexy® is a bio fungicide with the active ingredient *C. oleophila* (strain O). This product is applied by mixing with water and shaking the container throughout the application to achieve the best result (USEPA, 2000). This product must be applied on fruits before waxing and can be combined with imazalil and thiabendazole at rates of 50-550 ppm to improve decay control (USEPA, 2000).

1.3.4.3.4. Commercialization Challenges

Microorganisms and naturally occurring compounds are labelled as bio-fungicides once they are formulated for commercial use. Microorganisms are the main focus of research, whereas bio-fungicides have minimal progression in business and commercially in comparison to chemical fungicides. The main setback in the commercialization of bio-fungicides is the shelf-life stability and effectivity of the microorganism over time (Bazioli *et al*., 2019). The lack of field application reliability is a major stumbling block for the marketing of bio-fungicides (Bazioli *et al*., 2019). Yeasts are favoured due to their abilities and availabilities in the ecosystem; however, the process of commercializing bio-fungicides is extensive (Friemoser et al., 2019). The commercialization of a biocontrol agent depends on (i) suitability and effectiveness of a strain, (ii) the marketing of the product as it contains live organisms, (iii) the period of efficacy of the BCA, (iv) the strain is potent at low concentrations and (v) shelf-life (Hernández-Fernández *et al*., 2021).

The cost of production of bio-fungicides is often high which is a disadvantage as synthetic fungicides are relatively cheaper. Aspire® (active ingredient, *C. oleophila*) has been on the market for several years, however, it has faced setbacks due to inefficacy in field conditions making it difficult for market penetration (Sparado and Droby, 2016; Liu *et al*., 2019). In the EU, biopesticides are regulated as plant protection products under Regulation (EC) 1107/2009 of the European Parliament and of the Council responsible for placing plant protection products on the market. In comparison to the United-states, Brazil, or India, the EU has less biopesticides

and biofertilizers registered due to the complex and extensive protocol involved in registration (Damalas and Koutroubas, 2018; Hernández-Fernández *et al*., 2021). Other commercialized products based on yeasts include; Shemer® (*Metschnikowia fructicola*, Bayer, Germany) and Boni Protect® (*Aureobasidium pullulans*, Bio-protect, Germany) (Yánez-Mendizábal *et al*. 2012).

1.3.4.3.Bacteria as Biocontrol agents

Numerous bacteria strains have been used as biocontrol agents to inhibit pathogens causing postharvest diseases. Bacterial biocontrol agents including *B. subtlis*, *B. gladioli pv. agaricicola*, *Streptomyces spp* and *Paenibacillus brasilensis* are used by the citrus industry in integrated disease control (Yánez-Mendizábal *et al*., 2011; Chen *et al*., 2020). *Bacillus* species have been researched intensively over the years and they make promising biocontrol agents because of their high antifungal properties, and they occur naturally in the rhizosphere (Chen *et al*., 2016). Commercially, *Bacillus* spp are suitable for production because they maintain viability in long term storage (Pretorius *et al*., 2015; Dhumal *et al*., 2021). Mohammedi *et al*. (2017) reported that *B. subtilis* and *Agrobacterium radiobacter* can be used to inhibit the mycelial growth of *P. digitatum* and can be used as a biocontrol agent against the pathogen. Melon (*Cucumis melo* L.) was treated with *B. subtilis* Strain EXWB1 resulting in 77.2% inhibition of *Alternaria alternata* (Wang *et al.*, 2010). Mari *et al*. (1996) reported that *B. pumilus* and *B. amyloliquefaciens* inhibited the growth of grey mould disease caused by *B. cinerea* on tomatoes (*Solanum lycopersicum)* and pears (*Pyrus communis*). A few bacterial agents have been formulated and commercialized to manage postharvest diseases, products such as Bio-Save 10® (*Pseudomonas syringae* ESC-10, Jet harvest solutions, USA) (Yánez-Mendizábal *et al*. 2012).

1.3.4.3.1 Modes of Action

(i) Production of antimicrobial substances

A variety of *Bacillus* species produce antifungal lipopeptides grouped into three categories; surfactins, fengcins and iturins (Zeriouh *et al.*, 2011).; Guo *et al*., 2021). Lipopeptides (LPs) have been heavily investigated and studied because of low toxicity, antimicrobial activity and stability at high temperatures (Mandal *et al*., 2013). Surfactins are amphiphilic, with polar amino acid and a hydrocarbon chain, these substances have antifungal and antibacterial properties that have been reported to have effects on fungi such as *Fusarium* species and *Rhizoctonia solani* (Kumar *et al*., 2012). Arrebola *et al*., (2010). Investigated the use of *B. amyloliquefaciens* Strain PPCB044, which produced iturin lipopeptides that displayed

antagonism against *P. digitatum* on different citrus fruits. Ntushelo *et al*. (2019). discovered that iturin A killed conidia of a *Fusarium* sp. at its minimal inhibitory concentration of 50 µg/mL. Fengycins display antifungal properties and induce systemic resistance in plants (Chen *et al*., 2020), for example *B. subtilis* NCD-2 produced fengycin-type LPs reduced the population of *Rhizoctonia solani* in soil growing cotton plants (Guo *et al*., 2014). The pH/temperature stability and biodegradable nature of these bacteria make them ideal for development in agriculture and food production industries (Rodrigues *et al*., 2006).

(ii) Competition for Nutrients and Space

Wound pathogens must colonize wounds for infection to be successful and for the disease to spread. The efficacy of biocontrol agent is dependent on its ability to colonize the wound site on fruit surfaces effectively more than/before pathogens can survive in unfavourable conditions which is dependent on the initial concentration (Droby *et al*., 1992). El-Ghaouth *et al*. (2004) reported that the concentration that is most effective in controlling postharvest pathogens is 10^7-10^8 CFU/ml. Bacterial antagonists are said to take up nutrients faster than pathogens *in vitro*, which supresses spore germination of the pathogen (Droby and Chalutz., 1989; Lastochkina *et al*., 2019).

(iii) Induction of Systemic Resistance in Host Plants

The induction of systemic resistance (ISR) is an indirect mechanism of action, which is through the synthesis of metabolites with antifungal activity and defence response mechanisms. ISR can promote the activity of pathogenesis-related proteins such as phenylalanine ammonia-lyase (PAL) in plants (Chen *et al*., 2020). Plant growth promotion (PGP) is not a direct mechanism of action, but the impact of promoting plant growth improves the plants' defence system. PGP hormones gibberellic acid (GA3) and indole-3-acetic acid (IAA) influence nutrient availability (Shafi *et al*., 2017). Many reports of *B. subtilis* promoting plant growth by decreasing ethylene production and improving nutrient uptake (Harman, 2011; Lastochkina, 2019; Fonseca *et al*., 2022). *Bacillus* also produce volatile organic compounds (VOCs) like yeasts, which are antimicrobial substances. Gao *et al*., (2017) reported that *Bacillus velezensis* produce VOCs that significantly controlled the growth of *Botrytis cinerea* and *Alternaria solani. et al.* (2010) reported that 11 of 36 VOCs produced by *B. amyloliquefaciens* can inhibit fungal growth of *Fusarium oxysporum* f. sp. *niveum*. ISR caused by endophytic bacteria is efficient in controlling pathogens in storage conditions (Lastochkina *et al*., 2019). Tunsagool *et al.* (2019)

reported the effects of fengycin, iturin A and surfactin applied on mandarin fruits, initiated the production of defensive proteins that resulted in the limitation of green mould disease spread.

1.3.4.3.2. Commercialization of *Bacillus* spp Based Biocontrol Products

Bacillus subtilis strain B-3 was the first microorganism that was commercialized as a biocontrol product for postharvest control of *Monilinia fructicola* causing brown rot of peach (Pusey and Wilson, 1984). *Bacillus subtills* B-3 strain was incorporated into wax used in three pack house lines: packing lines in Byron (Georgia), Clemson (South Carolina) and commercial packhouse in Musella (Georgia). Many bacteria have been used as potential biocontrol agents in laboratory and field conditions (Pusey *et al*., 1988; Leelasuphakul *et al*., 2008; Tian *et al*.., 2020; Hammami *et al*., 2022). Serenade® (AgraQuest) based on the *B. subtilis* strain QST 713 is registered in the USA, for control of *Fusarium, Pythium* and *Phytophthora* genera on postharvest fruit and vegetable (Anon, 2010).

1.4. Integrated control

All methods of disease management strategies mentioned in this literature review have their downfalls. Safety demands increase for citrus exports requiring lower fungicide use and residues, while the expectation of receiving quality fruit remains. The use of biocontrol-based products provides control over *Penicillium* mould diseases, although the longevity of the efficacy these products has not provided reliable control in the past years. The use of physical control of blue and green mould alone is not efficient if infection has already occurred. However, the integration of all control methods has proven to be the most efficient method of disease management (Zhang *et al*., 2019; Ons *et al*., 2020). Scouting and removal of diseased fruit before harvest reduces the inoculum and therefore reduces the rate of disease spread (Berrie, 2019). Preharvest application of a thiophanate methyl (TPM) has shown control of green mould when applied one week before harvest (Zhang and Timmer, 2007). Combining generally regarded as safe (GRAS) chemicals with low toxicity with non-toxic alternatives such as bio-fungicides, hot water treatments, UV-radiation and synthetic elicitors can manage green and blue mould diseases on citrus (Janisiewicz and Korsten, 2002). Obagwu and Korsten (2003) reported the increase in control of green and blue moulds of citrus, when *B. subtilis* spp. were applied combined with sodium bicarbonate and hot water treatment (45 °C), resulting in 100% disease control on ‘Valencia’ and ‘Shamouti’ fruits.

