THE EVALUATION OF THE EFFECTS OF BIOLOGICAL CONTROL AGENTS AND MELATONIN AGAINST *FUSARIUM OXYSPORUM* INFECTING POTATOES

By

Londeka Akhona Mbatha

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Dissertation Summary

Potatoes (*Solanum tuberosum* L.) are essential to the South African agricultural industry. Postharvest diseases, including *Fusarium* dry rot (FDR) caused by *Fusarium oxysporum* (S.), are detrimental to the potato industry, resulting in yield and economic losses. Currently, fungicides are the primary means of controlling the disease commercially. However, the use of synthetic chemicals has reduced their efficacy against some *Fusarium* strains. Therefore, there is a need for safer, more sustainable, and environmentally friendly control methods for the management of postharvest fungal diseases such as FDR. Biological control agents (BCAs) and generally regarded as safe (GRAS) products have inhibitory effects against various pathogens and can be used as alternatives to synthetic chemicals. The aim of the study was to evaluate the efficacy of biological control agents (yeast and bacteria) and melatonin, individually and in combination, against *F. oxysporum in vitro* and *in vivo*. To evaluate the efficacy of the potential BCAs, 30 isolates were obtained from the exocarps and leaves of various crops, namely, potatoes (*Solanum tuberosum*), sweet potatoes (*Ipomoea batatas*), erect prickly pear (*Opuntia stricta*), Ganoderma mushrooms (*Ganoderma resinaceum*, *Ganoderma austroafricanum*,) and tomatoes (*Solanum lycopersicum*). The isolates were screened against *F. oxysporum* using the streaking method. Isolates NG1, NG2, NG3, NG4, and KG1 inhibited the growth of *F. oxysporum* on potato dextrose agar by more than 50% and were selected for secondary screening. In the secondary screening, isolates NGI (60%) and NG2 (62%) significantly inhibited mycelial growth of *F. oxysporum* on PDA compared to the control. *In vivo*, isolates NG1 and NG2 significantly reduced the severity of FDR on inoculated 'Sifra' potato tubers and had disease severity percentages of 47,2% and 46,1%, respectively, compared to the untreated control. BLAST prediction identified isolate NG1 as the bacterial species *Burkholderia cenocepacia* (strain LMG 16656) and isolate NG2 as *Bacillus amyloliquefaciens* (strain MPA 1034). The efficacy of melatonin was determined by screening six concentrations (0 µM; 1 µM; 10 µM; 15 µM; 50 µM and 100µM) of the phytohormone against *F. oxysporum* using the disc fusion method. The best- performing melatonin concentrations were selected for secondary screening and progressed to *in vivo* screening. Melatonin concentration of 100 µM had the highest inhibition percentage (40,83%) of *F. oxysporum in vitro* compared to the untreated control. The second and third best concentrations were 15 µM and 50 µM with inhibition percentages of 35,83% and 34,17%, respectively. The efficacy of melatonin as an antifungal agent, was significantly lower *in vitro*

compared to the *in vivo* results. *In vivo,* the 'Sifra' potato tubers treated with 100 μM showed the lowest FDR severity of 58,57%, followed by 50 μM and 15 μM with disease severity percentages of 59,54% and 60,31%, respectively. Subsequently, the effects of the integration of best-performing BCAs and melatonin were evaluated. BCAs, (*Burkholderia cenocepacia* and *Bacillus amyloliquefaciens*) were integrated with six melatonin concentrations and screened against *F. oxysporum in vitro* and *in vivo*. *In vitro,* treatment *Bamy* + MEL100 had the highest mycelial growth inhibition percentage (59,92%), followed by *Bamy* + MEL15 and *Bamy* + MEL50 with mycelial growth inhibition percentages of 56,12% and 55,27%, respectively. 'Sifra' potato tubers treated with treatment *Bamy* + 100 μM melatonin had the lowest disease severity of FDR of 50,61% compared to control treatment after 9 days at 25℃. *Bcen* + 100 μM melatonin and *Bamy* + 50 μM melatonin exhibited the disease severity percentages of 52,63% and 59,72%, respectively. Overall, tubers treated with a combination of melatonin and *B. amyloliquefaciens* showed less severe symptoms of FDR across all concentrations. Furthermore, the effects of the exogenous application of *B. amyloliquefaciens* and melatonin on the quality parameters of the potato tubers were evaluated. The treatments were administered to tubers using the dipping method and stored at ambient temperature for 14 days. The treated tubers were sampled and prepared for phenolic, protein, and ascorbic acid determination assays. The exogenous application of 100 μM melatonin combined with *B. amyloliquefaciens* was the most effective treatment with the highest phenolic content (144,1 mg GAE/g DW), and protein content (68 mg/g DM). Tubers treated with melatonin had the highest ascorbic acid content (5,48 mg AAE/100g DM). Findings indicated that the exogenous application of melatonin and *B. amyloliquefaciens* do not have adverse effects on the quality parameters of potato tubers. This study demonstrated for the first time, the efficacy of melatonin as an antifungal agent against *F. oxysporum* causing FDR on potatoes and the effectiveness of the combination of melatonin and the antagonistic microorganism, *B. amyloliquefaciens* against *F. oxysporum*.

Declaration

I, **Londeka Akhona Mbatha**, declare that:

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

(ii) This thesis has not been submitted for any degree or examination at any other university.

(iii) This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

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Where other written sources have been quoted, then:

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Signed:  Date: 04 December 2023

Londeka Akhona Mbatha (Student)

Signed:  Date: 04 December 2023

Dr Nokwazi Mbili (Supervisor)

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Sontshikazi!

Dedication

To my precious baby boy, Philasande “NINO” Mbatha

Contents

Chapter 1

Dissertation Introduction

1.1 Background

Potatoes (*Solanum tuberosum* L.) are one of the economically important crops grown in South Africa (SA) (FAOSTAT, 2021). They are significant contributors to the total gross value of vegetable production in SA, contributing approximately 2.3 million tons annually between 2010 and 2020 (DALRRD, 2022). The South African potato industry is spread over 52 407 hectares and comprises sixteen commercial growing regions (Denner *et al.*, 2012a). Overall, the industry produces approximately 42637.7 kg/ha annually, which has a value of R7.45 billion (FAOSTAT, 2021). Potato production contributes 45% (R14.35 billion) towards the total vegetable production in SA and 3% towards the total potato production of the top six potato producing African countries (FAO, 2022).

The production of this economically important vegetable is often hindered by pathogens that infect the plant both pre and postharvest, which result in significant yield and economic losses. Fungal diseases result in great yield losses in the agricultural sector. In developing and non-developed countries, up to 50% of the yield losses is due to fungal infections (Kahramanoğlu *et al*.,2020). This study investigates the most important postharvest fungal potato disease, *Fusarium* dry rot (FDR), caused by *Fusarium oxysporum*. The pathogen infects potato seeds and eventually the tubers as the infection progresses (Bojanowski *et al.*, 2013). The devastating symptoms of the disease include the reduction of tuber size, shelf-life, tuber quality, and overall yield of potatoes (Tiwari *et al.*, 2020).

The loss of sensitivity to the fungicides used to control the fungal pathogen and the increasing amount of toxic residues on the potato plant and tubers are also major contributors to the significant economic losses in the potato industry. Therefore, new and safer control strategies will inhibit disease infestation, increase potato yield, and overall potato tuber quality, and maximise profit. BCAS and GRAS products are promising means for controlling pre and postharvest diseases on vegetables (Janisiewicz and Korsten, 2002).

1.2 Problem statement

Using synthetic fungicides is the most inexpensive and easily accessible way of controlling fungal diseases (Jawed *et al.,* 2019). Fungicides used to control FDR can be applied both pre- and postharvest. Preharvest treatments are applied on the seeds before planting, and postharvest treatments are applied on the harvested tubers as a preventative control measure (Fravel *et al.*, 2005). In SA, fungicides such as benzimidazole, 2-aminobutane, imazalil, flusilazole, difenoconazole and thiabendazole are used to control FDR on ware and seed potatoes (Gachango *et al.*, 2012; Jawed *et al.*, 2019; Tiwari *et al.*, 2020). Thiabendazole is the most effective and excessively used synthetic chemical (Tiwari *et al.*, 2020). However, with rapid fungal mutation, introduction of new cultivars and excessive use of fungicides, these fungicides have lost their efficacy against some of the *Fusarium* species (Fravel *et al.*, 2005; Bojanowski *et al.*, 2013; Jawed *et al.*, 2019). Therefore, there is a need for alternative control strategies that are less prone to resistance and do not leave toxic residues on the potato tubers.

1.3 Justification

Biological control agents have been widely studied as potential control agents that can reduce disease infection with minimum toxic residues that cause undesirable effects on consumers (Tozlu *et al.*, 2018; Abd-Elgawad, 2020). Fungal diseases result in significant yield losses in the agricultural sector (Denner *et al.*, 2012). Ishii and Holloman (2015) reported that chemical fungicides are slowly losing efficacy against fungal pathogens. Previous studies showed the successful of integrating chemical and cultural control strategies to improve the efficacy of fungicides in controlling the disease (Zhang *et al*., 2017; Leng *et al*., 2022). However, this approach does not resolve the problem of harmful and toxic residues on the tubers and the insensitivity of pathogens towards synthetic fungicides (Pasche *et al.*, 2004). Furthermore, most harmful synthetic fungicides are being discontinued, creating a gap in the market for new alternative control agents (Runkle *et al.*, 2017; Gupta, 2018). Therefore, further studies on the use of non-toxic and safe biological control agents are needed to mitigate the problem.

Studies on BCAs have resulted in the formulation of target-specific BCAs, such as bio-insecticides, bio-fungicides, bio-stimulants, and bio-inoculants (Andermatt, 2022). These

products have different non-toxic and generally regarded as safe active ingredients, which include essential oils, non-pathogenic bacterial and fungal species, phytohormones, and plant extracts (Singh and Deverall, 1984; Moustafa-Farag *et al.*, 2019; Bhattacharya *et al.*, 2021). The BCAs have been proven to control and suppress diseases on different crops caused by different species. However, no biological control agent has been proven to have antifungal activity against *F. oxysporum* infecting potatoes.

Phytohormones are low molecular weight natural products that regulate the physiological and developmental processes of plants (Piotrowska and Bajguz, 2011; Fahad *et al.*, 2015) . Melatonin (N-acetyl-5-methoxytryptamine) has valuable characteristics that improve the physiological functions of plants, such as stress tolerance, promoting propagation, and growth and development (Nawaz *et al*., 2016). Studies have shown that melatonin has high efficacy as a recover-bioagent for chemically damaged soils and a natural antioxidant (Kolář and Macháčková, 2005). Amongst other attributes of melatonin in plants, literature has shown that melatonin concentration increases with exposure to stress, giving the plant stress resistance. The hormone upregulates antioxidant enzymes such as catalysts, peroxidases, and superoxide (Hardeland and Poeggeler, 2003; Nawaz *et al*., 2016). However, most studies on using melatonin as an antimicrobial agent have been reported against pathogens-infecting animals and humans. Very few studies have been conducted on pathogens infecting vegetable crops.

1.4 Research aim and objectives

The aim of the study was to evaluate the effect of various BCAs isolated from various plants and mushrooms and the effect of melatonin against *F. oxysporum* causing *Fusarium* dry rot in potatoes. The research objectives were as follows:

1. Evaluation of the *in vitro* and *in vivo* effects of BCAs against *F. oxysporum*
2. Evaluation of the *in vitro* and *in vivo* effects of melatonin against *F. oxysporum*
3. Evaluation of the integrated effects of BCAs and melatonin against *F. oxysporum*

1.5 Dissertation structure

The dissertation was written in the form of six independent chapters, where each chapter is focused on a specific objective of the research conducted, except for chapter 1 (dissertation introduction), chapter 2 (literature review), and chapter 6 (dissertation overview). Each chapter follows the format of a stand-alone research paper. There is, therefore, some unavoidable repetition of references, methods, and introductory information among chapters.

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Chapter 2

Management of *Fusarium* dry rot caused by *F. oxysporum* on potatoes: A review

Abstract

Potatoes (*Solanum tuberosum*) are the major contributors to the South African agricultural industry. Potato production is hindered by preharvest and postharvest factors that differ from region to region. Abiotic and biotic factors, climate, improper farming techniques, and poor postharvest handling practices are limiting factors in the potato industry. These limitations subsequently result in significant yield losses due to postharvest fungal diseases. *Fusarium oxysporum* is a fungal pathogen caused by *Fusarium* dry rot (FDR) on ware and seed potatoes. *F. oxysporum* produces mycotoxins which have harmful effects on human health. Overall, controlling the infection of *Fusarium* species is essential to ensure minimal mycotoxin contamination on potatoes. Current control strategies of FDR include using moderately resistant cultivars, fungicides, and integrated pest and disease control strategies. This review described the status of the use of control methods such as, chemical control, physical control, generally regarded as safe substances (GRAS), and the use of biological control agents (BCAs) against *F. oxysporum* on potatoes. Furthermore, this review highlights the symptoms, epidemiology, and control strategies of *Fusarium* dry rot with more focus on the use of BCAs and melatonin as alternative control measures of Fusarium dry rot caused by *F. oxysporum* on potatoes.

Keywords: Biological control, GRAS, Integration

2.1 Introduction

Potato (*Solanum tuberosum* L.) is the fourth leading crop commodity worldwide in global vegetable production (FAO, 2022). *S. tuberosum* falls under the *Solanaceae* family alongside other economically essential vegetables such as tomato (*S. lycorpersicum*) and eggplant (*S. melongena*) (Spooner and Hetterscheid, 2006; Shah *et al.*, 2013). Potatoes are cool-temperature crops native to the Peruvian-Bolivian Andes region of South America (Spooner and Hetterscheid, 2006). However, their domestic production only started in Europe in the seventeenth and eighteenth centuries and expanded to the rest of the world after the second half of the twentieth century (de Haan and Rodriguez, 2016). Due to the rapid spread and successful adaptation in different climatic areas, the crop has more than 5000 cultivars grown in more than 102 countries (Spooner *et al.*, 2014; Castañeda-Álvarez *et al.*, 2015). In 2020, China was the leading potato-producing country, producing 22% (78 237 000 tons) of the world's potato production, followed by India and Ukraine (FAO, 2022).

In 2020, the African region ranked fifth with a total output of 7.3% of the world's potato production (FAO, 2022). Despite the low ranking compared with the other regions, there has been a notable increase in the region's overall potato production since the increase of growing areas in 1990 (Jennings *et al.*, 2020). In Africa, the leading countries are Egypt, Algeria, and South Africa in the third position (FAO, 2022). Potatoes are economically important due to their significant role in improving food and nutritional security in developing countries in Africa, Latin America, and Asia (Devaux *et al.*, 2020). Potato production is hindered by preharvest and postharvest factors that differ from region to region. Abiotic and biotic factors, climate, improper farming techniques, and poor postharvest handling practices are limiting factors in the potato industry. These limitations subsequently result in significant yield losses both preharvest and postharvest. Studies show that approximately 40% of the world’s food is lost during the early stages of preharvest and processing (Beretta *et al.*, 2013).

Postharvest potato fungal diseases are a global problem in the potato industry (Savary *et al.*, 2012). *Fusarium* dry rot is one of the common postharvest diseases infecting potatoes. Yield losses related to this disease range from 6-25% in storage (Bojanowski *et al.*, 2013). Since different diseases can infect potatoes concurrently, this results in devastating losses. Management strategies are applied to control fungal infections in potato plants and tubers. The control strategies include using moderately resistant cultivars, fungicides, and integrated pest and disease strategies. Even though these control measures are employed to halt the spread and damage caused by fungal pathogens, the latter continue to be a massive problem in the potato and agricultural industries. This review describes the management strategies currently used to combat *Fusarium* dry rot in South Africa: chemical control, biological control agents, and cultural practices. Furthermore, the potential of alternative control agents will be explored. Postharvest management of fungal diseases is an essential aspect of the South African potato production. Hence, this review aims to highlight the symptoms, epidemiology, and control strategies of *Fusarium* dry rot with more focus on the use of BCAs and melatonin as alternative control measures of Fusarium dry rot caused by *F. oxysporum* on potatoes.

2.2 Nutritional and quality attributes of potato

Potato plants are herbaceous perennial plants with spiral and alternately arranged leaves. The herbaceous plant is cultivated for direct human consumption of its tubers as they are a rich source of dietary fiber, vitamin C, starch, antioxidants, and proteins (Westermann, 2005; Liu *et al.*, 2021; Xue *et al.*, 2023). Figure 2.1 shows the nutritional content of potatoes. Potato production significantly alleviates food insecurity, especially in less developed countries (Devaux *et al.*, 2020). Potatoes are also a good source of polyphenols and antioxidants that benefit human nutrition and health (Table 2.1) (Rasheed *et al.*, 2022). In SA, approximately 20% of the total annual potato production is processed into fresh and frozen chips and canned products (Visser, 2012). Among other commercial uses of potatoes are glycogen production, vegetative propagation, and alcohol production (Woolfe *et al.*, 1987; Kanter and Elkin, 2019).