1.5.Conclusion

Blue and green mould are among the most important postharvest diseases affecting the quality and yield of citrus fruit. The demand for safer, less toxic produce has been continuously expanding, calling on environmentally friendly alternatives that have realistic efficacy in managing postharvest diseases. Biocontrol agents have been widely investigated as a potential alternative to manage *Penicillium* spp. on citrus fruit.

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Chapter 2

Isolation and *in vitro* screening of potential biocontrol agents for the biological control of *Penicillium digitatum and Penicillium italicum*

Abstract

The objective of this study is to assess the potential antagonistic effects of isolated microorganisms on the growth of *Penicillium* blue and green mould in laboratory conditions. One hundred and two microbes were washed off the fruit and leaves of lemon and lime trees, and cultured to create pure colonies. These were screened against *P. digitatum* and *P. italicum,* the causal agents of green and blue moulds of citrus, respectively, *invitro* using a dual culture method. In the secondary screening, only seven (6.86%) of the isolates caused inhibition of the pathogens seven days after incubation. In a secondary screening of the best seven isolates using the dual culture assay Isolate UK37 caused the highest percentage inhibition of 98.83% against *P. digitatum* and 96.89% for *P. italicum,* followed by SCO13 (95.29% and 94.23%), respectively. Molecular techniques were used to identify the best two isolates from the secondary screening. The isolates were both identified as *Bacillus amyloliquefaciens.*

Keywords: Green mould, blue mould, *Bacillus amyloliquefaciens,* subtropical

2.1. Introduction

Citrus fruits are grown in more than 140 countries in tropical and subtropical areas. The most commercially important citrus fruit include oranges (*Citrus sinensis*), limes (*Citrus aurantiifolia*), lemons (*Citrus limon*) and grapefruit (*Citrus paradisi*) that are consumed as fresh produce and processed goods (Liu *et al*., 2012). Injuries occurring during packaging and transportation of citrus fruits cause wounds that allow fungal pathogens to infect the fruit (Talibi *et al*., 2014). A number of pathogens affect citrus production, including *Penicillium digitatum* (Pers.) Sacc and *Penicillium italicum* Wehmer, which are necrotrophic fungi that cause massive losses of untreated fruit globally (Kanetis *et al*., 2007; Diaz *et al*., 2020). *Penicillium* spp have a short life cycle of three to five days. Once a fruit is infected, the pathogens colonize the fruit quickly and then initiate the production of prolific quantities of conidia (Kanetis *et al*., 2007). Enzymes produced by the fungal mycelium degrade the fruit cell walls, causing infected fruit to shrink, and a soft water-soaked spot forms due to infected pericarp and mesocarp cells (Papoutis *et al*., 2019).

Control of citrus moulds has been dominated by the use of fungicides. However, due to resistance issues and consumer rejection of fungicide residues in citrus fruit, this control option is rapidly disappearing. Biological control using antagonistic microorganisms is an alternative for the control of citrus *Penicillium* moulds (Bhatta, 2022), which is non-toxic and unlikely to face resistance problems due to the multiple modes of action of most biocontrol agents. For example, yeast-based biocontrol agents (BCAs) such as *Candida membranifaciens* and *Candida oleophila* induce systemic resistance on grapefruit fruit peels (Droby *et al*., 2002; Terao *et al*., 2017). The yeast *Metschnikowia pulcherrima* produces iron chelators, which bind iron, the lack of which inhibits the growth of pathogens (Gore-Lloyed *et al*., 2019). The secretion of antifungal peptides is a mode of action used by a number of bacterial biocontrol agents against fungi (Montesinos, 2007).

In this chapter, 102 microbes were isolated from different parts of various citrus trees, screened using dual culture bioassays and the most effective isolates were identified using molecular techniques. The aim of this chapter was to isolate and screen microorganisms for their antagonistic abilities *against Penicillium sp*.

2.2. Materials and Methods

2.2.1. Fruit Material Used for Isolation of Potential Yeast and Bacterial Antagonists

Yeast and bacterial cells were isolated from the leaves and fruit peels of lemon, orange and lime trees from three sites; Ukulinga Farm, Ozwathini and Scottville located in Kwa-Zulu Natal. 50 grams of fruit peel were weighed and placed in a 250 ml flask with 100 ml of sterile distilled water. The flask was shaken in a water bath at 90 rpm at 30°C for one hour. Similarly, 5 grams of fruit tree leaves were weighed and placed in a 250 ml flask and treated as above. The microbial suspensions were plated onto yeast extract peptone dextrose agar (YPD) in a dilution series. Discrete colonies were then selected and inoculated onto potato dextrose agar (PDA) at 25 ℃ for seven days to obtain pure cultures of each microbial strain. The pure isolates were stored on PDA slants and placed in a fridge at 4°C for short term storage. The microbes were suspended in 60% glycerol and stored at -80℃ for long term storage

2.2.2. Pathogen isolation and morphology

Citrus fruit with powdery green and blue conidia visible on fruit skin were collected from supermarkets in Pietermaritzburg. Infected citrus fruits had water-soaked lesions, from which emerged green or blue conidia with white mycelia on the edges. Small sections of fruit displaying symptoms were cut and placed on PDA plates and incubated at 30 °C for 5 days. Single colonies were sub-cultured on PDA plates to obtain pure cultures. A light microscope (Carl Zeiss, Germany) was used to identify colonies of *P. digitatum* and *P. italicum*. The fungi were stored in the short term on PDA at 4 °C. Fungi were stored on PDA slants for medium term storage. A plug of each pathogen growing on PDA was inverted onto a PDA slant and stored at room temperature. Pure cultures were stored in glycerol stock (30%) into microfuge tubes at stored at -80℃ for long term storage.

2.2.3. *In vitro* Primary for Antifungal Activity of Biocontrol Agents Against *Penicillium* spp.

The 102 isolates were tested for their ability to inhibit the mycelial growth of *P. digitatum* and *P. italicum* on PDA plates. A three-day old mycelial plug (2mm x 2mm) of *P. digitatum* and *P. italicum* was inverted onto the centre of freshly prepared PDA plates. On opposite ends of a petri dish, approximately 1 cm away from the edge, isolates previously incubated at 25 ℃ for two days were streaked using an inoculating loop. Mycelial plugs of either *P. digitatum* or *P. italicum* were placed at the centre of a fresh PDA plate as a control treatment. The plates were sealed with parafilm and incubated at 25℃ for seven days. The experiment was repeated twice. The mycelial growth of the pathogen was measured after seven days and the zone of inhibition

was measured by the length between the microbe line and the *P. digitatum* and *P. italicum* colonies

2.2.4. Secondary *in vitro* screening of potential biocontrol against *P. digitatum and P. italicum.*

The best 10 performing microbes were selected for secondary screening based on the width of their zones of inhibition in the primary screening. A dual culture assay of selected isolated against P. *digitatum* was conducted to re-confirm their efficacy.

After seven days in the incubator, the width of the inhibition zone was measured to calculate the percentage inhibition using the formula below:

$$PI = \frac{Dc-Dt}{Dc} \times 100;$$

Where;

PI= Percent inhibition

Dc= diameter of control plate (*P. digitatum*)

Dt= diameter of *P. digitatum* in the dual test plate

2.2.5. Molecular identification of Potential Biocontrol Agents Isolates

The molecular identification of the isolated biocontrol agents was done according to Stephen et al., (1997). The analyses were conducted by Inqaba Biotec, South Africa. Genomic DNA was extracted from the cultures using the Quick-DNA^{TM} Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The ITS and 16S target region was amplified using the NEB OneTaq 2X Master mix (Catalogue No M0482S) with the primers presented in Table 2.1 and 2.2. The PCR products were run on a 1% agarose gel (CSL-AG500, Cleaver Scientific Ltd) stained with EZ-vision® Bluelight DNA Dye. The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrilliantDye^{TM} Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit^{TM}, Catalogue No. D4050). The purified fragments were analysed on an ABI 3500x1 Genetic Analyser (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample. CLC Bio Main Workbench was used to analyse the files with the samples generated by the ABI 3500XL Genetic Analyser and results were obtained by a BLAST search (NCBI). Only promising isolates from secondary screening were sent for identification.

Table 2.1. 16S RNA Primer Sequences used to identify bacterial isolates from *in vitro* studies

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

Table 2.2. ITS Primer Sequences used to identify yeast isolates from *in vivo* studies

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

2.2.5. Statistical Analysis

All experiments were set up in a completely randomized design. The data obtained were subjected to analysis of variance (ANOVA) using Genstat® 20th edition. Duncan Multiple Range Test (DMRT) at $P \leq 0.05$ was used to determine differences between treatments

2.3. Results

2.3.1. Primary *in vitro* screening of yeast and bacterial isolates against *Penicillium* spp.

A total of 102 microbes were isolated from lemon, orange and lime fruit and leaves, with 47 isolates obtained from leaves and 55 from fruit peel. All 102 isolates were screened against *P. digitatum* and *P. italicum* (Appendix 1). Of these, seven isolates (6.86%) inhibited the growth of *Penicillium* spp. after seven days of incubation.