Table 2.1: Health benefits of consuming potatoes

Health Benefit	**Beneficial Compound**	**Reference**
Anti-hyperlipidemic	Starch Fiber Glycoalkaloids Phenolics Potato protein and peptides	Visvanathan *et al* (2016); Burgos *et al* (2020); Raigond *et al* (2023)
Anti-inflammatory	Anthocyanins Glycoalkaloids Vitamin C	Konczak and Zhang (2004); Burgos *et al* (2020); Raigond *et al* (2023)
Anti-cancer	Chlorogenic acid Anthocyanins Glycoalkaloids Fiber	Reddivari *et al* (2010); Burgos *et al* (2020)
Anti-hypertensive	Anthocyanins Potassium Potato protein and peptides	Reddivari *et al* (2007); Burgos *et al* (2020)
Anti-obesity	Starch Phenolics Potato protein and peptides	Burgos *et al* (2020)

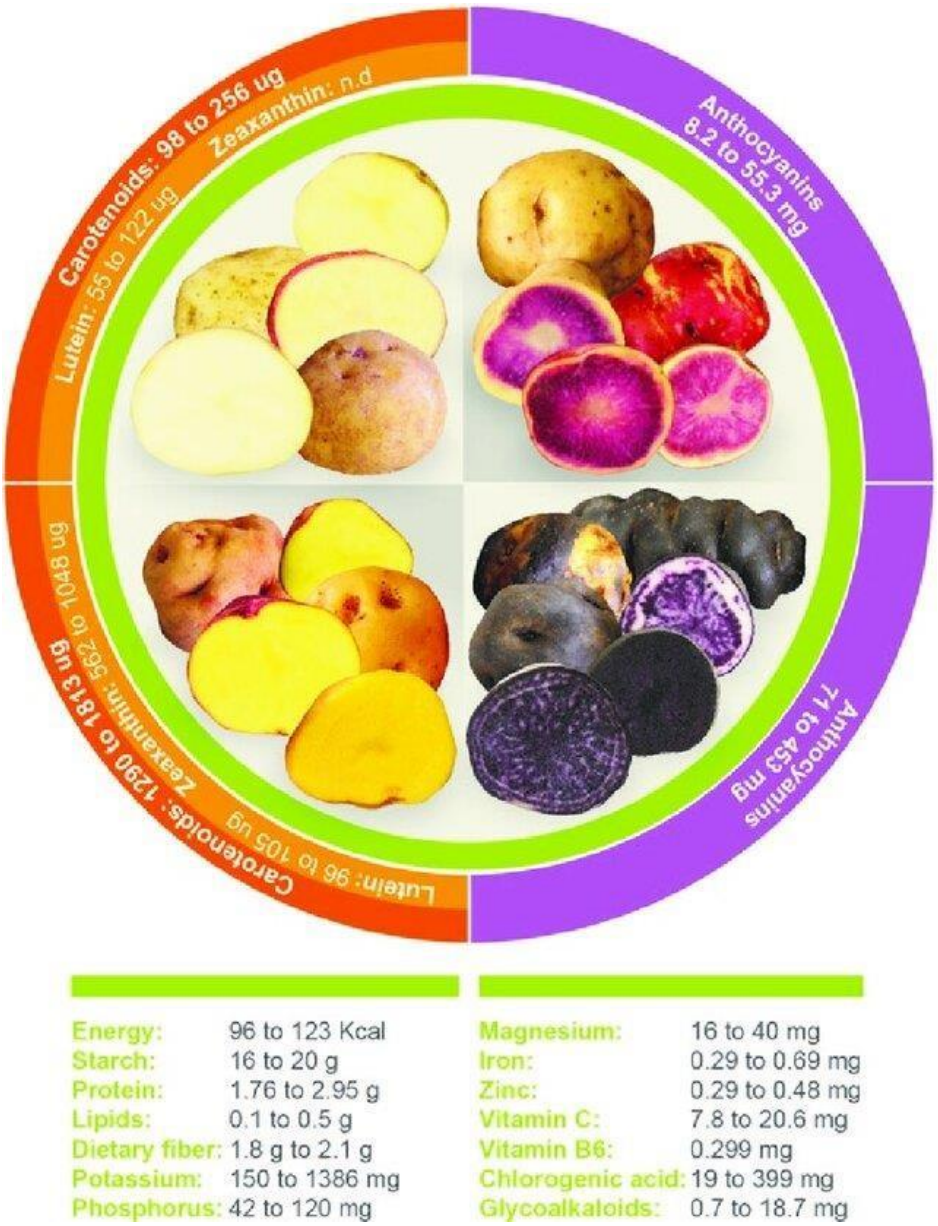

Figure 2.1: Graphic presentation of the nutritional content of potatoes based on their peel and flesh colour expressed in mg/100g Fresh Weight (Burgos *et al.*, 2020).

2.3 Potato production in South Africa

The production of potatoes is spread over 16 commercial growing regions, making up approximately 52 407 hectares of land (Denner *et al.*, 2012a). The SA potato industry plays a significant role in the agricultural sector (Denner *et al.*, 2012b). The top four leading SA production areas are Western Free State, Eastern Free State, Limpopo, and Sandveld (Denner *et al*., 2012b; DAFF, 2019). These regions produce approximately 70% of the country's total potato production and 11% of the total crop production in the continent (du Plessis, 2012; DAFF, 2019). Potato production is hindered by a plethora of abiotic and biotic factors that reduce total yield and overall tuber quality (Adaskaveg, 2012). Many diseases are caused by pests, viruses, bacterial and fungal pathogens that infect potatoes postharvest.

2.4 Preharvest and postharvest yield losses of potato

Potato diseases are widespread and are problematic in all growing regions. In SA, most common potato diseases are caused by fungal pathogens. Approximately 70% of the world's potato diseases have been recorded in South Africa (Denner *et al.*, 2012a). Therefore, it is of great importance to have alternative control measures for these diseases. There is a plethora of fungal species that cause disease in vegetables. These fungal species cause pre and postharvest infections, resulting in high yield and economic loss in the agricultural industry (Van Der Waals *et al*., 2016).

The negative effect of fungal diseases is severe in developing and non-developed countries, where up to 50% of the total yield losses occur due to postharvest and preharvest fungal infections (Kahramanoğlu *et al*., 2020). Amongst the most common fungal pathogens that infect potatoes globally, 16 are problematic in SA (Denner *et al.*, 2012a). These include *Colletotrichumcoccodes* (Wallr.), *Rhizoctonia solani* (Kühn.), *Alternaria alternata* (Fr.) Keissler, *Fusarium solani* var. *coeruleum*, *Phoma exigua* var. *foveata*, and *Alternaria solani* (S.), and *Fusarium oxysporum* (S.) (Denner *et al*., 2012a).

2.5 *Fusarium* dry rot

Fusarium dry rot (FDR) is a common soil and seed-borne disease infecting potatoes worldwide (Bojanowski *et al.*, 2013). The disease is caused by various *Fusarium* species, such as *F. solani*,

F. oxysporum, *F. sambucinun*, and *F. graminearum* (Tiwari *et al.*, 2020). However, *F. oxysporum*, *F. sambucinun*, and *F. solani* are the most common causal agents of FDR (Stefańczyk *et al.*, 2016; Li *et al.*, 2022). FDR results in significant economic losses due to growth suppression and postharvest yield losses (Bojanowski *et al.*, 2013; Tiwari *et al.*, 2020). *Fusarium* spp., including *F. oxysporum*, are mycotoxin-producing microorganisms (Tiwari *et al.*, 2020). The mycotoxins produced are pathogenicity factors that promote the development of symptoms in the tubers (Nguyen *et al.*, 2017). These toxins worsen the adverse effects of dry rot as they also negatively affect animals and humans if consumed (Stefańczyk *et al.*, 2016). The FDR is problematic in all 16 commercial growing regions, resulting in 5-15% annual postharvest losses (Denner *et al.*, 2012a). The disease is most problematic to seeds, potato cuttings and harvested tubers as spores can overwinter on infected soil debris.

2.5.1 Disease cycle and epidemiology

F. oxysporum, the causal agent of FDR, is a pre and postharvest pathogen. The disease can infect the potato seeds, resulting in seed decay, or infect the tuber, resulting in dry rot (Bojanowski *et al.*, 2013). Tuber infection is prevalent because the pathogen can over-winter in the soil and on infected potato seeds in the form of chlamydospores (Xue *et al.*, 2023). Figure 2.2 shows the disease cycle of *Fusarium* spp. causing FDR on potatoes. FDR disease occurs when tubers are infected after harvest under favorable conditions. Wounds and scars created during harvesting, handling, and transportation are the main points of infection (Yang *et al.*, 2020). The spores enter through the surface wounds of the tuber and develop hyphae that plug into the cells to initiate infection. As the infection spreads, the hyphae grow in the intercellular spaces of the live tuber cells and intracellular when the cells die (Tiwari *et al.*, 2020).

The spores spread from the infected plant debris to other tubers in storage and initiate infection where there are cuttings and surface wounds (Peters *et al.*, 2008). Sporulation and mycelial growth occur under optimum temperature and relative humidity levels. The optimum temperature for *F. oxysporum* ranges between 20-25℃ with relative humidity levels greater than 70% (Secor and Salas, 2001). However, studies have shown that dry rot can develop at cold storage at temperatures equal to and more significant than 10℃ even though at temperatures below 5℃, the infection is reduced (Bojanowski *et al.*, 2013). Extremely high temperatures above 40℃ have been proven to suppress the development of dry rot, causing pathogens completely (Daami-Rreamdi *et al.*, 2010; Tiwari *et al.*, 2020).

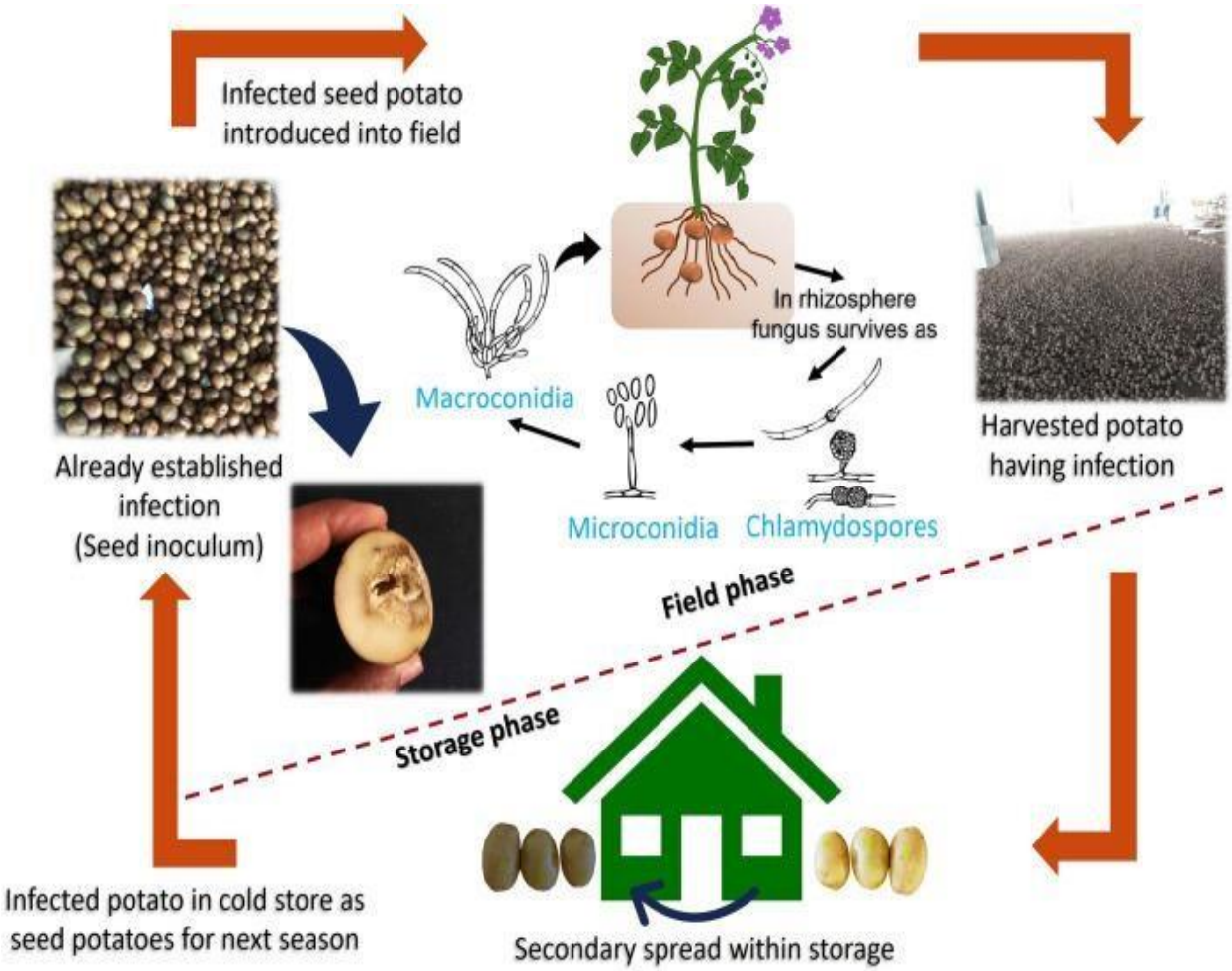

Figure 2.2: Disease cycle of *Fusarium* species on potato (Tiwari *et al.*, 2020).

2.5.2 Pathogen morphology and symptoms

F. oxysporum has ovoid-shaped microconidia produced in false heads on short monophilades and sickle-shaped septate macroconidia (Figures 2.3c and d). On potato dextrose agar (PDA), *F. oxysporum* cultures appear to have white aerial mycelium with purple-pink pigmentation (Tiwari *et al.*, 2020). The symptoms of FDR are mainly manifested on the outer and inner surfaces of the harvested tubers. The external symptoms of FDR appear as shallow dark depressions on the surface of the tuber, which can expand and become wrinkled in concentric rings as the underlying tissue dies (Secor and Salas, 2001). These symptoms grow and develop simultaneously, with the inner symptoms manifesting as brown-black internal tissue rot, as seen in Figure 2.3a and b below. Over time, the dead tissues become necrotic and produce mycelia with pigmentation variation from yellow to white to pink. Other factors contribute to the prevalence of the symptoms, including secondary infection by other postharvest and the presence of mycotoxins on infected tissue (Xue *et al.*, 2023).

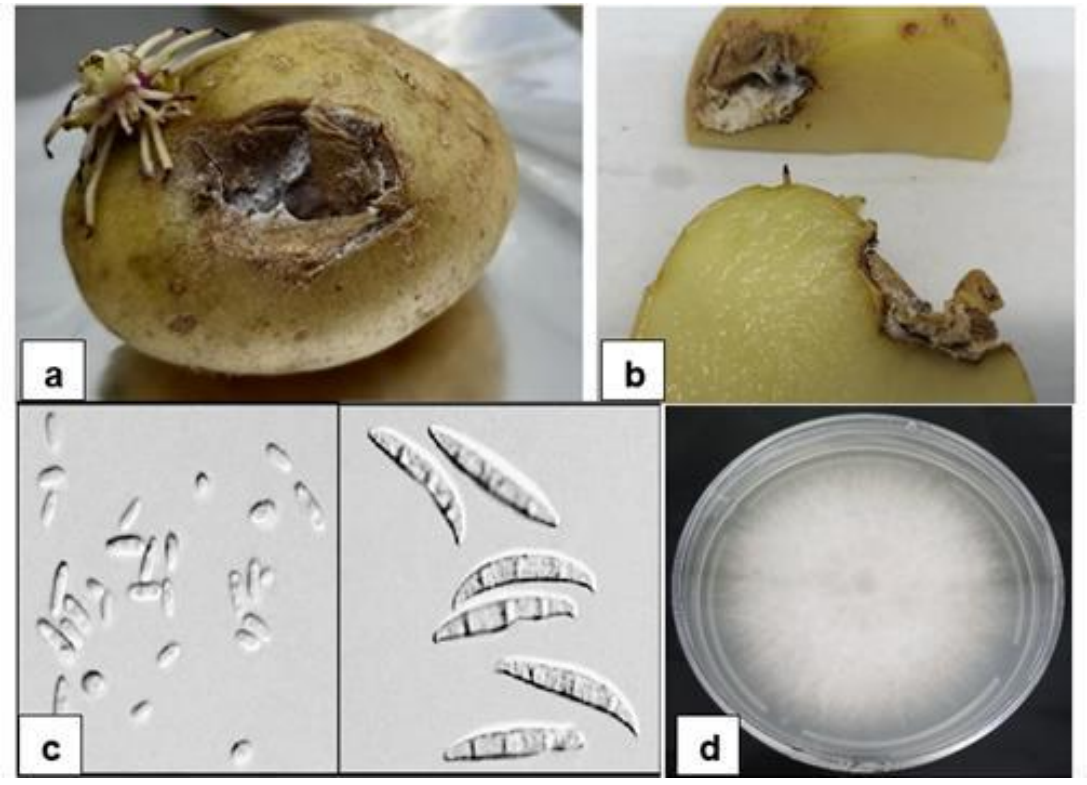

Figure 2.3: Symptoms of *Fusarium* dry rot on a potato tuber and micromorphological features of *F. oxysporum*. **a**: External dry necrotic lesion. **b**: Internal necrotic lesion. **c** microconidia and macroconidia of *F. oxysporum* (Fourie *et al.*, 2011). **d**: *F. oxysporum* on PDA.

2.5.3 Mycotoxins associated with the pathogenicity of *Fusarium* species on potatoes

Mycotoxins are secondary metabolites produced by mycotoxigenic fungi (Lee,2000; Go *et al.*, 2021). Fungal species from the *Aspergillus*, *Penicillium*, and *Fusarium* genera are the main fungal species that produce mycotoxins (El-Sayed *et al.*, 2022). These toxins are usually found on infected vegetable commodities in storage and can further contaminate by-products from infected vegetables (Nguyen *et al.*, 2017). Mycotoxins play a significant role in host-pathogen interactions, where they enhance the pathogenicity of the pathogen and aid in disease development (Xue *et al.*, 2023). The mycotoxins produced by *Fusarium* species fall under two distinct groups: non-trichothecene and trichothecene mycotoxins (Bojanowski *et al.*, 2013). Mycotoxins adversely affect humans and animals if ingested in large quantities and can cause mycotoxicosis (El-Sayed *et al.*, 2022). Other conditions induced by ingesting mycotoxins include kidney failure, liver cancer, brain damage, and nervous system malfunctioning (Richard, 2007; El-Sayed *et al.*, 2022).

Table 2.2: Mycotoxins associated with *Fusarium* dry rot caused by *F. oxysporum*

Toxin Group	**Toxin name**	**Reference**
Non-Trichothecene	Fusaric acid	Bacon *et al* (1996); El-Hassan *et al* (2007); Singh *et al* (2017)
	Fumonisin	
	Sambutoxin	
	Enniatin B	Kim and Lee (1994); Go *et al* (2021)
	Beauvericin	Kim and Lee (1994); Song *et al* (2008)
	Zearalenone	El-Hassan *et al* (2007); Song *et al* (2008)
Trichothecene	T-2 toxin	El-Hassan *et al* (2007)

The non-trichothecene mycotoxin group has the most toxins associated with FDR disease in potatoes. Fumonisin is a member of the non-trichothecene group with the most adverse effects on animals, plants, and humans (El-Hassan *et al.*, 2007). The toxin is reported to be hepatotoxic and carcinogenic, causing leukoencephalomalacia in horses, having phytotoxic effects on plants and causing oesophageal carcinoma in humans (Reddy *et al.*, 2010; Bojanowski *et al.*, 2013). Overall, controlling the infection of *Fusarium* species is essential to ensure minimal mycotoxin contamination.

2.6 Management strategies of *Fusarium* dry rot on potato

2.6.1 Cultural control

Potato diseases spread through improper in-farm and off-farm postharvest handling, resulting in significant yield loss. However, effective cultural measures minimize the infection and dispersal of such diseases (Adaskaveg *et al*., 2022). Using cultural control measures is the most economically and environmentally friendly control strategy. Cultural control practices for potato diseases include cultivating certified potato seeds and crop rotation (Hide and Read, 1991). Crop rotation is the most used cultural control method against soilborne diseases infecting potatoes (Denner *et al.*, 2012b; Honeycutt *et al.*, 1996). Furthermore, accurate and timely irrigation is a cultural control measure used to prevent over-irrigation that increases the susceptibility of the potatoes to *F. oxysporum* infection (Denner *et al*.,2012b).

Poor farming practices also contribute to the loss of tuber yield. Potatoes should be grown during the correct growing period (Denner *et* al.,2012b). Choosing a suitable cultivar and certified seeds reduces the risk of nematode and disease infestation. It is essential to ensure good storage and transportation measures to prevent losses. Wounds caused by mishandling and poor harvesting and storage strategies are the main entry points for postharvest pathogens (Fravel *et al.*, 2005). It is, therefore, essential to control and maintain the storage temperature and humidity levels to prevent diseases and maintain tuber quality. Avoiding prolonged exposure to excess moisture and allowing airflow in storage makes the condition unfavourable for pathogens and pests by slowing their life cycle (Tiwari *et al.*, 2020). Controlling the storage temperatures also allows the wounds to heal before pathogens and pests can infect them.

2.6.2 Physical control

Physical control is the management of postharvest plant diseases with the physical application of plant defense elicitors such as temperature and controlled atmosphere (Laguë *et al.*, 2001). Commonly used physical control measures on potatoes are hot water treatment (HWT) and ultraviolet-C (UV-C) (Xue *et al.*, 2023). HWT is the method where tubers are briefly exposed to specific temperatures to activate the defence mechanism of the tuber and reduce pathogen infection (Yang *et al.*, 2020). It has been used to reduce the effects of bacterial, fungal, and viral diseases on various crops (Zong *et al.*, 2010).

Studies show that potato tubers exposed to HWT significantly reduced the incidence of FDR caused by *F. solani* after being exposed to water at 57.5℃ for 20 minutes (Touba *et al.*, 2012; Shuzhen *et al.*, 2019), and *F. sulphureum* exposed to water at 45℃ for 10 minutes (Yang *et al.*, 2020). HWT does not only reduce the effects of pathogens on the tubers, but studies have also shown that the treatment increases the accumulation and activity of defence enzymes such as suberin polyphenolic, phenylalanine ammonia-lyase (PAL) and peroxidase (POD) on potato tubers and muskmelon fruits (Yuan *et al.*, 2013). HWT increases the antioxidant activity, total phenols, and H_2O_2 content (Yang *et al.*, 2020).

UV-C is used on both potato seeds and tubers to prevent infection (Xue *et al.*,2023). UV-C irradiation promotes disease resistance by inducing the defence response of the vegetable or fruit exposed to UV-C at specific wavelengths. The germicidal wavelength of ultraviolet light is between 240-280 nm. The exposure to UV-C (260 nm) at 34.5 kJm^{-2} significantly inhibited the growth of *P. carotovorum* f.sp. *carotovorum* and *F. solani* infecting potatoes (Rocha *et al.*, 2015; Xue *et al.*, 2023). UV-C irradiation induces a defence response to increase disease resistance. The exposure also increases the activity of plant defence enzymes such as catalase, peroxidase and PAL (Lin *et al.*, 2017). Physical control methods are often combined with other control methods, such as chemicals and biological control agents.

2.6.3 Chemical control

Synthetic chemicals are the most used disease control strategy in SA because they are more easily accessible and cost-effective (DAFF, 2019). Fungicides used to control FDR can be applied both pre- and postharvest. Preharvest treatments are applied on the seeds before planting, and postharvest treatments are applied on the harvested tubers as a preventative control measure (Fravel *et al.*, 2005). In SA, fungicides such as benzimidazole, 2-aminobutane, imazalil, flusilazole, difenoconazole and thiabendazole are used to control FDR on tubers and potato seeds (Gachango *et al.*, 2012a; Jawed *et al.*, 2019; Tiwari *et al.*, 2020). Thiabendazole is the most effective and excessively used synthetic chemical (Tiwari *et al.*, 2020).

However, with rapid fungal mutation, the introduction of new cultivars, and excessive use, the synthetic fungicides have lost their efficacy against some of the *Fusarium* species (Fravel *et al.*, 2005; Bojanowski *et al.*, 2013; Jawed *et al.*, 2019). *F. graminearum* and *F. asiaticum*, have

gained resistance to triazole and benzimidazole fungicides and more species are expected to gain resistance over time (de Chaves *et al.*, 2022). The problem with chemical fungicide resistance has led to the development and use of biological control agents (BCAs), GRAS substances and natural plant extracts as alternatives to chemical control strategies (Fravel *et al.*, 2005; Nguyen *et al.*, 2017).

2.6.4 Biological control

Biological control agents (BCAs) have been widely used to combat the adverse effects of carcinogenicity of chemical residues, which cause undesirable effects on the environment and consumers' health (Boro *et al*.,2022; Vatankhah *et al.*, 2019). BCAs control the spread and development of preharvest and postharvest fungal infections. BCAs prevent diseases using indirect or direct modes of action depending on the nature of the agents (Gachango *et al.*, 2012b). BCAs use the direct mode of action against pathogens by competition, antibiosis, and parasitism (Boro *et al*.,2022). In contrast, control agents with indirect methods of action control the disease by altering the pH of the soil and exhibiting direct toxicity to the fungus (Tiwari *et al.*, 2020).

Many studies that use BCAs as safer alternatives to chemical fungicides against *F. oxysporum* are listed in Table 2.3 below. However, only a few have been commercialized and registered as bio- fungicides due to lengthy formulation procedures and production costs. Registered yeast-based bio-fungicides used against fungal infections include Nexy® (*Candida oleophila* strain I-182), Protect® (*Aureobasidium pullulans* strain DSM 14940), Shemer® (*Metshnikowa fructicola* strain NRRL Y-30252) (Freimoser *et al.*, 2019). Registered bacteria-based bio-fungicides used against fungal infections include RhizoPlus® (*Bacillus subtilis* strain FZB24), YieldShield® (*B. pumulis* strain GB34), RhizoVital42® (*B. amyloliquefaciens* strain FBZ24) (Bonaterra *et al.*, 2022).

Table 2.3: Biological control agents used against *F. oxysporum* of potatoes

Category	Species	Strain	Reference
Bacteria	*Bacillus subtilis*	V26	Gachango *et al* (2012b); Khedher *et al* (2021)
		M-19	Recep *et al* (2009)
		M-34	Recep *et al* (2009)
		M-48	Recep *et al* (2009)
		M-58	Recep *et al* (2009)
		BA-140	Recep *et al* (2009)
		OSU-142	Recep *et al* (2009)
		10-4	Lastochkina *et al* (2020b)
		26D	Lastochkina *et al* (2020b)
	B. pumilis	M-38	Recep *et al* (2009)
	Burkholderia cepacia	OSU-7	Recep *et al* (2009)
	Pseudomonas putida	OSU-8	Recep *et al* (2009)
		VUPF506	Vatankhah *et al* (2019)
	P. fluorescens	VUPF40	Vatankhah *et al* (2019)
		VUPF44	Vatankhah *et al* (2019)
Fungi	*T. harzianum*	-	Daami-Rreamdi *et al* (2010); Jawed *et al* (2019); Saravanakumar *et al* (2017)
	T. viride	-	Daami-Rreamdi *et al* (2010)
	T. polysporum	-	Jawed *et al* (2019)
	Paecilomyces lilacinus	-	Jawed *et al* (2019)
	P. varioti	-	Jawed *et al* (2019)

2.6.5 Generally regarded as safe substances (GRAS)

GRAS products are of natural origin and therefore have no toxic residues compared to the effects of synthetic chemicals (Sharma *et al.*, 2018). Aromatic plants and essential oils are becoming a popular and effective way of controlling postharvest fungal diseases (Mohapatra *et al.*, 2014). Table 2.4 shows the studies that have antifungal effects against *Fusarium* species infecting potatoes and other vegetable crops. Essential oils are a natural and less toxic alternative to synthetic chemicals (Sreenivasa *et al.*, 2011). Essential oils have bioactive components that act on the fungal cell membranes' function and structure and inhibit the fungal species' growth and development (da Silva *et al.*, 2021). The essential oil mode of action targets the biosynthesis of the mycotoxins produced (Kavitha *et al.*, 2020). Perckzak *et al* (2019) reported that essential oils decrease the concentration of *Fusarium* mycotoxins. Limitations to the use of essential oils include their high volatility, poor solubility in water, and their effects on the flavour of vegetables and fruits (Kumar *et al.*, 2022). In addition to essential oils, other GRAS substances, such as edible coatings and phytohormones, can be used to control *Fusarium* diseases (Xue *et al.*, 2023).

Phytohormones are low molecular weight natural products that regulate the physiological and developmental processes of plants (Piotrowska and Bajguz, 2011; Fahad *et al.*, 2015). For this study, the focus will be on the potential of melatonin as a GRAS substance.

Table 2.4: Essential oils with antifungal activity against *F. oxysporum*

Essential Oil	Crop	Reference
Spearmint	Cucumber	Pattnaik *et al* (1996)
Cumin	Tomato	Hashem *et al* (2010)
Thyme	Tomato	Daferera *et al* (2003)
	Potato	Hamini-Kadar *et al* (2014)
	-	Bounar *et al* (2020)
Eucalyptus	Tomato	Pattnaik *et al* (1996)
Clove	Tomato	Hamini-Kadar *et al* (2014); Mohapatra *et al* (2014)
Basil	Cumin	Hashem *et al* (2010)
Oregano	Potato	Bounar *et al* (2020)
Geranium	Cumin	Hashem *et al* (2010)

2.6.6 Melatonin as a GRAS substance

Melatonin (N-acetyl-5-methoxytryptamine) is an organic compound found across many different kingdoms. This hormone was first discovered in the late 1950s in bovine pineal gland tissue (Lerner *et al.*, 1958; Moustafa-Farag *et al.*, 2019). In humans, melatonin regulates circadian rhythms such as moods, sleep, retina physiology, and immunological systems (Maronde and Stehle, 2007; Hardeland *et al.*, 2012; Carrillo-Vico *et al.*, 2013). Melatonin was only discovered in plants in 1995. Subsequently, studies have shown that it has beneficial effects against biotic and abiotic plant stressors. Melatonin acts as an antioxidant against reactive oxygen and nitrogen species. It is a protective agent against biotic and abiotic stressors (Zang *et al*., 2022). Melatonin reinforces plant physiological processes (germination, senescence, and ripening), primary and secondary metabolism, and plant hormone uptake (Zang *et al*., 2022). Melatonin also acts as a biostimulator against biotic and abiotic stress. Most studies have been done on the function of melatonin as an agent against abiotic stress, but studies on the efficacy of the phytohormone against plant pathogens are limited (Table 2.5).

Table 2.5: Melatonin concentration showing efficacy against viral, bacterial, and fungal plant pathogens

Crop	Target pathogen	Melatonin concentration (μM)	Reference
Solanum lycopersicum	Tobacco Mosaic Virus (TMV)	100	Zhao *et al* (2019)
Malus domestica	Apple Stem Grooving Virus (ASGV)	15	Chen *et al* (2019)
Arabidopsis thaliana	*Pseudomonas syringae*	1	Lee and Back (2016)
		10	Lee *et al* (2015)
		20	Qian *et al* (2015); Shi *et al* (2015)
		50	Zhao *et al* (2015)
Nicotiana benthamiana	*P. syringae*	1	Lee and Back (2016)
		10	Lee *et al* (2015)
Malus prunifolia	*Diplocarpon mali*	50-500	Yin *et al* (2013)
Musa acuminata	*F. oxysporum*	100	Wei *et al* (2017)
Fragania ananassa	*Botrytis cinerea* *Rhizopus stolonifer*	100	Aghdam and Fard (2017)
Citrullus lanatus	*Phytophthora capsici*	100	Mandal *et al* (2018)
Oryza sativa	*Rhizoctonia solani*	10	Li *et al* (2023)
S. tuberosum	*P. infestans*	10	Zhang *et al* (2017)

2.6.7 Integrated control strategies

Integration of control methods is commonly used to improve the effectiveness of the chosen control measures. Fungicides are often integrated with other control methods, such as BCAs, cultural methods, and GRAS substances, to reduce the amount of toxic residues on the crops and counteract their adverse effects on the environment. In a study by Zhang *et al* (2017), melatonin was integrated with a commercial fungicide used to control late blight diseases on potatoes. Combining the two treatments improved the efficacy of the fungicide against *Phytophthora infestans* (Mont.) and improved the shelf-life of the treated tubers (Zhang *et al.*, 2017).

Antagonist yeast species, *Wickerhamomyces anomalus* (Hansen.) was integrated with UV-C to manage *Alternaria tenuissima* (Kunze.) causing postharvest disease in potatoes (Leng *et al.*, 2022). Integrating treatments resulted in lower disease incidence. Integrating organic acid salicylic acid and *B. subtilis* significantly reduced the incidence of *P. infestans* and *F. oxysporum* causing potato diseases (Lastochkina *et al.*, 2020a). However, there is limited research on combining BCAs and melatonin as possible control agents against *F. oxysporum* causing FDR in potatoes.

2.7 Conclusion

F. oxysporum is a causal agent of a devastating postharvest disease of potatoes. The fungicide resistance problem has opened a gap for alternative, less toxic, environmentally friendly control measures. This review highlighted the currently used control measures and their shortfalls. This lays a foundation for further studies on using biological control agents and GRAS substances, such as melatonin, to control *F. oxysporum*.