2.3.2. *In vitro* secondary screening of citrus epiphytes against *P. digitatum*

The seven isolates were then used for secondary screening, selected on the diameter of zones of inhibition against *P. digitatum* and *P. italicum* during the screening trial. The majority of isolates were isolated from lemon tree leaves. The zones of inhibition measured are shown in Table 2.3.

Table 2.3: Average inhibition (%) of microbial antagonist isolates against *P. digitatum* after seven days. Means with the same letters are not significantly different according to Duncan's multiple range test (P=0.05).

Source of isolate	Isolate name	Mycelial growth (mm)	% Inhibition
***Citrus aurantiifolia* (leaves)**	SCO13	4.00ab	95.29
***Citrus lemon* (leaves)**	UK26	44.00d	48.24
***Citrus lemon* (leaves)**	UK29	41.33d	51.38
***Citrus lemon* (leaves)**	UK37	1.00a	98.83
***Citrus lemon* (peel)**	UK49	42.33d	50.2
***Citrus lemon* (peel)**	UK53	27.33c	67.85
***Citrus lemon* (peel)**	UK58	5.33b	93.73
	Control	85.00e	
P-value		<.001	
Fishers LSD		3.789	
CV		7	
SED		1.787	

Note: The values are presented as a mean of three replicates. Different letters represent significant differences at a 5% significance level, according to DMRT.

2.3.3. *In vitro* secondary screening of the most effective microbes against *P. digitatum*

The seven best microbes were selected based on the diameter of zones of inhibition against *P. digitatum* during the primary screening trial. Secondary screening of selected isolates provided inhibition percentages of 48.24 to 98.83%. Four out of the seven isolates were from citrus peels and three from citrus leaves (Table 2.3)

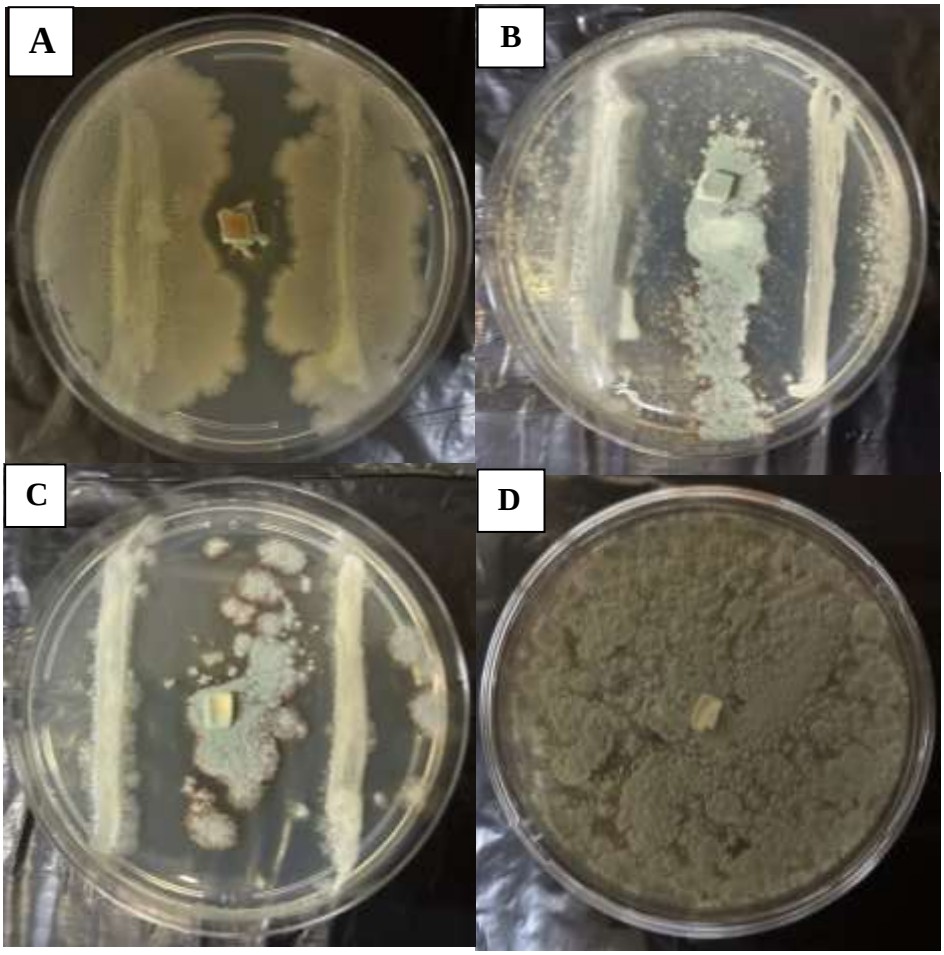

Figure 2.1: The inhibition of *P. digitatum* mycelial growth by the best epiphytic isolates SCO13 (A), UK58 (B) and UK37 (C) compared to the control plate with *P. digitatum* only (D) after seven days *in vitro* secondary screening.

Mycelial growth of *P. digitatum* was inhibited by the best isolates (Table 2.3). All three isolates were significantly different from each other based on the inhibition of *P. digitatum* mycelial growth after seven days (P<.001). Six isolates inhibited the mycelial growth of *P. digitatum* by more than 50% *in vitro* (Table 2.3). UK37 was the best isolate, with the highest percentage inhibition of 98.83% of the mycelial growth of the pathogen after seven days.

2.3.4. *In vitro* secondary screening of bacterial isolates against *P. italicum*

The secondary screening of selected epiphytic isolates provided mean inhibition percentages from 22.23-96.89%. as seen in Table 2.4.

Table 2.4: Secondary screening of microbial antagonist isolates against *P. italicum* after seven days. Means with the same letters are not significantly different according to Duncan's multiple range test (P=0.05)

Source of Isolate	Isolate name	Mycelial growth (%)	%Inhibition
***Citrus aurantiifolia* (leaves)**	SCO13	4.33a	94.23
***Citrus lemon* (leaves)**	UK26	46.67b	37.77
***Citrus lemon* (leaves)**	UK29	45.00b	40
***Citrus lemon* (leaves)**	UK37	2.33a	96.89
***Citrus lemon* (peel)**	UK49	58.33c	22.23
***Citrus lemon* (pee)**	UK53	45.67b	39.11
***Citrus lemon* (peel)**	UK58	5.33a	92.89
	Control	75.*00*d	
P-value		<.001	
Fishers LSD		3.870	
CV%		6.3	
SED		1.826	

Note: The values are presented as an average of three replicates. Different letters represent significant differences at a 5% significance level, according to DMRT.

Mycelial growth of *P. italicum* was inhibited by the best epiphytic isolates (Table 2.4). The three best performing isolates were not significantly different from each other (UK37, SCO13 and UK58) but were significantly different from the rest of the isolates on the inhibition of the mycelial growth of *P. italicum* after seven days (P<.001). Only three isolates inhibited the mycelial growth of the fungus by more than 50% *in vitro* (Table 2.4). UK37 was the best isolate with the highest percentage inhibition of 96.89% of the mycelial growth of the pathogen after seven days.

2.3.5. Identification of bacterial and yeast isolates using molecular techniques

The best two isolates were identified using the 16S RNA primer sequences (Table 2.5). The two best isolates SCO13 and UK37 were both identified as *Bacillus amyloliquefaciens*.

Table 2.5: Molecular identification of the best two bacterial isolates using the 16S primer sequence.

Sample	Name of Predicted organism	Request ID	Similarity
SCO13	*Bacillus amyloliquefaciens*	GGNAFBZD012	100
UK37	*Bacillus amyloliquefaciens*	GGNANMUX01R	100

Two yeast isolates previously tested against *P. digitatum* for their efficacy were provided for *in vivo* screening and were identified using the ITS primer sequences. The yeasts were both identified as *Meyerozyma guilliermondii*.

Table 2.6: Molecular identification of the two yeast isolates using the ITS primer sequence.

Sample	Name of Predicted organism	Request ID	% Identity
B13	*Meyerozyma guilliermondii*	G8DY03RJ013	99.67 %
M1	*Meyerozyma guilliermondii*	G8DY3T18013	100 %

2.4 Discussion

This study aimed to isolate and screen the best performing antagonists from citrus fruit and leaves to inhibit the growth of *P. digitatum* and *P. italicum*. One hundred and two isolates were screened using the dual culture assay method for inhibitory effects against *P. digitatum* and *P. italicum* under *in vitro* conditions. Most isolates could not control the mycelial growth of the fungal pathogens. From 102 isolates, seven isolates were selected for secondary screening, in which two isolates, SCO13 and UK37, demonstrated the highest levels of inhibition of the pathogens. *B. amyloliquefaciens* B (UK37) caused the most inhibition of green mould (98.83%) and blue mould (96.89%). *B. amyloliquefaciens* A (SCO13) was the second-best isolate

inhibiting the mycelial spread of green and blue mould by 95.29% and 94.23%. These isolates were then identified as *Bacillus amyloliquefaciens* using their 16S rRNA genes.