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Chapter 3

Effects of biological control agents against *Fusarium oxysporum*

Abstract

Potatoes (*Solanum tuberosum* L.) are essential to the South African agricultural industry. Postharvest diseases, including *Fusarium* dry rot (FDR) caused by *F. oxysporum* are detrimental to the potato industry, resulting in yield and economic losses. Biological control agents such as yeast, bacteria, and antagonistic fungi have inhibitory effects on pathogens and can be used as alternatives to synthetic chemicals. The aim of the study was to evaluate the efficacy of antagonistic yeast and bacterial species against *F. oxysporum in vitro* and *in vivo*. 30 isolates were obtained from the exocarps and leaves of various crops, namely, potatoes (*Solanum tuberosum*), sweet potatoes (*Ipomoea batatas*), erect prickly pear (*Opuntia stricta*), Ganoderma mushrooms (*Ganoderma resinaceum*, *Ganoderma austroafricanum*,) and tomatoes (*Solanum lycopersicum*). The isolates were screened against *F. oxysporum* using the streaking method. In the primary screening, isolates NG1, NG2, NG3, NG4, and KG1 inhibited the growth of *F. oxysporum* on PDA by more than 50% and progressed to secondary screening. Isolates NG1 and NG2 significantly reduced the severity of FDR on inoculated tubers and had inhibition percentages of 60% and 62%, respectively. BLAST prediction identified isolate NG1 as the bacterial species *Burkholderia cenocepacia* (strain LMG 16656) and isolate NG2 as *Bacillus amyloliquefaciens* (strain MPA 1034). Scanning electron imaging of the interactions of *B. amyloliquefaciens* and *B. cenocepacia* with *F. oxysporum* showed mycelial deformation, poor microconidia formation, and mycelium shrinking. Based on these findings, *B. amyloliquefaciens* and *B. cenocepacia* can be used as biological control agents to control FDR caused by *F. oxysporum* on potato.

Keywords: Potato, Postharvest, *Bacillus amyloliquefaciens*, *Burkholderia cenocepacia*

3.1 Introduction

Potato (*Solanum tuberosum* L.) are essential to the South African agricultural industry. They are the fourth leading crop commodity worldwide in global production (FAO, 2022). The crop is cultivated for direct human consumption of its tubers as they are a rich source of dietary fiber, vitamin C, starch, and proteins (Westermann, 2005). With the rapid growth in their cultivation and consumption, potatoes have become significant contributors to improving food and nutritional security in most developing countries in Latin America, Asia, and Africa (Devaux *et al.*, 2020). Hence, the undoubtedly economic importance of potatoes in South Africa (SA) and globally. However, postharvest fungal diseases hinder potato production, which reduces their aesthetic value, overall quality, and marketability.

Postharvest diseases, including *Fusarium* dry rot (FDR), are detrimental to the potato industry, resulting in significant yield losses. Synthetic fungicides are used to control the occurrence of these fungal infections caused by *Fusarium* species (Fravel *et al.*, 2005). In SA, fungicides such as benzimidazole, 2-aminobutane, imazalil, flusilazole, difenoconazole and thiabendazole are used to control FDR on tubers and potato seeds (Gachango *et al.*, 2012a; Jawed *et al.*, 2019; Tiwari *et al.*, 2020). Thiabendazole is the most effective and excessively used synthetic chemical (Tiwari *et al.*, 2020). However, with rapid fungal mutation, introduction of new cultivars, and excessive use, the fungicides have lost efficacy against some of the *Fusarium* species (Fravel *et al.*, 2005; Jawed *et al.*, 2019; de Chaves *et al.*, 2022).

Synthetic fungicide resistance has led to the development and use of biological control agents (BCAs) and natural plant extracts as alternatives to chemical control strategies (Fravel *et al.*, 2005; Nguyen *et al.*, 2017). BCAs are microbial species such as yeast, bacteria, and antagonistic fungi that have inhibitory effects on pathogens (Boro *et al.*,2022). They inhibit the growth of the pathogen by either inducing plant resistance or suppressing the growth of the target pathogen (Li *et al.*, 2022). Different microbial species have been proven to control numerous bacterial and fungal pathogens, including those infecting potatoes (Recep *et al.*, 2009; Jawed *et al.*, 2019; Lastochkina *et al.*, 2020a; Khedher *et al.*, 2021).

The successful use of BCAs as alternative control has resulted in the formulation of bio-bactericides and bio-fungicides, which have bacteria, antagonistic fungi, and yeast as safer active ingredients compared to synthetic chemicals (Miastkowska *et al.*, 2020). Registered yeast-based

biofungicides used against fungal infections include Nexy® (*Candida oleophila* strain I-182), Protect® (*Aurebasidium pullulans* strain DSM 14940), Shemer® (*Metshnikowa fructicola* strain NRRL Y-30252) (Freimoser *et al.*, 2019). Registered bacteria-based biofungicides used against fungal infections include RhizoPlus® (*Bacillus subtilis* strain FZB24), YiledSheild® (*B. pumulis* strain GB34), RhizoVital42® (*B. amyloliquefaciens* strain FBZ24) (Bonaterra *et al.*, 2022). However, the commercialization process of BCA formulations has limitations, resulting in fewer options in the market (Robin and Marchand, 2019). Currently, there is no registered bio-fungicide used to control FDR infecting potatoes in SA. The aim of this study was to evaluate the efficacy of BCAs against *F. oxysporum in vitro* and *in vivo*.

3.2 Materials and methods

3.2.1 Isolation of biological control agents

The BCAs were obtained from the exocarps and leaves of randomly selected variety of crops, namely, potatoes (*Solanum tuberosum*), sweet potatoes (*Ipomoea batatas*), erect prickly pear (*Opuntia stricta*), Ganoderma mushrooms (*Ganoderma resinaceum* and *Ganoderma austroafricanum*), and tomatoes (*Solanum lycopersicum*). The samples were washed with water and 7 5 % sodium hypochlorite (Jik) to remove contaminants on their surfaces. Approximately 10 g of each plant was excised from respective parts of the plants. The various plant part samples were transferred into 250 ml conical flasks with 100 ml of distilled water. The flasks were then placed in a water bath and shaken for one hour at 130 rpm and 28°C to isolate yeast species and 80°C to isolate bacterial species. Serial dilutions were done to obtain different concentrations (10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}) from the stock solution for each sample. Each sample was plated on yeast extract peptone dextrose agar (YEPDA) and potato dextrose agar (PDA) at 28°C for two days. The colonies showing typical morphological yeast and bacterial features were sub-cultured onto fresh potato dextrose agar (PDA) and incubated at 28°C. The pure cultures of the unidentified species were stored in 20% glycerol at -80°C.

3.2.2 Pathogen isolation

F. oxysporum was isolated from potato tubers, showing typical symptoms of FDR disease. The infected parts of the tubers were excised and washed following a three-step wash regime using

70% ethanol and distilled water to remove any surface contaminations. The small cuttings were then plated on freshly prepared PDA plates and incubated at 25°C for five days. Mycelial plugs (3 mm x 3 mm) were aseptically excised from the edges of the actively growing mycelia and sub-cultured onto fresh PDA plates for apparent radial growth and incubated at 25°C. Wet mounts were aseptically prepared from 7-day-old *F. oxysporum* cultures, and the morphology was confirmed using light microscopy. The pathogen was suspended in 20% glycerol by gently scraping off the mycelia from the agar plates and stored at -80°C until further used.

3.2.3 Pathogenicity test of *Fusarium oxysporum*

The pathogenicity of *F. oxysporum* was tested on 'Sifra' potato tubers according to methods by Jawed *et al* (2019) with minor amendments. Tubers were washed with distilled water to remove soil debris, sterilised with 70% ethanol to remove contaminants from their surface, and then air-dried. Four (6 mm width x 6 mm depth) wounds were made on the upper surface of each tuber using a sterile cork-borer. Agar plugs were excised from the edges of actively growing 10-day-old cultures and placed on each wound. The wounds on the control tubers were only inoculated with sterile agar plugs. The treated tubers were neatly boxed and wrapped with thick, black plastic bags. The relative humidity level was kept high (~75%) by adding a damp paper towel to each box before storing them at 25 ℃ for three weeks. The trial was repeated twice with three replicates and five tubers per treatment. On the 21st day post-inoculation, disease incidence was calculated using the formula below.

$$\text{Disease incidence (\%)} = (\text{No. infected wounds} \div \text{Total No. of wounds}) \times 100 \quad (1)$$

To prove Koch's postulate, tissue showing typical symptoms of FDR was cut from wounds with disease incidence of FDR ranging between 75- 100%. The small cuttings were then plated on PDA and incubated at 25°C for five days. Mycelial plugs (3 mm x 3 mm) were aseptically cut from the edges of the growing mycelia and sub-cultured onto fresh PDA plates for apparent radial growth. The inoculated PDA plates were stored at 25°C for seven days. Wet mounts were aseptically prepared from 7-day-old cultures of the pathogens, and the morphology and structure were confirmed using light microscopy.

3.2.4 In vitro screening of biological control agents against *F. oxysporum*

The different isolates were screened against *F. oxysporum* using the dual culture method under the primary and secondary *in vitro* screening trials. The different BCA isolates were aseptically streak-plated on freshly prepared PDA using an inoculating loop and incubated at 28℃ for three days before being screened against *F. oxysporum.* Mycelial plugs (3 mm x 3 mm) were aseptically excised from the edges of actively growing 5-day-old *F. oxysporum* cultures and placed at the centre of freshly prepared PDA plates. Each isolate was introduced to the pathogen by streaking on either side of the plug approximately 3 cm from the mycelial plug. Only the mycelial plugs were plated at the centre of the PDA plates for the control plates. All the plates were sealed with parafilm and incubated at 25℃ for nine days. The trial was repeated twice with three replicates per treatment. The diameter of the radial mycelial growth of *F. oxysporum* was measured at 5,7, and 9 days post-inoculation. The primary screening trial data was used to calculatethe inhibition percentage using the following formula.

$$\text{Mycelial inhibition (\%)} = (dc-dt) \div dc \times 100 \quad (2)$$

where, dc= colony diameter (mm) of control, dt = colony diameter (mm) of treatment

A scale was developed to group the BCAs into four groups based on their inhibition percentages, where Class 1 had the highest inhibition percentage and Class 4 had the lowest (Table 3.1). The isolates falling under Class 1 and 2 progressed to the secondary screening trial. The secondary screening trial was conducted using the same methods as the primary one. Both trials were repeated thrice.

Table 3.1: Classification scale for grouping biological control agents according to their mycelial growth inhibition percentages

BCA ranking	Ranges of average percentage inhibition
Class 1	76 – 100%
Class 2	51 – 75%
Class 3	26 – 50%
Class 4	0 – 25%

3.2.5 In vivo screening of biological control agents against *F. oxysporum*

The top five BCAs, based on mycelial growth inhibition percentages from the *in vitro* secondary screening trial, were screened against *F. oxysporum* under postharvest *in vivo* conditions. The efficacy of the BCA isolates as potential preventative agents was tested on 'Sifra' potato tubers. Tubers were washed with distilled water to remove soil debris, sterilised with 70% ethanol to remove contaminants from their surface, and then air-dried. Thereafter, four (7 mm diameter x 7 mm depth) equidistant wounds were made on the upper surface of each tuber using a sterile cork-borer and allowed to dry for 2 hours under the laminar flow. The different BCA cultures were suspended in distilled water and transferred into 250 ml conical flasks. The cell suspension of each culture was then adjusted to a concentration of 1×10^8 cells/ml using the hemocytometer. The flasks were sealed with foil and stored at 4℃ to avoid contamination and culture degradation.

The inoculum was introduced as mycelial plugs into the wounds, whereas the BCAs were introduced to the tubers using the dipping method. The wounded tubers were dipped in each BCA isolate suspension for ten minutes and allowed to air dry for two hours before mycelial plugs excised from 7-day-old cultures of *F. oxysporum* were placed into each wound. The treated tubers were neatly boxed and wrapped with thick, black plastic bags. The relative humidity (~75%) levels were kept high by adding a damp paper towel to each box before storing them at 25 ℃ for 21 days. The trial was repeated twice with four replicates per treatment. The disease severity was estimated using the methodology described by Mejdoub-Trabelsi *et al* (2015) with minor modifications. The maximal width and depth were measured for each wound and used to calculate pathogen penetration (*p*) using the formula below as described by Lapwood *et al* (1984).

$$\text{Pathogen penetration (mm)} = \left[\frac{w}{2} + (d - 7)\right] \div 2 \qquad (3)$$

Where *w* is the maximal width of the lesion and *d* is the maximal depth of the lesion.

The pathogen penetration rate was used to determine the severity of the dry rot on the tubers using the scale below:

- Moderately severe $p \leq 5$ mm
- Severe 5 mm < p < 7 mm
- Highly severe $p \geq 7$ mm

3.2.6 Molecular identification of biological control agents

The best two isolates that significantly inhibited the growth of *F. oxysporum* were identified according to Altschul *et al* (1997). Genetic DNA was extracted from the pathogen cultures using the Quick-DNATM Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S target region was amplified using the OneTaq@ Quick load @ 2X Master mix (NEB, Catalogue No M0486) with the primers presented in Table 3.2. The PCR products were run on a gel extracted with the ZymocleanTM Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrillianDyeTM Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up KitTM, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500x1 Genetic Analyser (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample. CLC Bio Main Workbench v7.6 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).

Table 3.2:16S Primers sequences

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

3.2.7 Statistical analysis

The data collected were subjected to GenStat 23rd edition to analyse variance (ANOVA). According to Duncan's multiple range test, mean separation was done at a 5% significance level.

3.2.8 Scanning electron microscopy studies of the interactions of BCA isolates with *F. oxysporum*

The best two isolates that significantly inhibited the growth of *F. oxysporum* were observed under the scanning electron microscope at the Microscopy and Microanalysis Unit, University of KwaZulu-Natal, Pietermaritzburg. Samples were excised from dual culture PDA plates. The samples were excised to evaluate the interactions between *F. oxysporum* and the best-performing

BCA isolates. The samples were held for 2 hours in a fixation of 3% buffered glutaraldehyde and buffer washed in 0.05M sodium cacodylate buffer twice 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. In the final stage of dehydration, the samples were washed three times with 100% ethanol for 10 minutes. The samples were transferred to the Quorum K850 critical drying point dryer (CPD) basket under 100% ethanol.

During CPD, the ethanol was replaced with liquid CO_2. The CO_2 was heated and pressurized to its critical point at which the liquid converted to gas without the damaging effects of surface tension on the samples, resulting in dry, intact samples. The dried samples were then carefully mounted onto SEM stubs using black double-sided tape. The sample stubs were then transferred to the Quorum Q150R ES sputter coater. In this step, the samples were made conductive to the electron beam with two layers of gold and palladium coats and allowed to dry before being viewed with the Zeiss EVO LS15 scanning electron microscope.

3.3 Results

3.3.1 Biological control agent isolation

Thirty potential BCA species were successfully isolated from various plants. Four potential BCA species were successfully isolated from the *Ganoderma resinaceum* mushroom, two were obtained from *Solanum lycopersicum*, and three isolates were obtained from *Ipomea batatas*. *Solanum tuberosum* and *Opuntia stricta* had seven isolates, and *Ganoderma austroafricanum* had the highest number of BCA isolates, nine species (Table 1).

Table 1: Primary screening of biological control agents against *F. oxysporum in vitro* after 7 days at 25°C

Source of isolation	Treatment	Species Type	MGI (%)	Mean + SE (mm)	BCA Class
	Control			79,00±0,58	
Ganoderma resinaceum	KgX	Bacteria	22,78	61,00±0,58	4
Ganoderma resinaceum	Kg1	Bacteria	59,92	31,67±0,33	2
Ganoderma resinaceum	Kg4	Bacteria	57,81	33,33±0,33	2
Ganoderma resinaceum	Kg5	Bacteria	2,53	77,00±0,58	4
Solanum tuberosum	LPPY1	Yeast	42,19	45,67±1,86	3
Solanum tuberosum	LPPY2	Yeast	42,62	45,33±0,88	4
Solanum tuberosum	LPPY3	Yeast	13,92	69,00±1,73	4
Solanum tuberosum	LPPY4	Yeast	35,44	51,00±1,00	3
Solanum tuberosum	LPPY5	Yeast	36,29	50,33±0,88	3
Solanum tuberosum	LPPY6	Yeast	14,77	67,33±0,67	4
Solanum tuberosum	LPPY7	Yeast	21,94	61,67±1,20	4
Ipomea batatas	LSPY1	Yeast	41,35	46,33±2,85	3
Ipomea batatas	LSPY2	Yeast	40,93	46,67±4,41	3
Ipomea batatas	LSPY3	Yeast	21,94	61,67±1,20	4
Solanum lycopersicum	LTPY1	Yeast	37,55	49,33±0,67	3
Solanum lycopersicum	LTPY2	Yeast	22,78	61,00±1,00	4
Ganoderma austroafricanum	Mg3	Bacteria	55,27	35,33±0,88	2
Optica stricta	MeRo2y	Yeast	35,02	51,33±0,67	3
Optica stricta	MeRo3y	Yeast	43,46	44,67±0,33	3
Optica stricta	MeRo4y	Yeast	33,76	52,33±1,45	3
Optica stricta	MeRo5ya	Yeast	49,37	40,00±2,08	2
Ganoderma austroafricanum	NG1X	Bacteria	42,19	45,67±1,20	3
Ganoderma austroafricanum	NG1	Bacteria	60,34	31,33±0,33	2
Ganoderma austroafricanum	NG2	Bacteria	56,96	34,00±0,58	2
Ganoderma austroafricanum	NG3X	Bacteria	21,94	61,67±0,88	3
Ganoderma austroafricanum	NG3	Bacteria	59,49	32,00±1,00	3
Ganoderma austroafricanum	NG4	Bacteria	52,32	37,67±1,45	4
Ganoderma austroafricanum	NG5	Bacteria	48,95	40,33±2,60	4
Ganoderma austroafricanum	R1Y1	Yeast	37,97	49,00±0,58	4
Optica stricta	YAL	Yeast	47,68	41,33±0,67	4

3.3.2 Pathogenicity testing of *F. oxysporum*

The results showed a 100% disease incidence rate for *Fusarium* dry rot on potato tubers. All the inoculated wounds on the tubers showed typical symptoms of *Fusarium* dry rot, such as spongy dead tissue, cavities with white aerial mycelium, and dark brown flesh around the infected area (Figure 3.1). To prove identity by Koch's postulate, the isolates from the diseased parts from the tuber showed typical morphological traits of *F. oxysporum* under the light microscope (Figure 3.2).

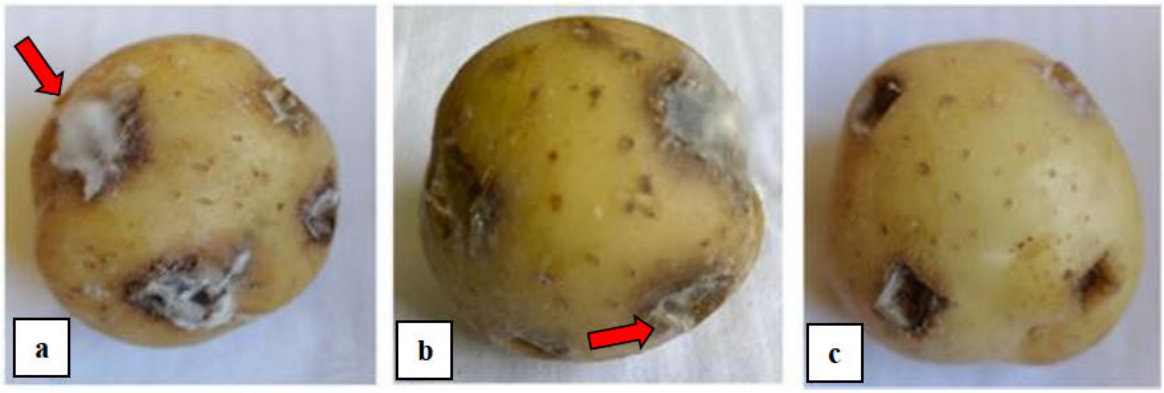

Figure 3.1: Potato tubers showing typical symptoms of FDR disease 21 days post-inoculation at 25°C. **a:** fluffy whitish-yellow mycelium shown with arrows; **b**: dark brown region around the infected area andcavity with dead spongy tissue shown with arrow. **c**: untreated control.

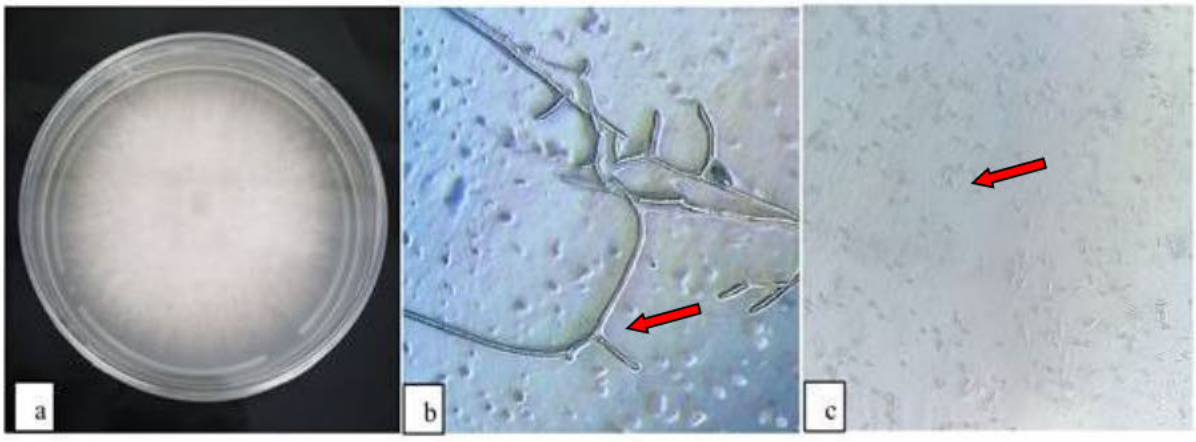

Figure 3.2: Macroscopic and microscope features of *F. oxysporum*. **a**: white-pinkish mycelia on PDA. **b**: septate mycelia branching at a 90° angle under the light microscope (40x) shown with arrow. **c**: ovoid- shaped microconidia 40x magnification shown with an arrow.

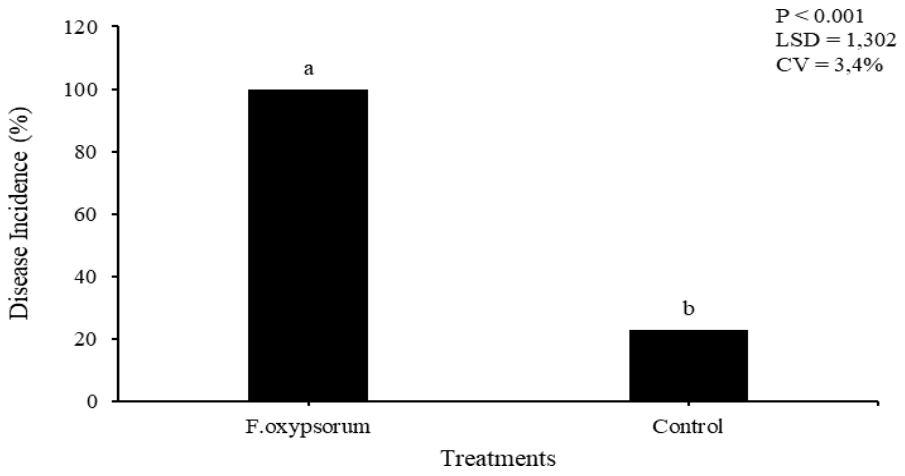

Figure 3.3: Disease incidence of *F. oxysporum* on Sifra potato tubers 21 days post-inoculation. Different letters indicate significant differences at a 5% significance level according to Duncan's multiple range test.

3.3.3 In vitro screening of BCAs against *F. oxysporum*

A total of 30 isolates were screened against *F. oxysporum* as potential BCAs. Primary screening results showed that 43% of the isolates fall under Class 3, with mycelial growth inhibition percentages >25% but <50%. Only 20% of the isolates fall under Class 2, with mycelial growth inhibition percentages >50% but <75% (Appendix Table 1). Isolates falling under class 2 were progressed to a secondary screening trial. Secondary screening results showed significant differences in the mycelial growth inhibition percentages of Class 2 BCAs after nine days (P-value < 0.001). The best-performing isolate was NG2, with an inhibition percentage of 62%, followed by NG1 and KG1, with 60% and 56% inhibition percentages, respectively (Table 3.3).

Table 3.3: Mean mycelial growth inhibition percentage (MGI) and mean mycelial growth (MMG) of *F. oxysporum* secondary screening against Class 2 BCA at 25℃.

Treatments	5 DPI		7 DPI		9 DPI	
	MMG (mm)	MGI (%)	MMG (mm)	MGI (%)	MMG (mm)	MGI (%)
Kg1	30^{ab}	32	31^{a}	52	35^{b}	56
Kg4	32^{b}	27	34^{b}	48	38^{c}	52
Mg3	32^{b}	28	36^{bc}	46	41^{d}	48
NG1	30^{ab}	31	32^{a}	52	32^{a}	60
NG2	28^{b}	35	30^{a}	54	30^{a}	62
NG3	31^{ab}	29	34^{b}	48	37^{bc}	54
NG4	32^{b}	27	36^{c}	45	38^{c}	52
Control	44^{c}	0	66^{d}	0	80^{e}	0
P-value	<0,001	-	<0,001	-	<0,001	-
LSD	2,548	-	1,870	-	2,737	-
CV (%)	4,5	-	2,9	-	3,8	-

Means with thesame letters within a column are not significantly different at 5% level of significance Duncan's multiple range test (DMRT).

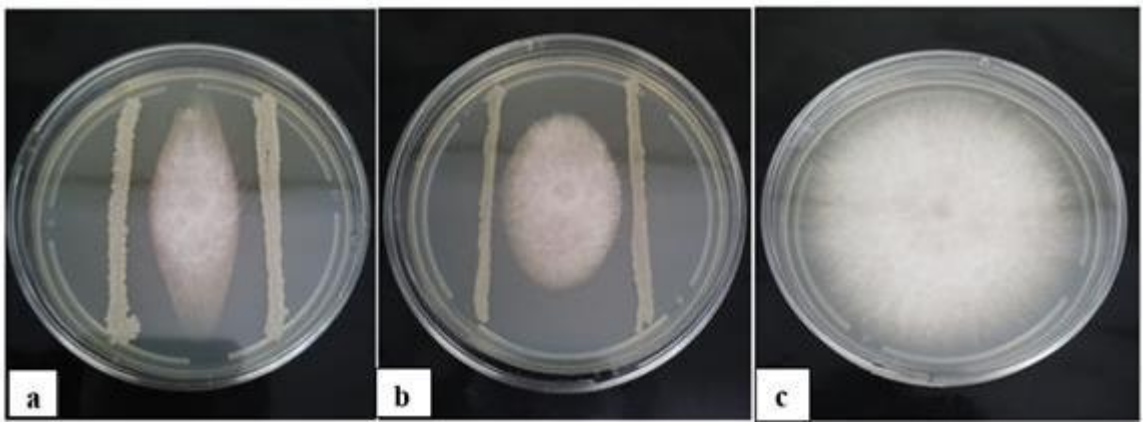

Figure 3.4: Mycelial growth inhibition of *F. oxysporum* by best two BCA isolates after nine days at 25℃ in the secondary screening trial. **a**: Interaction between isolate NG2 and *F. oxysporum* on PDA. **b**: Interaction between isolate NG1 and *F. oxysporum* on PDA. **c**: *F. oxysporum* in PDA.

3.3.4 In vivo screening of BCA against *F. oxysporum*

Five BCA isolates were screened against *F. oxysporum* under *in vivo* conditions. There were significant differences in the disease severity on tubers treated with different BCAs at a 5% significance level.

Disease severity of *F. oxysporum* was expressed in two methods: Lesion diameter and pathogen penetration methods. Tubers treated with NG2 showed the lowest FDR severity based on lesion diameter and pathogen penetration (Figure 3.5 and Figure 3.6). The disease severity percentage was 46.1%, and the pathogen penetration value was 5.39 mm. The tubers treated with NG2 were considered moderately severe according to the pathogen penetration scale. Isolate NG1 had the second lowest FDR severity with a pathogen penetration value of 6,20 mm and was ranked as severe according to the scale. The disease severity of isolate NG1 based on lesion diameter was 47.2%. The other treatments, NG3, NG4 and KG1, were highly severe, with Pathogen penetration values greater than 7 mm and disease severity percentages of 76,8%, 95,5% and 96,5%, respectively (Table 3.4 and Figure 3.5).

Table 3.4: Disease severity of potato tubers treated with Class 2 BCAs 21 days post-inoculation at 25°C in the secondary screening trial.

Treatment	Mean lesion diameter (mm)	Disease severity (%)
NG1	13,4 [a]	47,2
NG2	13,1 [a]	46,1
NG3	21,8 [b]	76,8
NG4	27 [c]	95,1
KG1	27,4 [c]	96,5
Control	28,4 [c]	100
P-value	<0,001	-
CV %	6,0	-
LSD	1,947	-

Means with the same letters within the same column are not significantly different at a 5% significance level according to DMRT.

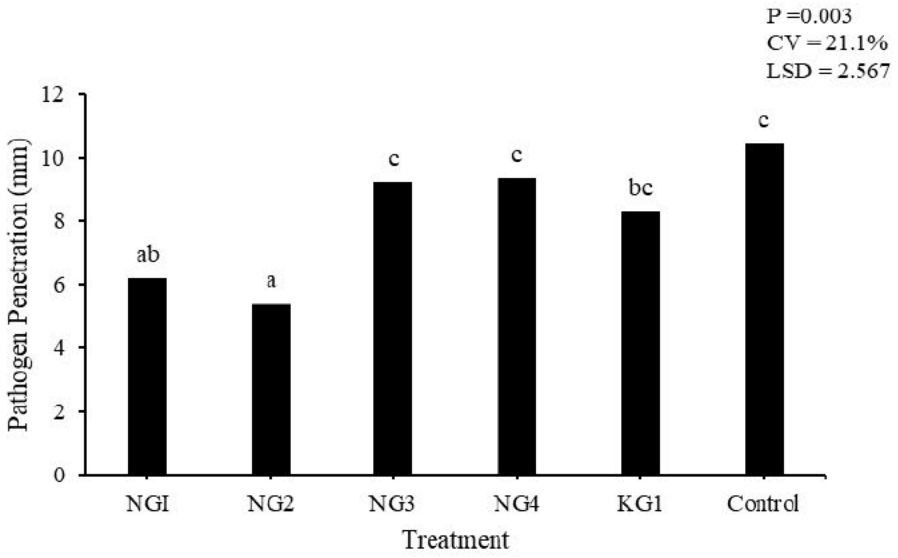

Figure 3.5: Pathogen penetration of *Fusarium* dry rot on potato tubers treated with Class 2 BCAs 21 days post-inoculation at 25°C in the secondary screening trial. Means with the same letters are not significantly different at 5% level of significance according to DMRT.

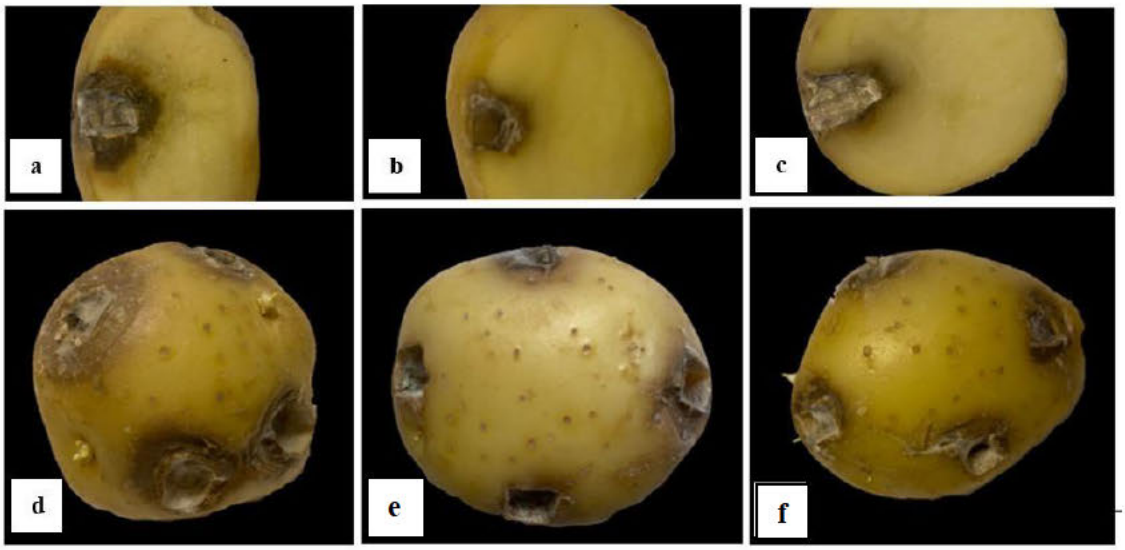

Figure 3.6: Effects of the two BCA isolates on tubers inoculated with *F. oxysporum* 21 days post-inoculation. **a** and **d**: untreated control. **b** and **e**: tuber treated with isolate NG2. **c** and **f**: tuber treated with isolate NG1.

3.3.5 Identification of biological control agents

The top two BCA isolates from the *in vivo* screening were identified using BLAST (Table 3.5). Isolates NG1 and NG2 were identified using the primers mentioned in section 1.2.6 (Table 3.2). The identity percentage was 99.76% for isolate NG1 and 99.66% for isolate NG2. *Burkholderia cenocepacia* (V.) is a gram-negative bacterial species from the *Burkholderia* genus and *Burkholderiaceae* family (Figure 3.7a). *Bacillus amyloliquefaciens* (P.) is a gram-negative bacterial species from the *Bacillus* genus and *Bacillaceae* family (Figure 3.7b).

Table 3.5: BLAST prediction results showing similarity between isolate sequences and sequences within the NCBI database 2023.