The fungal pathogens did not grow towards the bacteria, which suggests that the bacteria released volatile organic compounds (VOCs), antibiotics or lytic enzymes, inhibiting the mycelial spread of the fungi (Maldonado *et al*., 2010; Lai *et al*., 2012). The two isolates of *B. amyloliquefaciens* are potential biocontrol agents for the control of *P. digitatum* and *P. italicum*. Cawoy *et al*. (2015) reported that some *Bacillus* species can devote 8% of their genetic potential to synthesize a wide range of antimicrobial compounds such as lipopeptides and lytic enzymes that are necessary for the inhibition of pathogen growth. *Bacillus* species have been reported to produce lytic enzymes that actively degrade the cell wall of pathogens. For example, an antagonistic strain *B. subtilis* NSRS 89-24 synthesized lytic enzymes that eroded the cell walls of sheath blight pathogens due to the production of extracellular chitinase (Leelasuphakul *et al*., 2006; Swain *et al*., 2008). Green mould disease on mandarin was successfully supressed by *B. subtilis* ABS-S14, which synthesized three cyclic lipopeptides that were antifungal (Waewthongrak *et al*., 2014). The *Bacillus* genus has been widely studied for their antifungal and antimicrobial activities, which has led to investigations and intensive research in their mechanisms of action against pathogens. The production of LPs is common with *Bacillus sp*. when interacting with pathogens, for example, *B. amyloliquefaciens* produces the antifungal adjuvants, iturins and fengycins when interacting with *Pythium aphanidermatum* and *Fusarium oxysporum* (Cawoy *et al*., 2015).

Penicillium italicum produce secondary metabolites during infection that increase the pathogen's virulence and disease spread. Studies have reported natural products produced by *P. italicum* such as 5,6-dihydroxy-4-methoxy-2H-pyran-2-one, which has been classified as a mycotoxin by the Human Metabolome Database (Faid and Tantaoui-Elaraki, 1989). *Penicillium digitatum* causes substantial economic losses for the citrus industry, and can cause an allergic response due to the air-borne spores (Moss *et al*., 2008). These fungi are two of the main pathogens responsible for postharvest diseases in citrus fruit. To prevent and manage their spread, a number of control methods are needed. The most common control method has been the use of synthetic fungicides which has been efficient in controlling green and blue mould of citrus. However, the residues of these chemicals affect the environment and are toxic to human health (Kanashiro *et al*., 2020). Some strains of *P. digitatum* and *P. italicum* have developed resistance to the fungicides, requiring higher dosage and different mixes of the chemicals (Torres and Tuset., 2011). As alternatives, antagonistic yeasts and bacteria can be used in

combination with physical methods such as UV and hot water treatment to control the infection and spread of these fungi (Sharma *et al*., 2009; Kanashiro *et al*., 2020).

In this study, two isolates of *B. amyloliquefaciens* were identified and demonstrated their efficacy in inhibiting *P. digitatum* and *P. italicum in vitro*. The *M. guilliermondii* species displayed a low percentage of inhibition and did not control the spread of *P. digitatum* and *P. italicum* moulds. These isolates will be further tested to assess their ability to control *P. digitatum* and *P. italicum* under *in vivo* conditions on citrus fruit.

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Chapter 3

The Effect of *Bacillus amyloliquefaciens* and *Meyerozyma guilliermondii* for the Postharvest Management of *Penicillium digitatum* and *Penicillium italicum* on 'Valencia' Oranges

Abstract

In this study the efficacy of two bacterial strains of *Bacillus amyloliquefaciens* and two yeast strain of *Meyerozyma guilliermondii* were tested as biocontrol agents against *Penicillium digitatum* and *P. italicum* on Valencia oranges. *Bacillus amyloliquefaciens* strain SCO13 and strain UK37 showed potential as biological control agents against *P. italicum* with the average disease incidence 35.87% and 51.37%, respectively. The disease incidence was reduced by SCO13 and strain UK37 by 19.54% and 39.53%, respectively. *Meyerozyma guilliermondii* Strain B13 and Strain M1 caused the highest mean percentage of 57.17% and 62.33% of blue mould disease. Valencia fruits treated with *M. guilliermondi*i Strain B13 and Strain M1 displayed high disease incidence of 56.60% and 59.70%, respectively. The interaction of the two strains of *B. amyloliquefaciens* and two strains of *M. guilliermondii* with *P. digitatum* were observed using scanning electron microscopy (SEM). The *Bacillus* spp. inhibited the formation of conidia of *P. digitatum*, while *Meyerozyma* spp. had minor visible effects, like the bending of hyphae *in vitro* assay for seven days at 25℃.

Keywords: sugar content, limonene, *Meyerozyma* spp.

3.1. Introduction

Citrus production is divided into fresh and processed markets, where oranges represent half of the total production of citrus which is estimated to be over 98 million tons (USDA, 2021). Processed products such as fruit juice, dried pulp, essential oils and syrups are key components on the economic standing of citrus production (Bhatta, 2022). The success of South Africa in high value export markets, especially in Europe relies on the cultivars, traceability, logistics, marketing and phytosanitary standards (Cramer and Chisoro-Dube, 2021).

Green mould and blue disease, caused by *Penicillium digitatum* (Pers.) Sacc and *Penicillium italicum* Wehmer, are destructive pathogens that cost the citrus industry billions of rands each year. Maturity and the amount of inoculum are factors that influence disease severity directly, as mature fruits are more susceptible to the pathogens (Papoutis *et al*., 2019). These *Penicillium* spp. have a high degree of host specification as they do not occur naturally on other fruits other than the family Rutaceae (Barkai-Golan, 2001). *Penicillium digitatum* has a short life cycle of four to five days and secretes enzymes that quickly degrade citrus fruit. The fungus sporulates prolifically and spreads rapidly to healthy fruits in packaging, storage and transportation (Yang *et al*., 2019; Iturrieta-González *et al.*, 2022). Contamination of fruits occurs when the conidia encounter a wound where nutrients are available to trigger germination (Lahlali *et al*., 2006). Millions of airborne conidia are produced, and fruit is completely covered five days post infection. These conidia can cause an allergic reaction in humans (Moss, 2008). Furthermore, *P. digitatum* can produce citrinin which has carcinogenic effects to animals and humans (Flajs, and Peraica, 2009). Infection in humans is uncommon, with only one report of *P. digitatum* infection, resulting in fatal pneumonia for a patient in Japan in 2013 (Oshikata *et al*, 2013).

The interest of consumers in organic food and goods has increased significantly over the years, creating a general expectation for consumers in having alternative organic products at all supermarkets. Biological control has been investigated as an alternative for disease control for many decades. However, the use of yeast and bacteria as biocontrol agents to control plant pathogens has not been investigated as much as fungal agents (Sui *et al*., 2015). Gutter and Littauer (1953) undertook one of the first studies to isolate a strain of *Bacillus subtilis* that inhibited ten post-harvest pathogens causing decay on citrus. The use of yeast-based biocontrol agents (BCAs) has proven to be advantageous in integrated disease management and control of diseases. Yeasts have the ability to adhere onto the cuticle of fruits which gives them an advantage as biological control agents (Ferraz *et al*., 2016). *Bacillus* strains have the advantage

of forming endospores, which are long-lived, tough structures (Tsotetsi *et al*., 2022). This means that *Bacillus*-based biocontrol agents tend to have a long shelf-life, and are relatively easy to produce and formulate commercially. This study was aimed to determine if *Bacillus amyloliquefaciens* strains and *Meyerozyma guilliermondii* decrease green and blue moulds of citrus *in vivo.*

3.2. Materials and Methods

3.2.1 Pathogenicity Test of *P. digitatum* and *P. italicum* on Citrus Fruit

Pathogenicity tests were conducted by using the methods described by Abraha *et al*. (2010). Citrus fruit orange cv. 'Valencia' was collected from Donavale Farm in Wartburg, KwaZulu-Natal. Pathogens were isolated from diseased fruits (Chapter 2, 2.2.2). The surface of fruits was disinfected using 70% ethanol and then left to dry overnight in the laboratory at room temperature. Three replicates of 3 fruits per replicate for each treatment was used. The trial was repeated twice. An area of approximately 20-25 mm length and 10 mm width of fruit skin was lightly shaved using sandpaper as a simulation of a natural wound. This was done on opposite sides of each fruit. Fruits were inoculated with 2 ml of conidia suspension of *Penicillium* of concentrations 1 x10^5, 1 x 10^6 and 1 x 10^7 conidia ml^{-1}. Two fruits were inoculated with distilled water to serve as a control. Inoculated fruits were kept at room temperature for ten days. Ten days post inoculation, the lesion diameter was measured, and the mean length and width of each lesion was used to determine the lesion diameter.

3.2.2. *In vivo* screening of *B. amyloliquefaciens* and *M. guilliermondii* strains against *Penicillium* moulds on oranges

Healthy, unwounded fruits were harvested from the Donavale Farm in Pietermaritzburg, KwaZulu-Natal, South Africa for the experiments. Each fruit was dipped in 70% ethanol for one minute and dried to disinfect the surface. The control fruit was inoculated with a conidial suspension that was prepared using conidia from a 7-day old colony of *P. digitatum* or *P. italicum.* Petri dishes containing sporulating pathogens were flooded with 10 ml of distilled water and stirred with a glass rod. A haemocytometer was used to enumerate the conidial suspension concentration and adjusted to 1 x 10^6 conidia/ml. The wound was dipped into cell suspensions of each isolate of 1 x 10^6.

For the biocontrol treatments, the *B. amyloliquefaciens* strains stored in 60% glycerol solution were sub-cultured onto Petri dishes containing potato dextrose agar (PDA) media and incubated for 72 hours at 30℃. A 500 ml bacterial suspension was prepared using sterile

distilled water. Serial dilutions of 1 x 10^{-1} to 1 x 10^{-6} was prepared from the bacterial suspension. The final suspension was adjusted to 10^6 cells ml^{-1}. Yeast suspensions were prepared as bacterial suspensions. The two yeast strains of *Meyerozyma guilliermondii were* previously isolated by Dr M.J Morris (Andermatt-PHP (Pty) Ltd). B13 isolated by Abraham *et al* (2010) was also used a one of the treatments in previous studies were also used against the fungal pathogens in the *in vivo* trial.

The biocontrol assay was conducted using sandpaper to scrape fruits on opposite sides (2mm in width and 4mm length) to simulate a natural wound. Fruits were dipped into suspensions of the biocontrol agents (1 x 10^6 cells/ml) for one minute and left to dry for 3 hrs. After 3 hrs the fruits were inoculated with *P. digitatum* and *P. italicum* suspension by dipping the fruits in one of the two pathogen inocula for 1 minute. The inoculated fruit were placed into boxes and wrapped in plastic bags for 10 days at room temperature. Disease severity was measured after days 3, 6 and 10 post-inoculations. Three fruits were used for each replicated, replicated three times and the experiment was repeated twice.

Ten days post inoculation the results were measured and recorded to calculate the percentage disease inhibition using the formula below:

$$L = \frac{C - T}{C} \text{ X } 100$$

Where;

C= average growth of pathogen in control

L= Inhibition of radial mycelial growth

T= average radial growth in presence of antagonists

3.2.3. Scanning Electron Microscopy Observations of Interactions Between *Penicillium* spp. and Biocontrol Agents *in vitro* and *in vivo*

The biocontrol agents that successfully inhibited *Penicillium* spp. in dual culture (Chapter 2, 2.3.1) and on 'Valencia fruit' (3.3.2) were grown on a freshly prepared PDA petri dish. A mycelial disc (2 mm x 2 mm) was placed at the centre of the PDA plate and each biocontrol agent was streaked 1 cm away from the mycelial plug on both sides. For an *in vivo* trial, orange fruits were disinfected with 70% ethanol, wounded and inoculated with the biocontrol agents and the pathogens as discussed above. The inoculated PDA plates and fruits were incubated at 25°C. After 7 days, mycelial growth and sporulation of *P. digitatum* and *P. italicum* was

observed under a scanning electron microscope (SEM) (Zeiss EVO LS15, Carl Zeiss NTS Ltd., Germany). The SEM unit is in the Microscopy and Microanalysis Unit, University of KwaZulu Natal, Pietermaritzburg, South Africa. Samples were cut from inoculated PDA plates and fruit samples, placed for 2 hours in 3% buffered glutaraldehyde, then and washed twice in 0.05M sodium cacodylate buffer for 5 minutes. The samples were then dehydrated with approximately 2 mL aliquots of 10%; 30%; 50% and 70% ethanol for 10 minutes per concentration. The samples were rinsed three times with 100% ethanol for 10 minutes to complete the dehydration process. The samples were placed in the basket of a critical drying point dryer (Model K850, Quorum supplies, East Sussex, United Kingdom) (CPD) in 100% ethanol. The ethanol was replaced with liquid carbon dioxide (CO_2) during CPD. The liquid CO_2 was heated and pressurized to the critical point at which point the liquid turned into a gas without damaging the samples due to surface tension, leaving the samples dry and undamaged. Using black double-sided tape, the dried samples were carefully mounted onto SEM stubs. The sample stubs were transferred to a sputter coater (Model Q150RS ES, Quorum supplies, East Sussex, United Kingdom). In this step, the samples were coated twice with gold and palladium to make them reflective of an electron beam. After drying, the samples were examined under the Zeiss EVO LS15 SEM.

3.2.4. Statistical analysis

All experiments were set up in a completely randomized design. The data obtained were subjected to analysis of variance (ANOVA) using Genstat® 20th edition. Duncan Multiple Range Test (DMRT) at $P \leq 0.05$ was used to determine differences between treatments.

3.3. Results

3.3.1. Pathogenicity test of *P. digitatum* and *P. italicum* on 'Valencia' fruits.

The pathogenicity test showed that *P. digitatum* and *P italicum* were pathogenic to 'Valencia' oranges 7 days post inoculation. Treatments with the same letter are not significantly different according to Duncan's Multiple range test (P=0.005). There was no significant difference in green mould incidence of oranges dipped in a conidial suspension of *P. digitatum* at concentrations 1 x 10^5 and 1 x 10^6 conidia/mL with P-value <0.001 (Figure 3.1. a). However, there was a significant difference in blue mould incidence significant difference in blue mould incidence of oranges dipped in a conidial suspension of *P. digitatum* at concentrations 1 x 10^5 and 1 x 10^6 conidia/mL with P-value <0.001.

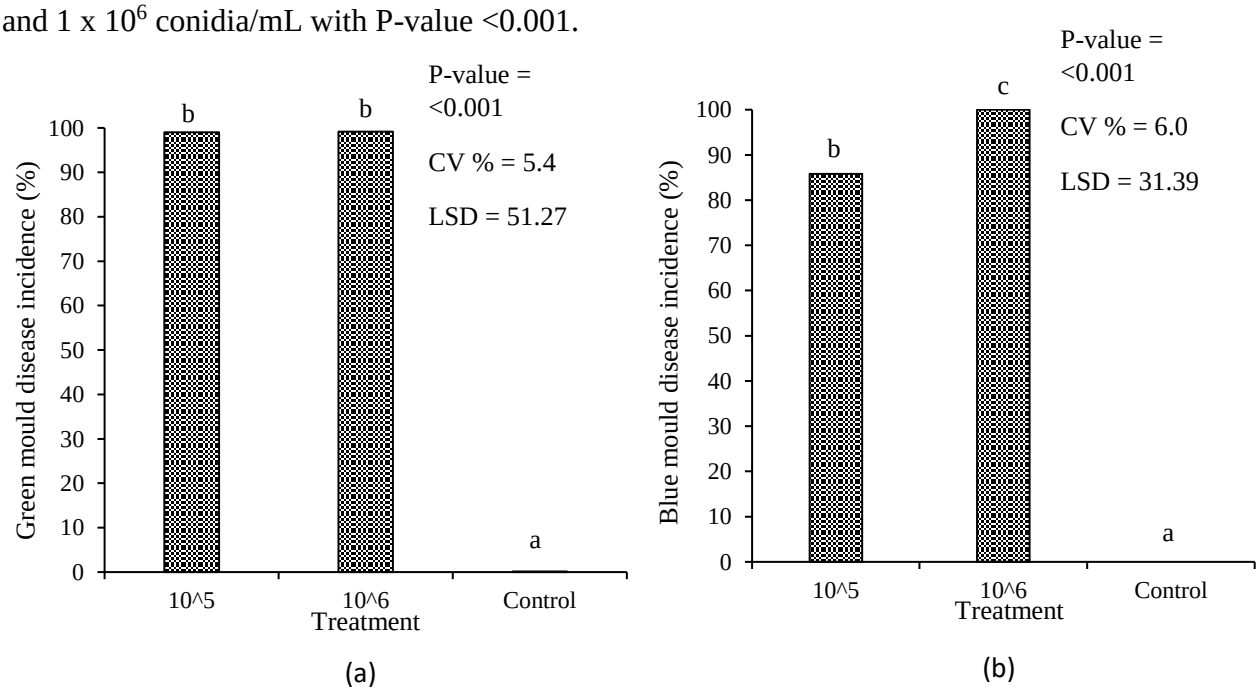

Figure 3.1: Pathogenicity test on *P. digitatum* and *P. italicum* on 'Valencia' oranges for seven days at 25 ℃

3.3.2. *In vivo* Screening for Antifungal Activity of Biocontrol Agents Against *P. italicum*

Orange fruits were observed for blue mould disease incidence 10 days post inoculation at room temperature (Figure 3.3). Fruits treated with SCO13 (35.87%), UK37 (51.37%) had the lowest disease incidence of *P. italicum* compared to the pathogen control treatment (100%). There was no significant difference between two *B. amyloliquefaciens* treatments SCO13 and UK37, respectively.