Isolate	**Predicted Organism**	**Species Type**	**Strain**	**Accession number**
NG1	*Burkholderia cenocepacia*	Bacteria	LMG 16656	CP034547.1
NG2	*Bacillus amyloliquefaciens*	Bacteria	MPA 1034	NR_117946.1

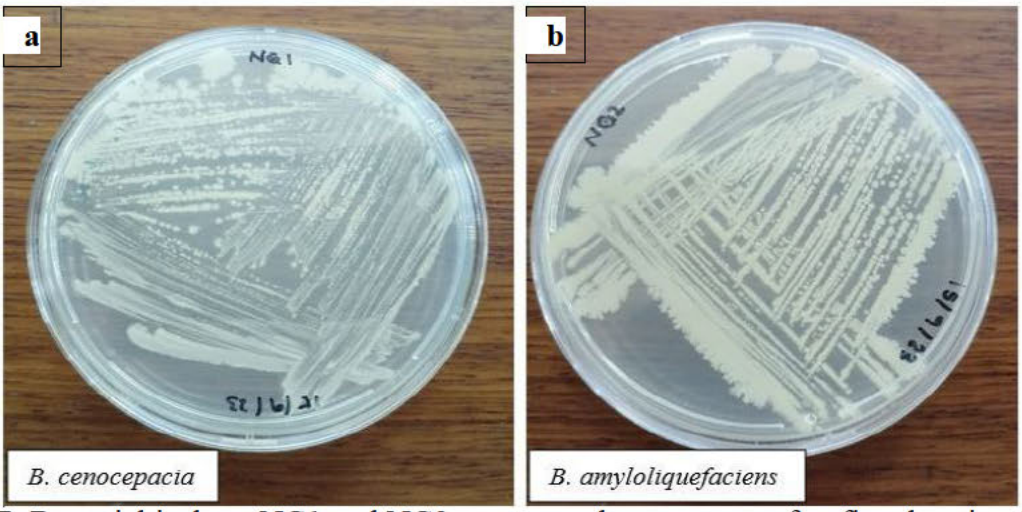

Figure 3.7: Bacterial isolates NG1 and NG2 on potato dextrose agar after five days incubated at 28°C. **a:** *B. cenocepacia*. **b:** *B. amyloliquefaciens.*

3.3.6 Scanning electron microscopy studies of the interactions of BCA isolates with *F. oxysporum*

SEM imaging was conducted to determine the mode of action of the BCA isolates screened against *F. oxysporum* under *in vitro* conditions. Samples were excised from PDA plates stored at 25°C for nine days to observe the microscopic effects of isolates NG1 (*B. cenocepacia*) and NG2 (*B. amyloliquefaciens*) on the growth of *F. oxysporum* under the SEM. The SEM images (Figures 3.8a and c) showed varying rates of mycelial deformation, poor microconidia formation, and shrinking on the samples exposed to the NG2 (*B. amyloliquefaciens*) and NG1(*B. cenocepacia*) for nine days at 25°C. Furthermore, morphological features were observed in Figures 3.8c and d, macroconidia, and microconidia of *F. oxysporum*.

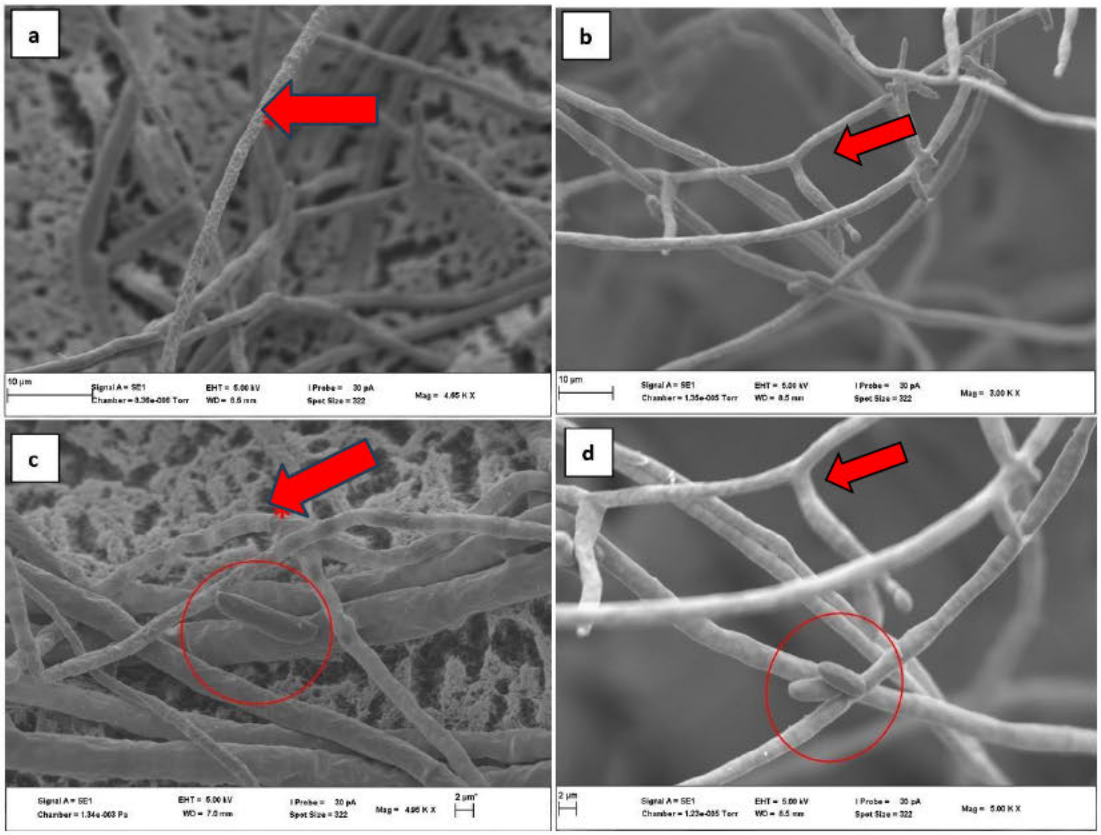

Figure 3.8: Effects of the best two BCA isolates on *F. oxysporum* under the SEM (~5000x). **a**: Shrinking effect of isolate *B. amyloliquefaciens* on *F. oxysporum* mycelia shown with an arrow. **b** and **d**: Mycelia branching at 90° angle obtained from untreated control shown with an arrow. **c**: Shrinking effect of *B. cenocepacia* on *F. oxysporum* mycelia shown with an arrow.

3.4 Discussion

F. oxysporum is the causal agent of FDR disease in potatoes, resulting in significant yield losses. The study aimed to evaluate the efficacy of different yeast and bacterial isolates as potential BCAs that can inhibit the growth of *F. oxysporum in vitro* and *in vivo*. In this study, isolates NG1 and NG2, isolated from Ganoderma mushroom, significantly reduced the adverse effects of *F. oxysporum in vitro* and on actual potato tubers. These isolates were identified as bacterial species *B. cenocepacia* and *B. amyloliquefaciens*, respectively. Many BCAs have been proven to reduce the adverse effects of various *Fusarium* species infecting different crops, including *F. oxysporum* (Nguvo and Gao, 2019).

Various bacterial species have antimicrobial effects against *F. oxysporum* species, causing disease in cucumbers, cereal, tomatoes, and bananas (Recep *et al.*, 2009; Abdelrahman *et al.*, 2016; Raza *et al.*, 2017). *B. cenocepacia* is the most common and prominent member of the *Burkholderia cepacia* complex (Bcc) (Ryall *et al.*, 2008; Wong *et al.*, 2016). The Bcc is a group of nine closely related bacterium with a wide range of functions ranging from opportunistic human and plant pathogens to biological control agents (Parke and Gurian-Sherman, 2001). Some Bcc species have inhibitory effects against soilborne and foliar fungal pathogens, such as *Phytium, Fusarium*, and foliar *Alternaria* species (Mussa, 2011; Bijitha and Bhai, 2019).

The Bcc species also have strains that have inhibitory effects against postharvest plant pathogens such as *Botrytis cinerea*, *Penicillium expansum*, and *F. oxysporum* (Parke and Gurian-Sherman, 2001; Recep *et al.*, 2009; Kilani-Feki and Jaoua, 2011; Zeidan *et al.*, 2019). The mode of action of Bcc species includes the production of secondary metabolites, such as antibiotics that have antifungal properties. The antibiotics produced by Bcc species include cepacin, cepacidine, phenazine, and pyrrolnitrin (Elshafie *et al.*, 2017; Elshafie and Camele, 2021). Despite the findings of the study that show the inhibitory effects of *B. cenocepacia* strain LMG 16656 against *F. oxysporum*, the strain cannot be used as BCA on ware potatoes because it is a human pathogen. *B. cenocepacia* strain LMG 16656 causes respiratory diseases in immune-compromised persons with cystic fibrosis (Scoffone *et al.*, 2017).

It, therefore, cannot be regarded as safe to use as a postharvest treatment on potatoes. On the other hand, *B. amyloliquefaciens* is a member of the *Bacillus* genus, which has been extensively studied for its use as BCAs (Khedher *et al.*, 2021; Bonaterra *et al.*, 2022). *Bacillus* species use antibiosis and the production of inhibitory secondary metabolites as their mode of action (Yuan *et al.*, 2012; Anckaert *et al.*, 2021). The SEM images (Figure 3.8c and d) showed the effects of the suspected volatile compounds on the hyphae of *F. oxysporum*. Wu *et al* (2019) demonstrated that *B. amyloliquefaciens* strain L3 significantly reduced the growth of *F. oxysporum* f.sp. *niveum* causing *Fusarium* wilt on watermelon. In their study, they identified 2-nonanone and 2-heptanone as some of the volatile compounds produced by *B. amyloliquefaciens* strain L3 responsible for the antifungal effects of the BCA.

The volatile compounds produced by *Bacillus* species also act as plant growth promoters when exposed to plants preharvest (Yuan *et al.*, 2012). *B. amyloliquefaciens* strain FZB42 has been

proven to produce antibiotics such as iturin A, surfactin, bacillomycin D, and fengycin, which have antimicrobial effects that act against the growth of fungal species (Chowdhury *et al.*, 2015a). Lin *et al* (2018) demonstrated that these secondary metabolites are secreted by other strains of *B. amyloliquefaciens* as well (Li *et al.*, 2015; Lin *et al.*, 2018; Luo *et al.*, 2022). In a similar study, *B. amyloliquefaciens* strain Ba01 significantly inhibited the growth of *Streptomyces scabies* infecting potatoes (Lin *et al.*, 2018). The production of volatile compounds is the mode of action that enables the antifungal activity of *B. amyloliquefaciens*. The antimicrobial activity of *B. amyloliquefaciens* has led to the development of bioagents using various strains of the bacterial species. *B. amyloliquefaciens* strain F2B42 is commercially used as an active ingredient for bio-fungicides, bio-bacteriocides, and growth stimulators (Chowdhury *et al.*, 2015b).

3.5 Conclusion

The aim of the study was to evaluate the efficacy of various BCAs as potential control agents against *F. oxysporum in vitro* and *in vivo*. Based on the findings of this study, it can be concluded that *B. cenocepacia* strain LMG and *B. amyloliquefaciens* strain MPA 1034 have antifungal and antimicrobial effects against *F. oxysporum*. However, since *B. cenocepacia* strain LMG 16656 is a human pathogen, only *B. amyloliquefaciens* strain MPA 1034 can be recommended for use as an alternative to synthetic chemicals to control FDR on potato tubers.

3.6 References

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Chapter 4

Effects of melatonin against *Fusarium oxysporum* infecting potatoes

Abstract

Potatoes (*Solanum tuberosum* L.) are essential to the South African agricultural industry. *Fusarium* dry rot (FDR) caused by *Fusarium oxysporum* is a devastating potato disease resulting in yield and economic losses. Generally regarded as safe (GRAS) products such as phytohormone melatonin have inhibitory effects on pathogens and can be used an alternative to synthetic chemical fungicides. The aim of the study was to evaluate the efficacy of melatonin against *F. oxysporum in vitro* and *in vivo*. Six melatonin concentrations (0 μM, 1 μM, 10 μM, 15 μM, 50 μM, and 100 μM) were against *F. oxysporum*. Melatonin concentration 100 μM had the highest mycelial growth inhibition percentage, with a rating of 40,83% *in vitro*. The second and third best concentrations were 15 μM and 50 μM with inhibition percentages of 35,83% and 34,17%, respectively. The efficacy of melatonin as an antifungal agent, was significantly lower *in vitro* compared to the *in vivo* results. *In vivo*, the 'Sifra' potato tubers treated with 100 μM showed the lowest FDR severity of 58,57%, followed by 50 μM and 15 μM with disease severity percentages of 59,54% and 60,31%, respectively. Melatonin concentration 100 μM significantly reduced the pathogen penetration to 6,52 mm *in vivo* compared to the untreated control which had a pathogen penetration value of 15,23 mm. Scanning electron imaging of the interactions between *F. oxysporum* and melatonin concentrations 100 μM and 15 μM showed mycelial deformation, low sporulation rate, and shrinking of *F. oxysporum* mycelia. The results obtained from this study indicate that melatonin has antifungal effects against *F. oxysporum* at the concentration of 100 μM. Melatonin at this concentration can be recommended for use as an alternative control strategy against *F. oxysporum* causing FDR disease in potatoes.

Keywords: Postharvest, *Solanum tuberosum*, 100 μM melatonin

4.1 Introduction

Potato (*Solanum tuberosum* L.) is the fourth leading crop commodity worldwide in global production (FAO, 2022). The crop is cultivated for direct human consumption of its tubers as they are a rich source of dietary fiber, vitamin C, starch, and proteins (Westermann, 2005). However, the production of potatoes is hindered by postharvest fungal diseases, such as *Fusarium* dry rot (FDR), that result in significant yield and subsequent economic losses. In SA, fungicides such as benzimidazole, 2-aminobutane, imazalil, flusilazole, difenoconazole and thiabendazole are to control FDR on tubers and potato seeds (Gachango *et al.*, 2012a; Jawed *et al.*, 2019; Tiwari *et al.*, 2020). However, synthetic chemicals leave amounts of toxic residues that harm human health and pose export limitations (Sharma *et al.*, 2018).

This has led to exploring alternative control methods such as using biological control agents, natural plant extracts, and generally regarded as safe (GRAS) products. GRAS products, such as edible coatings, organic and inorganic salts, organic and inorganic acids, and phytohormones, can control *Fusarium* diseases (Xue *et al.*, 2023). Melatonin (N-acetyl-5-methoxytryptamine) is a low molecular weight organic compound found across many different kingdoms (Zang *et al.*, 2022). This hormone was first discovered in the late 1950s in bovine pineal gland tissue (Lerner *et al.*, 1958; Nawaz *et al.*, 2016). Melatonin regulates circadian rhythms such as moods, sleep, retina physiology and immunological systems (Maronde and Stehle, 2007; Hardeland *et al.*, 2012; Carrillo-Vico *et al.*, 2013).

The first discovery of melatonin outside the animal kingdom was in a dinoflagellate alga called *Lingulodinium polyedrum* (Kolář and Macháčková, 2005). Shortly after that, plant melatonin was discovered in 1995 in Japanese morning glory (*Ipomoea nil*) (Tan *et al.*, 2012). Three methods drove the discovery of melatonin, radioimmune assay, gas chromatography-mass spectrometry, and liquid chromatography-mass spectrometry (Lewy and Markey, 1978; Dubbels *et al.*, 1995; Shiu *et al.*, 1999). These methods led to the discovery of phytomelatonin in other flowers and edible crops such as tobacco (*Nicotiana tobacum*), cucumber (*Cucumis sativus*), banana (*Musa acuminata*), beetroot (*Beta vulgaris*), and tomatoes (*Solanum lycopersicum*) (Kolář and Macháčková, 2005). Even though the hormone was also proven to be present and endogenous to other *Solanaceae* crops, it was not found in potatoes. The absence of this hormone from the crop indicates that potatoes lack the protective and beneficial attributes of melatonin.

Melatonin has valuable characteristics that contribute to improving the physiological functions of the plant, such as stress tolerance, promoting propagation, and growth and development (Nawaz *et al.*, 2016). Studies have shown that melatonin has high efficacy as a recover-bioagent for chemically damaged soils and a natural antioxidant (Kolář and Macháčková, 2005). Amongst other attributes of melatonin in plants, literature has shown that melatonin concentration increases with exposure to stress, giving the plant stress resistance (Zang *et al.*, 2022). Melatonin has been widely used for its medicinal benefits for humans and animals (Sánchez-Barceló *et al.*, 2010; Agathokleous *et al.*, 2019). Most research on melatonin application on food crops is on fruits than on vegetables (Xu *et al.*, 2019; Wang *et al.*, 2020). Recent research has shown that melatonin induces resistance against plant pathogens such as *Alternaria*, *Pseudomonas,* and *Fusarium* species (Kong *et al.*, 2021). However, there is still limited information on the use of melatonin as a postharvest treatment on potatoes. The aim of the study was to evaluate the inhibitory effects of melatonin on *F. oxysporum in vitro* and *in vivo*.

4.2 Materials and methods

4.2.1 Melatonin preparation

Melatonin powder, ≥ 98% (TLC) (Sigma-Aldrich), was used for the experiments. A 30 mg/ml stock solution was prepared by dissolving 30 mg of melatonin powder in 99.9% ethanol and suspended in double autoclaved distilled water according to the formula below. The melatonin stock solution was re-suspended in autoclaved distilled water to prepare solutions of different concentrations.

100 ml Stock solution = (0,03g melatonin + 300 µl ethanol) + 700 ml water (1)

4.2.2 In vitro screening of melatonin against *F. oxysporum*

Melatonin was screened against *F. oxysporum* using the disc fusion method. The concentrations tested were 0 µM, 1 µM, 10 µM, 15 µM, 50 µM, and 100 µM. Two autoclaved 6.5 mm discs were placed equidistant on fresh PDA petri dishes. Mycelial plugs (3 mm) from five-day-old *F. oxysporum* cultures were placed at the centre of the petri dish. Aliquots (10 µl) of the different concentrations of the emulsified melatonin were pipetted onto each disk, immediately sealed with parafilm, and stored at 25°C. The trial was repeated twice with three replicates per

treatment. The diameters of the radial mycelial growth of the fungus were measured on days 5, 7, and 9 post-inoculation. The mycelial growth inhibition percentage was calculated using the formula below.

Mycelial inhibition (%) = (dc-dt) ÷ dc ×100 (2)

Where, dc= colony diameter(mm) of control, dt = colony diameter (mm) of treatment.

4.2.3 In vivo screening of melatonin against *F. oxysporum*

All four melatonin concentrations screened under the *in vitro* trial were screened against *F. oxysporum in vivo* on 'Sifra' potato tubers. The different concentrations were screened against *F. oxysporum* as a preventative control measure. Tubers were washed with autoclaved distilled water to remove soil debris, sterilized with 70% ethanol to remove contaminants from their surface, and then air-dried. Four (7 mm width x 7 mm depth) wounds were made on the upper surface of each tuber using a sterile scalpel. Each wound was flooded with 20 µl of each melatonin concentration and allowed to air dry for two hours under the laminar flow before mycelial plugs excised from 10-day-old cultures were placed into each wound.

To imitate dark storage, the treated tubers were neatly boxed and wrapped with thick, black plastic bags. The relative humidity levels were kept high (~75%) by adding a damp paper towel to each box before storing them at 25 ℃ for 21 days. The efficacy of the treatments was measured by their ability to reduce disease severity. The disease severity was estimated using the methodology described by Mejdoub-Trabelsi *et al* (2015) with minor modifications. The maximal width and depth were measured for each wound and used to calculate pathogen penetration (*p*) using the formula below as described by Lapwood *et al* (1984).

$$\text{Pathogen penetration (mm)} = \left[\frac{w}{2} + (d - 7)\right] \div 2 \quad (3)$$

Where, *w* is the maximal width of the lesion and *d* is the maximal depth of the lesion. Pathogen penetration rate was used to determine the severity of the dry rot on the tubers using the scale below:

- Moderately severe $P \leq 5$ mm

- Severe 5 mm < *P* < 7 mm
- Highly severe *P* ≥ 7 mm

4.2.4 Scanning electron microscopy studies of the interactions of melatonin with *F. oxsyporum*

The studies were conducted at the Microscopy and Microanalysis Unit, University of KwaZulu-Natal. Samples were excised from dual culture PDA plates to evaluate the interaction between *F. oxysporum* and the best-performing melatonin concentrations. The tissue samples were held for 2 hours in a fixation of 3% buffered glutaraldehyde and buffer washed in 0.05M sodium cacodylate buffer twice 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. In the final stage of dehydration, the samples were washed three times with 100% ethanol for 10 minutes. The samples were transferred to the Quorum K850 critical drying point dryer (CPD) basket under 100% ethanol.

During CPD, the ethanol was replaced with liquid CO_2. The CO_2 was heated and pressurized to its critical point, at which the liquid converted to gas without the damaging effects of surface tension on the samples, resulting in dry, intact samples. The dried samples were then carefully mounted onto SEM stubs using black double-sided tape. The sample stubs were then transferred to the Quorum Q150R ES sputter coater. In this step, the samples were made conductive to the electron beam with two layers of gold and palladium coats and allowed to dry before being viewed with the Zeiss EVO LS15 scanning electron microscope.

4.2.5 Statistical analysis

The data collected was subjected to GenStat 23rd edition to analyse variance (ANOVA). Mean separation was done according to Duncan's multiple range test at a 5% significance level.

4.3 Results

4.3.1 In vitro screening of melatonin against *F. oxysporum*

The different melatonin concentrations showed varying inhibition rates on the mycelial growth of *F. oxysporum in vitro*. There was a significant difference in the effects of the different melatonin concentrations on *F. oxysporum* (P-value <0,001). At nine days post-inoculation, the melatonin concentration of 100 μM (MEL100) had the highest mycelial growth inhibition percentage, with a rating of 40,83%. The second and third best concentrations were 15 μM (MEL15) and 50 μM (MEL50) with inhibition percentages of 35,83% and 34,17%, respectively (Table 4.1 and Figure 4.1). Melatonin concentrations 0 μM (MEL0) and 1 μM (MEL1) had the lowest inhibition percentage, with a mycelial growth inhibition percentage of less than 10%.

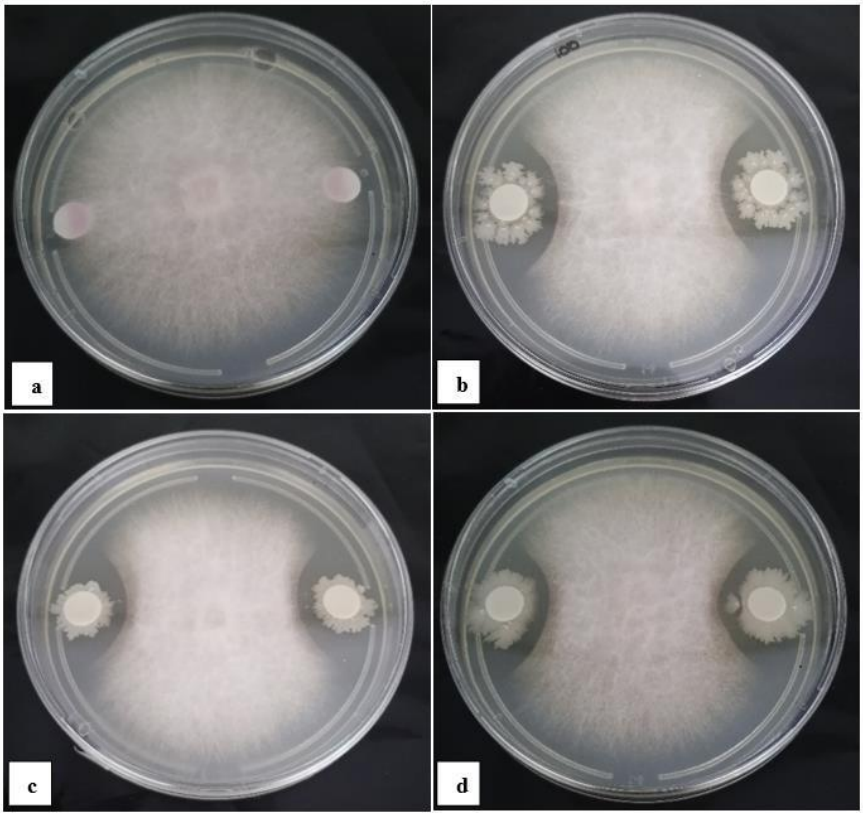

Figure 4.1: Mycelial growth inhibition of *F. oxysporum* by best three concentrations nine days post-inoculation at secondary screening 25°C. **a**: *F. oxysporum* only on PDA. **b**: Interaction between MEL100 and *F. oxysporum* on PDA. **c**: Interaction between concentration MEL50 and *F. oxysporum* on PDA. **d**: Interaction between concentration MEL15 and *F. oxysporum* on PDA.

Table 4.1: Mean mycelial growth inhibition percentage (MGI) and mean mycelial growth (MMG) of *F. oxysporum* secondary screening against melatonin concentrations at 25℃.

Treatments	5 DPI		7 DPI		9 DPI	
	MMG (mm)	**MGI (%)**	**MMG (mm)**	**MGI (%)**	**MMG (mm)**	**MGI (%)**
MEL0	48,33^{c}	2,62	57,00^{b}	16,52	78,00^{c}	2,92
MEL1	48,67^{c}	2,48	60,00^{b}	14,59	73,67^{c}	7,92
MEL10	44,00^{b}	12,01	48,00^{a}	31,76	58,67^{b}	26,67
MEL15	43,00^{b}	14,02	49,00^{a}	30,35	51,33^{ab}	35,83
MEL50	42,33^{ab}	15,33	49,00^{a}	30,32	52,67^{ab}	34,17
MEL100	38,67^{a}	22,56	46,67^{a}	33,65	47,33^{a}	40,83
Control	50,00^{c}	0,00	70,33^{c}	0,00	80,00^{c}	0,00
P-value	<0,001	-	<0,001	-	<0,001	-
LSD	3,990	-	6,162	-	7,595	-
CV (%)	5,1	-	6,5	-	6,9	-

Means with the same letters within a column are not significantly different at 5% level of significance according to DMRT.

4.3.2 In vivo screening of melatonin against *F. oxysporum*

The six concentrations of melatonin screened against *F. oxysporum* on potato tubers had varying effects on the severity of *Fusarium* dry rot 21 DPI. The disease severity of *F. oxysporum* was expressed in two methods: Lesion diameter and pathogen penetration methods. Tubers treated with 100 μM showed the lowest FDR severity with 58,57%, followed by 50 μM and 15 μM with disease severity percentages of 59,54% and 60,31%, respectively. The effects of 0 μM and 1 μM were not significantly different from the control treatment. The tubers treated with melatonin concentration of 100 μM had the lowest pathogen penetration value of 6,52 mm followed by 10 μM and 15 μM with 7,87 mm and 8,38 mm, respectively (Table 4.2 and Figure 4.2). Tubers treated with 100 μM were considered moderately severe according to the scale in section 1.2.5 above (Figure 4.3).

Figure 4.2: Effects of the melatonin on tubers inoculated with *F. oxysporum* 21 days post-inoculation. **a** and **d**: pathogen-only control. **b** and **e**: tuber treated with 100 μM. **c** and **f**: tuber treated with 15 μM melatonin.

Table 4.2: Disease severity *Fusarium* dry rot of potato tubers treated with different melatonin concentrations 21 days post-inoculation.

Melatonin Concentration (μM)	**Mean lesion diameter (mm)**	**Disease Severity (%)**
0	29,75^{c}	91,71
1	30,75^{cd}	94,80
10	22^{b}	67,82
15	19,56^{ab}	60,30
50	19,31^{a}	59,53
100	19^{a}	58,57
Control	32,44^{d}	100
P-value	<0,001	-
CV %	7,0	-
LSD	2,542	-

Means with the same letters within a column are not significantly different at 5% level of significance according to Duncan's multiple range test (DMRT).

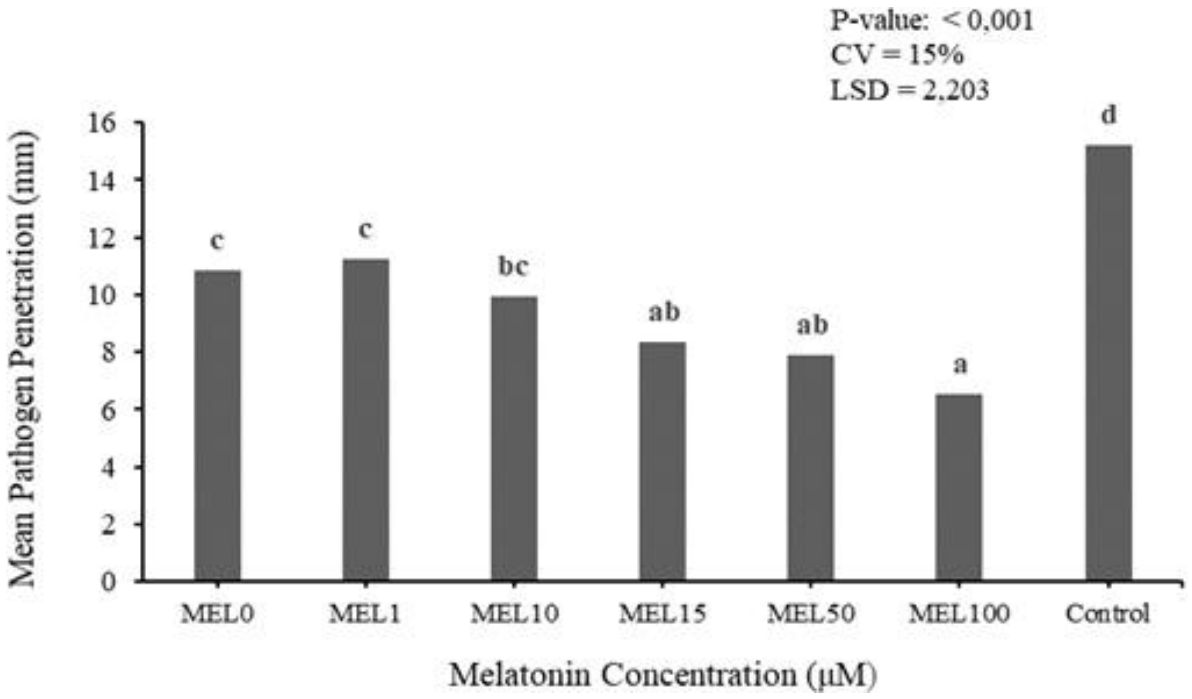

Figure 4.3: Average pathogen penetration of wounds treated with different melatonin concentrations 21 days post-inoculation. Means with the same letters are not significantly different at a 5% level of significance in Duncan's multiple range test (DMRT).

4.3.3 Scanning electron microscopy studies of the interactions of melatonin and *F. oxysporum*

SEM imaging was conducted to determine the possible mode of action of the melatonin concentrations screened against *F. oxysporum* under *in vitro* conditions. Samples were excised from PDA plates stored at 25℃ for nine days to observe the microscopic effects of the best two melatonin concentrations (100 µM and 15 µM) on the growth of *F. oxysporum* under the SEM. The SEM images (Figures 4.4c and d) show varying rates of mycelial deformation, low sporulation rate, and shrinking on the samples exposed to the different melatonin concentrations for nine days at 25℃.

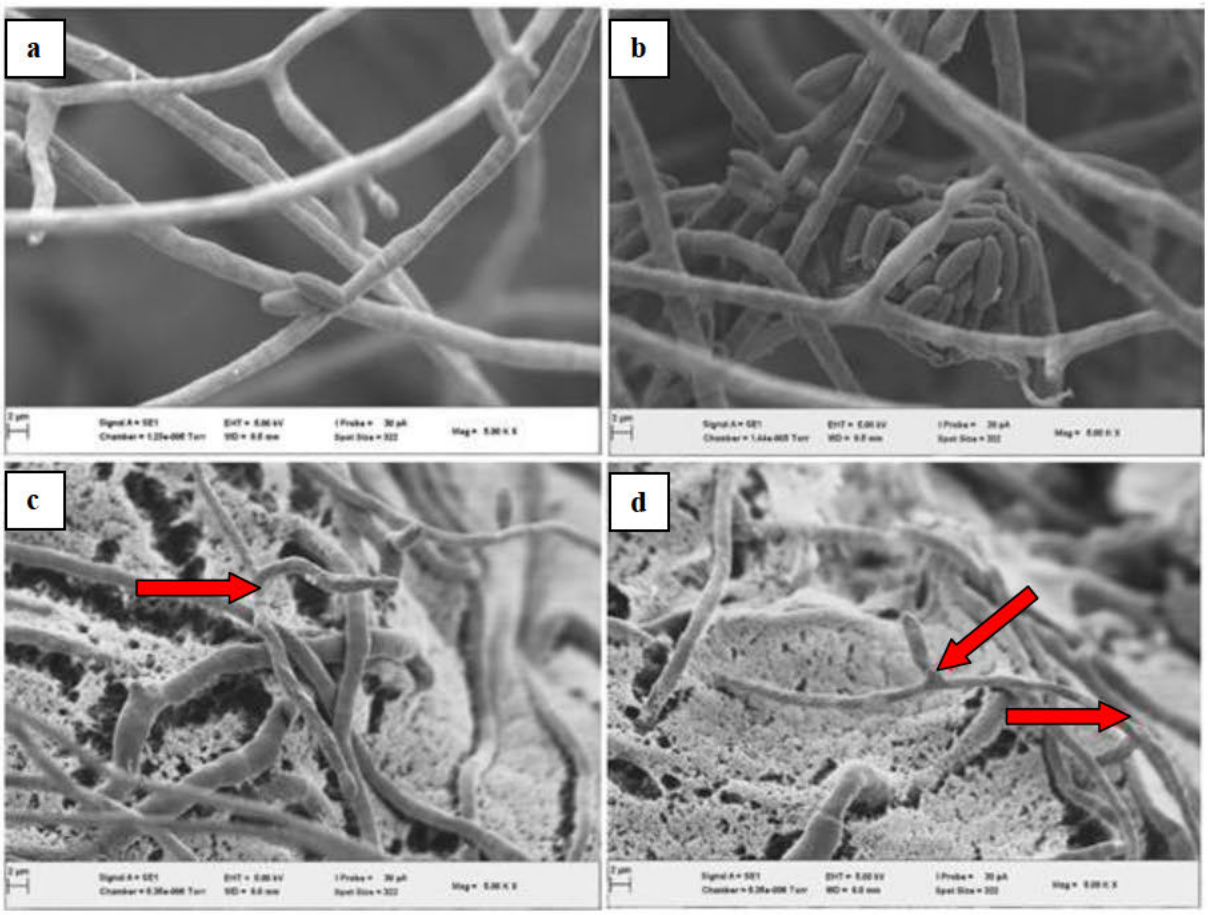

Figure 4.4: Effects of Melatonin on *F. oxysporum* under the SEM (≈ 5000x). **a** and **b**: Untreated *F. oxysporum* mycelia branching at 90° angle. **c**: Shrinking effect of melatonin concentration 15 µM on *F. oxysporum* mycelia shown with an arrow. **d**: Shrinking effect of melatonin concentration 100 µM on *F.oxysporum* mycelia shown with an arrow.