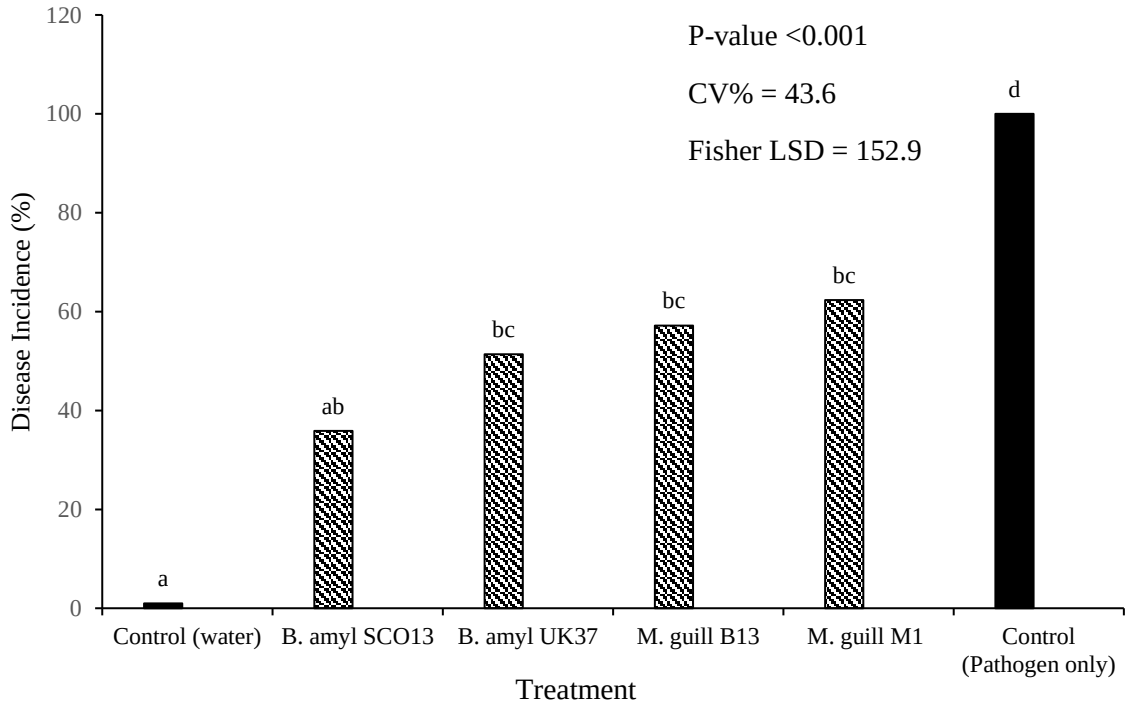

Figure 3.3: Disease incidence of *P. italicum* on Valencia oranges 10 days post inoculation at room temperature. Means with the same letters are not significantly different according to Duncan's multiple range test (*P*=0.05).

Antagonistic microorganisms inhibited *P. italicum* on oranges during *in vivo* screening (Figure 3.3 B, C and D) compared to control fruits (Figure 3.3 A). Fruits inoculated with *P. italicum* only (control) (Figure 3.3 A) showed high disease incidence compared to fruits treated with antagonistic microorganism 10 days post inoculation.

Figure 3.4: Disease incidence of blue mould on Valenica oranges treated with the B *amyloliquefaciens* Strain UK37 (B), *M. guilliermondii* Strain M1 (C), *M. guilliermondii* Strain B13 (D) and *B. amyloliquefaciens* Strain SCO13 (E), compared to the control (A) 10 days post inoculation at 25 °C.

3.3.3. *In vivo* screening for antifungal activity of biocontrol agents against *P. digitatum*

Orange fruits were observed for green mould disease incidence, 10 days post inoculation at room temperature (Figure 3.5). Fruits treated with *B. amyloliquefaciens* Strain SCO13 (19.54%) displayed the lowest disease inhibition of *P. italicum* compared to the pathogen control treatment (100%).

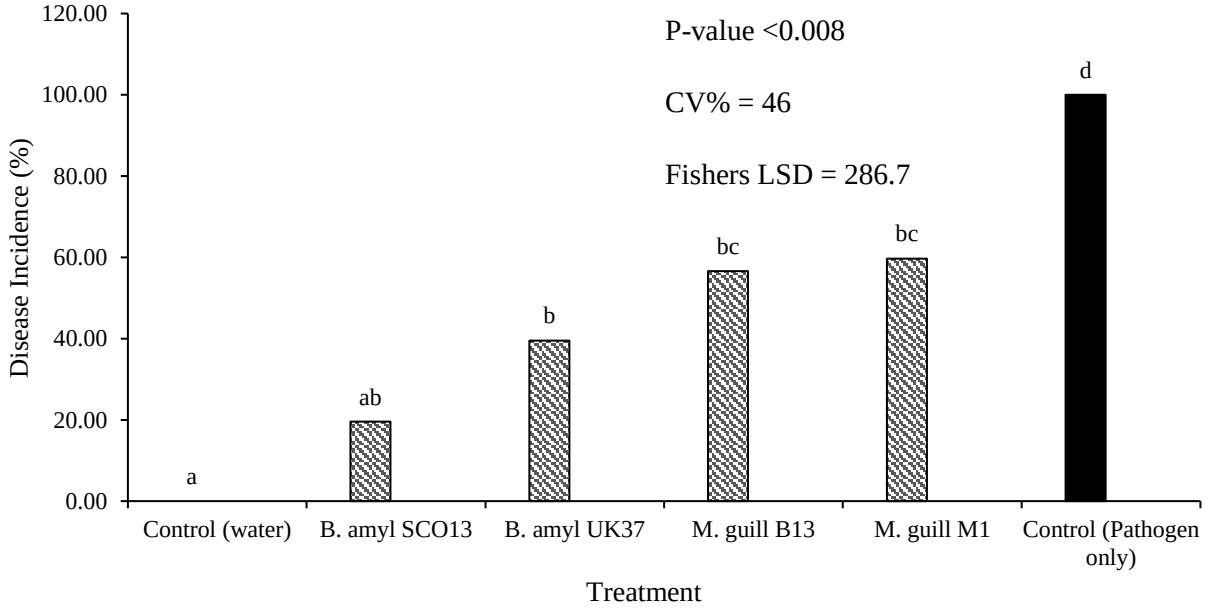

Figure 3.5: Incidence of green mould disease on 'Valencia' oranges 10 days post inoculation at room temperature. Means with the same letters are not significantly different according to Duncan's multiple range test (*P*=0.05).

Figure 3.6: Disease incidence of green mould on Valenica oranges treated with the best biocontrol agent isolates *B. amyloliquefaciens* Strain UK37 (B) and *B. amyloliquefaciens* Strain SCO13 (C), *M. guilliermondii* Strain B13(D), *M. guilliermondii* Strain M1 (E) compared to the control (A) 10 days post inoculation at 25 ℃.

3.4. Scanning Electron Microscopy Analysis of The Interaction Between *P. digitatum* and Biocontrol Agents

3.4.1. The interaction of biocontrol agents and *P. digitatum* growing alongside on PDA plates stored at 25℃ for 7 seven days.

Mycelial growth and conidia of *P. digitatum* alongside *B. amyloliquefaciens* Strain SCO13/*B. amyloliquefaciens* Strain UK37 were observed using the SEM (Figure 3.7) after 7 days at 25 ℃. The interaction and mode of action of biocontrol agents on *P. digitatum* revealed that the biocontrol agents had an effect on the morphology of the mycelia and conidia of the fungus compared to the control (Figure 3.7 B, Cand D) compared to the control (Figure 3.7, A).

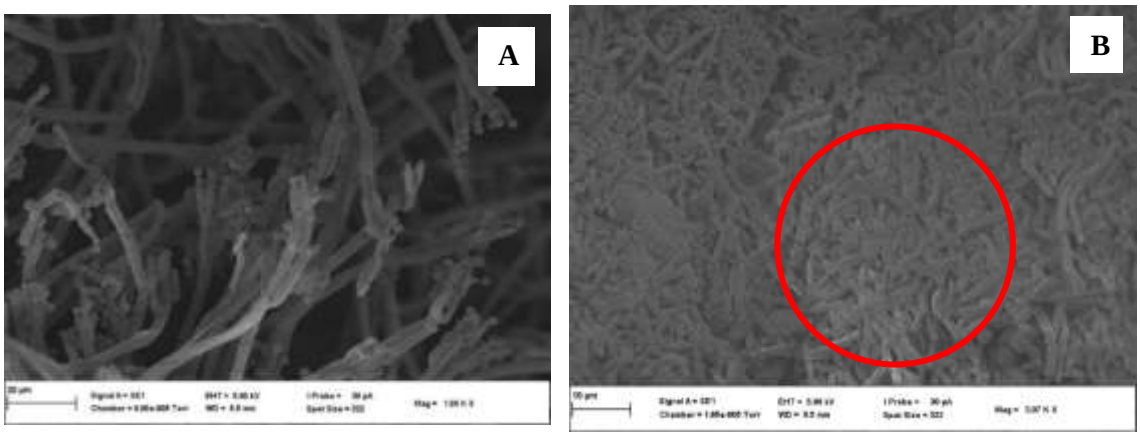

Figure 3.7: *B. amyloliquefaciens* Strain SCO13 completely colonizing the surface, against *P. digitatum* (B) on the mycelia and hyphae of *P. digitatum* (A) at 25 ℃ for 7 days.

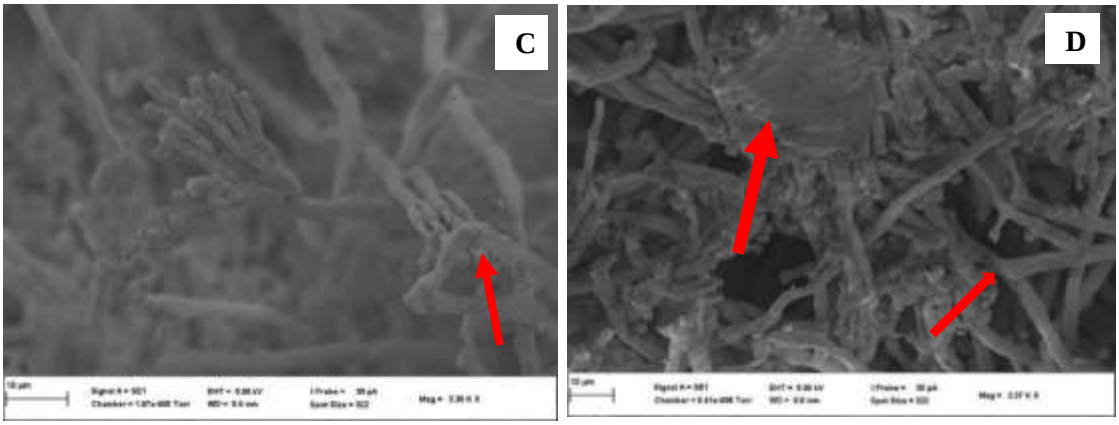

Figure 3.8: Interaction between *P. digitatum* and *B. amyloliquefaciens* Strain UK37 (C) and *M guilliermondii* Strain M1 (D). Shrivelled hyphae of *P. digitatum* (arrowed).

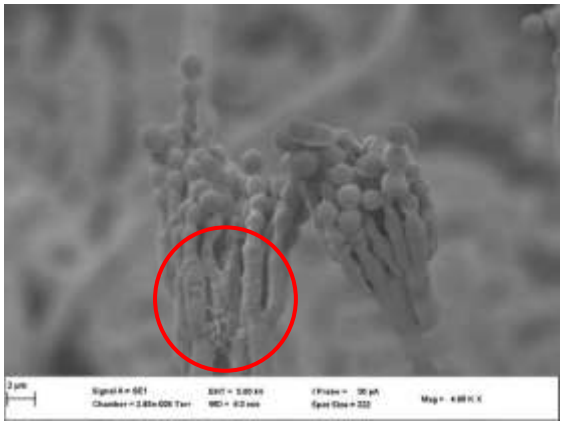

Figure 3. 9: Scanning electron micrograph showing growth of antagonists sticking on hyphae of *P. digitatum*. (circled).

3.4.2. SEM micrographs showing the interaction of biocontrol agents and *P. digitatum* on the surface of orange fruits ten days post inoculation, stored at room temperature. SEM micrographs below show the effect of biocontrol agents on the hyphae of *P. digitatum*.

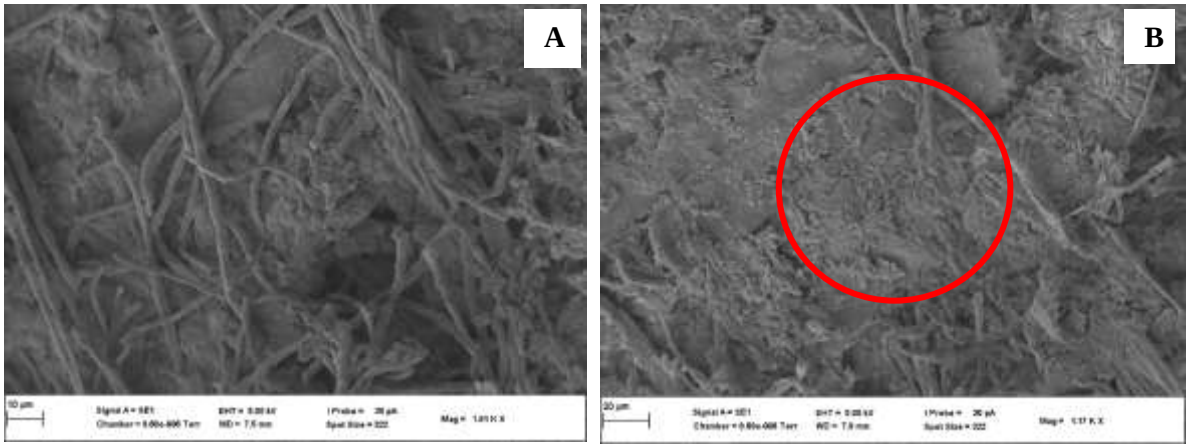

Figure 3.10: Scanning electron micrograph showing hyphae and conidia of *P. digitatum* on the surface of 'Valencia' fruits in the control treatment (A). The effect of *B. amyloliquefaciens* on the hyphae of *P. digitatum*, with no conidia present (B)

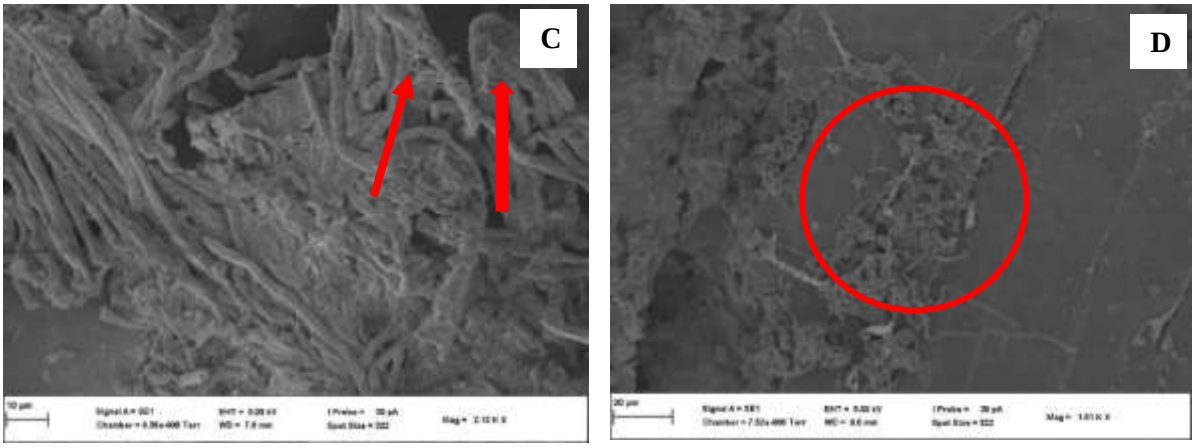

Figure 3.11: Scanning electron micrograph showing shrivelled hyphae (arrowed) of *P. digitatum* with no visible conidia of the pathogen on 'Valenica' fruit treated with *M. guilliermondii* Strain M1 (C). Yeast cells of *M. guilliermondii* Strain B13 colonizing surface of 'Valencia' fruits (circled) with no sight of hyphae and conidia of *P. digitatum.* (D), Control (A).

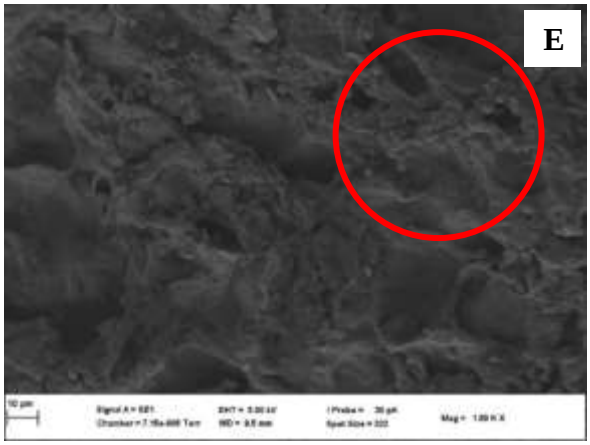

Figure 3.12: No visible hyphae of *P. digitatum* visible on orange fruit treated with *B. amyloliquefaciens* Strain UK37, Control (A).

3.3. Discussion

Biological control has been proven to be one of the most promising alternatives in reducing synthetic fungicides for managing postharvest diseases on fruits and vegetables, because it aligns with the objective of sustainable agriculture as it uses the natural cycles of the environment to achieve disease management (Sparado and Guillino, 2003). This study aimed to control green and blue mould diseases on oranges using two strains of *B. amyloliquefaciens* and two yeast species, *M. guilliermondii* and *Candida fermentati* strain B13. In this study *B. amyloliquefaciens* A (SCO13) had the best efficacy in controlling green mould and blue mould decay of oranges *in vivo.* The effect of each of the strains to inhibit *P. digitatum* growth was

studied using scanning electron microscopy. The morphological changes, including the bending of hyphae is associated with the presence of antifungal compounds. *Bacillus* species produce an array of antimicrobial substances such as iturins, surfactins and fengycins that play a major role in supressing pathogens (Lastochkina *et al*., 2019). The effects of these substances have been reported against *Fusarium graminearum, Fusarium oxysporum, Fusarium solani* and *Rhizoctonia solani* (Kumar *et al*., 2012; Yuan *et al*., 2012)

Bacillus amyloliquefaciens A (SCO13) was the best treatment and displayed lowest disease inhibition of *P. italicum* and *P. digitatum* by 35.87 % and 19.54 % respectively. *Meyerozyma guilliermondii* is a potential biocontrol agent due its survival ability as it grows in a wide range of temperatures and in varied osmotic environments (Saligkarias *et al.*, 2002). However, *M. guilliermondii* M1 showed the least control of *P. italicum* and *P. digitatum* with disease incidence of 62.33 % and 59.70 % at 25 ℃ for seven days. For example, *B. amyloliquefaciens* strain HF-01 provided 90% control of *Penicillium* moulds without changing the quality of fruits (Hao *et al*., 2011). Thus, it can be concluded that bacteria agents are better at managing blue and green mould disease of citrus when compared to yeasts. However, the *Bacillus* spp. were isolated in this study, whereas the yeast species were previously isolated and stored in 60% glycerol at -80 ℃, which could have had an impact on their efficacy *in vivo*. Abraham *et al.* (2010) isolated yeast B13 *Candida fermentati* and tested for efficacy against *P. digitatum* found that when B13 was applied at 1 x 10^8 cells/ml it completely controlled *P. digitatum*. Thus, it can be concluded that the efficacy of the yeast may have been degraded by storage and contamination within the past thirteen years. In this study yeast isolate B13 was applied at 1 x 10^6 cells/ml which may have reduced the efficacy of the isolate against both fungal pathogens as there was less concentration of the antagonist. Lower concentration was used, as commercial products are required to have a low concentration with high efficacy against pathogens.

Over-all all of the treatments tested in this study did not completely control both fungal pathogens as almost all of the treatments had disease incidence greater than 30%. Citrus fruits have a high sugar content and contain nutrients that fungi use to enhance their efficacy and speed up the infection process thus increasing disease incidence and severity (Lilly and Barnett 1953; Cheng *et al*., 2020). The pathogenicity of *P. digitatum* in comparison with the pathogenicity of *P. italicum* could be explained by the lower percentages of inhibition of *P. digitatum*. In wounds that develop during the harvesting process, volatiles such as myrcene, limonene and alpha and beta pinene are released from ruptured oil glands of citrus fruit that

promote the germination of *P. digitatum* conidia (Droby *et al*., 2008). *Bacillus amyloliquefaciens* strain A has the potential to effectively control the growth of *P. italicum* and reduces the spread of green mould disease, on 'Valencia' fruit. These *Bacillus* spp. can be integrated with control methods to effectively control blue and green mould disease on citrus fruit.

3.4. References

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Dissertation Overview

South Africa is the second largest exporter of citrus following Spain, and is responsible for 10% of global citrus exports. The success of the SA citrus industry relies on the production of high-quality fresh fruit that require expensive industrial processes and sophisticated technologies to meet the quality and phytosanitary standards of importers (Chisoro and Roberts., 2023). South Africa produces mainly soft citrus, orange varieties, lemons and limes, which are considered to be high-value citrus products (Chisoro and Roberts, 2023). The vulnerability of high-value citrus fruit to postharvest diseases is a global challenge. Use of biocontrol agents in disease management practices to achieve complete protection of oranges from two fungal pathogens was investigated in this study.

Bacterial and yeast-based biocontrol agents have been reported previously, with a few strains being commercially produced for the control of *Penicillium* moulds of citrus (Ladaniya 2010; de Cunha *et al*., 2020). In general, yeast and bacterial strains are isolated from various sources, including from citrus fruit and leaves, and then screened for their potential as postharvest biocontrol agents (Liu *et al*., 2013). This study aimed to investigate the effect of yeast and bacteria on the growth and disease spread *Penicillium* moulds caused by *Penicillium italicum* and *Penicillium digitatum in vitro* and *in vivo*.

Summary of Significant Findings

Chapter 2: Isolation and *in vitro* Screening of Potential Biocontrol Agents for The Biological Control of *Penicillium digitatum and Penicillium italicum*

Major findings:

- Two isolates showed potential as biocontrol agents, from the 102 isolates originally isolated, which confirms the necessity of isolating many microorganisms to find isolates with high efficacy.
- Strains SCO13 and UK37 successfully inhibited the mycelial growth of *P. italicum in vitro* by 94.23% and 96.89%, respectively.
- Strains SCO13 and UK37 successfully inhibited the mycelial growth of *P. digitatum in vitro* by 95.29% and 98.83%, respectively.
- These two strains were identified as isolates of *Bacillus amyloliquefaciens.*
- The two yeast isolates that were evaluated in the *in vivo* trial were identified as strains of *Meyerozyma guilliermondii*.

- *Bacillus* spp.. typically compete for space and nutrients, thus supressing the growth of the pathogens. However, some strains also secrete adjuvants that destroy the mycelia of fungi.

Chapter 3: Screening of two strains of *Bacillus amyloliquefaciens* and two antagonistic yeasts for the postharvest management of *P. digitatum* and *P. italicum* on "Valencia" oranges

Major findings:

- The two strains of *P. digitatum* and *P. italicum* strains used in the trials were highly pathogenic.
- The two strains of *B. amyloliquefaciens* provided better control of *P. digitatum* and *P. italicum* than the two strains of *M. guilliermondii in vivo.*
- *Bacillus amyloliquefaciens* Strain SCO13 and Strain UK37 were able to inhibit the spread of blue mould by 80.36% and 60.47%, respectively.
- *Bacillus amyloliquefaciens* Strain SCO13 and Strain UK37 were able to inhibit green mould disease spread by 64.13% and 48.63%, respectively.
- Scanning electron microscope (SEM) images show the presence of *Bacillus* cells and *M. guilliermondii* spores *in vitro* and on the surface of orange fruits. The SEM images of the mycelial growth of *P. digitatum* in the presence of the two bacteria showed shrivelled hyphae and a reduced number of conidial spores, both *in vitro* and *in vivo*.
- Disease incidence in the controls for both pathogens on all treatments was 100%

Recommendation and conclusion

To minimise blue and green mould on citrus, affordable, non-toxic methods are needed. Biocontrol agents used in this study shown inhibitory effects on blue and green mould, although none of the treatments achieved complete control of the pathogens. Exploring the different pH for the bacterial cultures to assess their efficacy at optimum levels which can provide clarity on the efficacy of the *Bacillus* spp. To further investigate the finding of this study, the investigation of coatings incorporated with *B. amyloliquefaciens* Strains SCO13 and UK37 on the shelf-life, antioxidant activity, phenolics and quality of oranges would be useful. Investigations of their mode of action against fungi would also be useful.

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Appendix 1

1.1.Mycelial growth of *P. digitatum* during primary screening isolates after seven days *in vitro*

Treatment	**Avg mycelial growth of *P.digitatum* (mm)**
Control	85
UK20	79
UK21	64
UK22	81
UK23	62
UK24	59
UK25	61
UK26	54
UK27	46
UK28	56
UK29	45
UK30	66.67
UK31	71
UK32	74
UK33	42
UK34	39
UK35	26
UK36	47.33
UK37	1
UK38	28
UK39	25
UK40	41
UK41	33
UK42	45
UK43	47.33
UK44	30
UK45	34
UK46	59
UK47	78
UK48	74
UK49	46
UK50	56
UK51	42
UK52	54
UK53	74
UK54	71
UK55	63
UK56	34
UK57	59
UK58	7
UK59	27.67
UK60	30
UK61	35
UK62	68
UK63	27
UK64	53
UK65	38
UK66	59
UK67	45

UK68	23
SCO1	20
SCO2	15.67
SCO3	20
SCO4	24
SCO5	27
SCO6	28
SCO7	33
SCO8	21
SCO9	27
SCO10	39.33
SCO11	47
SCO12	44
SCO13	1
SCO14	22
SCO15	37
SCO16	49
SCO17	54
SCO18	24
SCO19	45
SCO20	42
SCO21	66
SCO22	68
SCO23	69
SCO24	77.33
SCO25	21
OZW1	81
OZW2	79
OZW3	71
OZW4	69
OZW5	64
OZW6	60
OZW7	59
OZW8	43
OZW9	39
OZW10	62
OZW11	65
OZW12	68
OZW13	61
OZW14	72
OZW15	77.33
OZW16	55
OZW17	54
OZW18	32
OZW19	69
OZW20	78
OZW21	71
OZW22	63
OZW23	65
OZW24	80
OZW25	79
OZW26	56
OZW27	48

Table 1.2. Mycelial growth of *P. italicum* during primary screening isolates after seven days *in vitro*

Treatment	**Avg. mycelial growth of *P. italicum* (mm)**
Control	75
UK20	70
UK21	66
UK22	58
UK23	45
UK24	36
UK25	69
UK26	61
UK27	56
UK28	32
UK29	45
UK30	44
UK31	49
UK32	42
UK33	49
UK34	58
UK35	34
UK36	67.67
UK37	2
UK38	13
UK39	38
UK40	23
UK41	44
UK42	16
UK43	19
UK44	21
UK45	22
UK46	26
UK47	28
UK48	34
UK49	46
UK50	38
UK51	18
UK52	13.33
UK53	29
UK54	36
UK55	45
UK56	15
UK57	18
UK58	5
UK59	36
UK60	13
UK61	66
UK62	46
UK63	69
UK64	56
UK65	21
UK66	26
UK67	35
UK68	44

SCO1	20
SCO2	13
SCO3	25
SCO4	46
SCO5	80
SCO6	66
SCO7	12
SCO8	34
SCO9	16
SCO10	49
SCO11	55
SCO12	59
SCO13	2
SCO14	40
SCO15	65
SCO16	69
SCO17	33
SCO18	48
SCO19	78
SCO20	45
SCO21	62
SCO22	69
SCO23	70
SCO24	70
SCO25	53
OZW1	67
OZW2	64
OZW3	63
OZW4	54
OZW5	46
OZW6	41
OZW7	36
OZW8	38
OZW9	25
OZW10	42
OZW11	45
OZW12	58
OZW13	44
OZW14	62
OZW15	58
OZW16	39
OZW17	22
OZW18	26
OZW19	50
OZW20	64
OZW21	53
OZW22	47.33
OZW23	49
OZW24	62
OZW25	52
OZW26	33
OZW27	28