4.4 Discussion

F. oxysporum is the causal agent of FDR disease in potatoes, resulting in significant yield losses. Biological control agents and GRAS substances have been widely studied for their potential as alternative control agents that can be used as safer and environmentally friendly alternatives to synthetic chemicals. GRAS substances like phytohormones have antimicrobial properties that inhibit the growth of fungal diseases (Xue *et al.*, 2023). The study aimed to evaluate the inhibitory effects of different melatonin concentrations against *F. oxysporum* infecting potatoes *in vitro* and *in vivo*. In this study, the efficacy of melatonin as an antifungal agent increased with

the increase in the concentration. Findings showed that a melatonin concentration of 100 μM was the most effective in both the *in vitro* and *in vivo* trials. Melatonin at 100 μM have been proven to have efficacy against *F. oxysporum* causing *Fusarium* wilt on banana, *B. cinerea*, *Rhizoctonia solani,* and *Phytophthora capsici* (Aghdam and Fard, 2017; Wei *et al*., 2017; Mandal *et al.*, 2018). The efficacy of melatonin is highly dependent on the concentration and the type of crop it is used on. Melatonin at 1000 μM has been reported to have spoilage effects on strawberry fruits (Aghdam and Fard, 2017).

However, the same concentration significantly reduced the occurrence of late blight disease on potato without damaging the tuber (Zhang *et al.*, 2017; Xu *et al.*, 2019). The inhibitory effects of melatonin were more prominent in the *in vivo* trial compared to the *in vitro* screening trial. The results correlated with those from a study by Li *et al* (2019) and Zang *et al* (2022), where melatonin concentrations had less efficacy against *B. cinerea in vitro* but significantly reduced the occurrence of the disease on tomato fruits. The results may be influenced by the mode of action of melatonin, which is closely related to the promotion of defence-related enzymes of the host which are absent in the *in vitro* studies. Melatonin plays a significant role in promoting plant response against abiotic and biotic stress, including inhibiting the growth of pathogens (Wang *et al.*, 2020; Zhao *et al.*, 2021).

Melatonin enhances the activity of defense-related enzymes which suppress disease development (Mandal *et al*.,2018). The exogenous application of melatonin upregulates the antioxidant enzymes such as catalysts, peroxidase, and superoxide (Hardeland and Poeggeler, 2003; Nawaz *et al.*, 2016; Zang *et al.*, 2022). Other studies have demonstrated how the exogenous application of melatonin on watermelon upregulated the expression of defense genes involved in effector immunity- mediated defenses (Mandal *et al.*, 2018).

The SEM micrographs (Figure 4.4c and d) show no microconidia formation on the samples treated with melatonin. The micrographs also show the shrinking effects of melatonin on the growth of *F. oxysporum* hyphae, which concurs with the results obtained by Zhao *et al* (2011). Their findings demonstrated how melatonin stunted the growth of the hyphae of *P. infestans* by inhibiting hyphal elongation and denaturing the internal cellular structures of the fungus (Zhao *et al.*, 2011; Zhang *et al.*, 2017). The mode of action of melatonin includes the alteration of the cellular structure of the pathogen. Li *et al* (2023) demonstrated that melatonin inhibited the

growth of rice fungal pathogens, *R. solani*, *Magnaporthe oryzae*, and *Ustilaginoidea virens* by suppressing the formation of the fungal appressoria and conidia.

4.5 Conclusion

The aim of this study was to evaluate the inhibitory effects of melatonin against *F. oxysporum in vitro* and *in vivo*. The findings of this study demonstrated the antifungal effects of melatonin against *F. oxysporum*. The concentrations used in this study have not been previously used against *F. oxysporum* or exogenously applied on potato tubers to prevent FDR. Based on these results, it can be concluded that melatonin, at a concentration of 100 μM can be used as a GRAS postharvest preventative treatment against *F. oxysporum* causing FDR of potatoes.

4.6 References

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Chapter 5

Evaluation of the integrated effects of melatonin, *Burkholderia cenocepacia,* and *Bacillus amyloliquefaciens* on postharvest quality of potato tubers

Abstract

Fusarium oxysporum is the causal agent of *Fusarium* dry rot (FDR), which results in postharvest potato losses. Biological control agents and phytohormone melatonin have antifungal effects on fungal pathogens and can be used as alternatives to synthetic chemical fungicides. The aim of the study was to evaluate the integrated effects of melatonin, *Burkholderia cenocepacia* and *Bacillus amyloliquefaciens* on the postharvest quality of potato tubers. The BCAs were integrated with six melatonin concentrations (0 μM, 1 μM, 10 μM, 15 μM, 50 μM, and 100 μM) and screened against *F. oxysporum in vitro* and *in vivo*. In the *in vitro* screening trial, treatment *Bamy* + MEL100 had the highest mycelial growth inhibition percentage (59,92%), followed by *Bamy* + MEL15 and *Bamy* + MEL50 with mycelial growth inhibition percentages of 56,12% and55,27%, respectively. Potato tubers treated with *Bamy* + MEL100 had the lowest severity of FDR with a disease severity percentage of 50,61% and a pathogen penetration value of 6,39 mm. *Bcen* + MEL100 and *Bamy* + MEL50 showed disease severity percentages of 52,63% and 59,72%, respectively. Overall, tubers treated with a combination of melatonin and *B. amyloliquefaciens* showed less severe symptoms of FDR across all concentrations. Furthermore, the effects of the treatments on the antioxidant activity, phenolic content, ascorbic acid content, and protein content on treated potato tubers were evaluated. The exogenous application of melatonin at a concentration of 100 μM melatonin combined with *B. amyloliquefaciens* was the most effective treatment with the highest phenolic content (144,1 mg GAE/g DW), and protein content (68 mg/g DM) compared to the untreated tubers. Tubers treated with melatonin had the highest ascorbic acid content (5,48 mg AAE/100g DM) compared to the untreated tubers. Based on these findings, it can be concluded that *B. amyloliquefaciens* and melatonin at 100 μM concentration, can be used in combination to inhibit the growth of *F. oxysporum*. The combined treatment can be recommended for use as an alternative control strategy for FDR caused by *F. oxysporum* that will not negatively affect the quality of the potato tubers.

Keywords: Biological control agents, *Solanum tuberosum*, Integration

5.1 Introduction

Potatoes (*Solanum tuberosum* L.) are significant contributors to the improvement of food and nutritional security in most developing countries in Latin America, Asia, and Africa (Devaux *et al.*, 2020). The crop is cultivated for direct human consumption of its tubers as they are a rich source of carbohydrates, vitamin C, and proteins (Westermann, 2005; Zaheer and Akhtar, 2016). Potato tubers contain nutritional and non-nutritional compounds that have health benefits to human health, such as anthocyanins, phenolics, fiber, starch, glycoalkaloids, and proteinase inhibitors (Westermann, 2005; Visvanathan *et al.*, 2016; Burgos *et al.*, 2020). The health benefits of consuming potatoes include but are not limited to, anti-inflammatory, anti-hypersensitive, and anti-hyperlipidemic effects against chronic diseases (Burgos *et al.*, 2020; Raigond *et al.*, 2023).

The content of these compounds is affected by various factors such as cultivar type, postharvest processing, storage temperature, and storage duration (Ngceni, 2019). Therefore, it is vital to preserve the postharvest quality of the tubers to maintain the nutritional benefits of their consumption. *Fusarium* dry rot (FDR) is a devastating postharvest potato disease. The disease is caused by various *Fusarium* species, including *F. oxysporum,* the most common causal agent of the disease in South Africa. The pathogen infects the potato seed, resulting in seed decay, or infects the tuber, resulting in dry rot (Bojanowski *et al.*, 2013). Synthetic fungicides are used to treat and prevent FDR disease in potatoes (Fravel *et al.*, 2005). However, with rapid fungal mutation, introduction of new cultivars, and excessive use, the fungicides have lost their efficacy in controlling the disease (Fravel *et al.*, 2005; Jawed *et al.*, 2019). Chemical fungicide resistance has led to the development and use of biological control agents (BCAs), and generally regarded as safe substances (GRAS) as alternative control strategies (Fravel *et al.*, 2005; Nguyen *et al.*, 2017).

BCAs are microbial species such as yeast, bacteria, and antagonistic fungi that have inhibitory effects on pathogens (Boro *et al*.,2022). They inhibit the growth of the pathogen by either inducing plant resistance or suppressing the growth of the target pathogen (Li *et al.*, 2022). Different microbial species have been proven to control numerous bacterial and fungal pathogens, including those infecting potatoes (Recep *et al.*, 2009; Jawed *et al.*, 2019; Lastochkina *et al.*, 2020; Khedher *et al.*, 2021). Melatonin (N-acetyl-5-methoxytryptamine) is a

low molecular weight, GRAS organic compound found across many different kingdoms (Zang *et al.*, 2022). Melatonin has valuable characteristics that contribute to improving the physiological functions of the plant, such as biotic and abiotic stress tolerance, promoting propagation, growth, and development of plants (Nawaz *et al.*, 2016). Studies have shown that melatonin has high efficacy as a recover-bioagent for chemically damaged soils and a natural antioxidant (Kolář and Macháčková, 2005). The hormone upregulates the antioxidant enzymes such as catalysts, peroxidases, and superoxide (Hardeland and Poeggeler, 2003; Nawaz *et al.*, 2016; Zang *et al.*, 2022). Melatonin has been reported to have antimicrobial against some pathogens causing disease on food crops (Wei *et al.*, 2017; Chen *et al.*, 2019; Zhao *et al.*, 2019).

Integration of control methods is commonly used to improve the effectiveness of the chosen control measures. Fungicides are often integrated with other control methods, such as BCAs, cultural methods and GRAS products, to reduce the amount of toxic residues on the crops and counteract their adverse effects on the environment (Zang *et al.*, 2022). However, there is limited information on the use of melatonin integrated with BCAs. The aim of the study was to evaluate the integrated effects of melatonin and biological control agents, (*Burkholderia cenocepacia* and *Bacillus amyloliquefaciens*) against *F. oxysporum* causing FDR on potatoes, and the effects of the treatments on the postharvest tuber quality.

5.2 Materials and methods

5.2.1 Melatonin preparation

Melatonin powder, ≥ 98% (TLC) (Sigma-Aldrich), was used for the experiments. A 30 mg/ml stock solution was prepared by dissolving 30 mg of melatonin powder in 99.9% ethanol and suspended in double autoclaved distilled water according to formula 1 below. The melatonin stock solution was re-suspended in autoclaved distilled water to prepare working solutions of different concentrations. The concentrations tested were 0 μM, 1 μM, 10 μM, 15 μM, 50 μM, and 100 μM.

100 ml Stock solution = (0,03g melatonin + 300 μl ethanol) + 700 ml water (1)

5.2.2 Preparation of BCA isolate suspensions

Two BCA isolates, *Burkaholderia cenocepacia* strain LMG 16656 (*Bcen*) and *Bacillus amyloliquefaciens* strain MPA 1034 (*Bamy*), were integrated with the different melatonin concentrations. BCA suspensions were prepared from five plates of each BCA (*Bcen* and *Bamy*) cultures incubated at 28℃ for 72 hours. Each BCA culture was suspended in distilled water and transferred into 250 ml conical flasks. The cell suspension of each culture was then adjusted to a concentration of 1×10^8 cells/ml using the hemocytometer. The flasks were sealed with foil and stored at 4℃ to avoid contamination and culture degradation.

5.2.3 In vitro screening of melatonin and BCAs against *F. oxysporum*

The integrated effects of melatonin and BCAs were screened against *F. oxysporum* using the disc fusion method. Two autoclaved filter paper discs were placed equidistant on fresh PDA petri dishes. Mycelial plugs (3 mm diameter) from five-day-old *F. oxysporum* cultures were placed at the centre of the petri dish. Aliquots (5 µl) of the different concentrations of the emulsified melatonin were pipetted onto each disc and allowed to dry for 5 minutes before 5 µl of the BCA suspension was also pipetted onto each disc. The plates were then immediately sealed with parafilm and stored at 25°C. The trial was repeated twice with three replicates per treatment. The diameters of the radial mycelial growth of the fungus were measured on days 5, 7, 9, and 11 post-inoculation. The mycelial growth inhibition percentage was calculated using the formula below.

$$\text{Mycelial inhibition (\%)} = (dc-dt) \div dc \times 100 \quad (2)$$

Where, dc= colony diameter(mm) of control, dt = colony diameter (mm) of treatment.

5.2.4 In vivo screening of melatonin and BCAs against *F. oxysporum*

The preventative effect of the top three concentrations with the highest growth inhibition rates in the *in vitro* trial were selected and screened against *F. oxysporum in vivo* on ‘Sifra’ potato tubers. Tubers were washed with autoclaved distilled water to remove soil debris, sterilized with 70% ethanol to remove contaminants from their surface, and then air-dried. Four (5 mm diameter x 2,5 mm depth) wounds were made on the upper surface of each tuber using a sterile cork-borer. Each wound was flooded with 1 ml of each melatonin concentration, allowed to air dry for 5

minutes, and then flooded with 1 ml of the BCA suspension. Thereafter, the treated potato tubers were allowed to air dry for two hours under the laminar flow before a mycelial plug excised from 7-day-old cultures was placed into each wound. The control tubers were only inoculated with the *F. oxysporum* mycelial plug. The trial was repeated twice with four replicates per treatment. The inoculated tubers were stored at room temperature (~25°C) for 21 days, and the humidity levels were kept high (~75%) by adding a damp paper towel to each box and covering it with a black plastic bag. The efficacy of the treatments was measured by their ability to reduce disease severity. The disease severity was estimated using the methodology described by Mejoub-Trabelsi *et al* (2015) with minor modifications. The maximal width and depth were measured for each wound and used to calculate pathogen penetration (*p*) using the formula described by Lap-wood *et al* (1984).

$$\text{Pathogen penetration (mm)} = \left[\frac{w}{2} + (d - 7)\right] \div 2 \quad (3)$$

Where *w* is the maximal width of the lesion and *d* is the maximal depth of the lesion. The pathogen penetration rate was used to determine the severity of the dry rot on the tubers using the scale below:

- Moderately severe $p \leq 5$ mm
- Severe 5 mm < p < 7 mm
- Highly severe $p \geq 7$ mm

5.2.5 Determination of the effects of melatonin and *Bacillus amyloliquefaciens* on the postharvestquality of potatoes

5.2.5.1 Preparation of samples

Potato tubers (*cv.* "Sifra") were washed with distilled water and sterilised with 70% ethanol to remove contaminants from their surface. The tubers were subjected to three treatments, melatonin (100 μM) only, BCA (*B. amyloliquefaciens*) only, integration (melatonin 100 μM + *B. amyloliquefaciens*), and control (untreated). The tubers were dipped into their respective treatment for 10 minutes and air-dried for 2 hours. The treated tubers were then placed at ambient temperature (±25°C) to mimic shelf-life conditions for 14 days. Potato tubers were sampled on days 0, 7, and 14. Sampled potato tubers were diced and freeze-dried using the Vir

Tis BenchTop Pro freeze drier (SP Scientific, Warminster) before being ground to powder and stored at -80℃.

5.2.5.2 Determination of phenolic content

The total phenolic content was determined according to the Folin-Ciocalteu assay described by Wang *et al* (2011) with minor modifications. Freeze-dried potato powder (0.5 g) was weighed and transferred into centrifuge tubes and 25 ml of 80 % acetone was added. The samples were centrifuged at 10 000 rpm for 30 minutes at 5°C precooled centrifuge (Avanti J-HC, Beckman Coulter). After centrifugation, 1 ml of the supernatant and 2.5 ml of 0.2 N Folin-Ciocalteu reagent were simultaneously added into test tubes, and the tubes were allowed to stand for 5 minutes. Thereafter, 4 ml of sodium carbonate (Na_2CO_3) was added, followed by incubation for 30 minutes at 80°C. After incubation, tubes were placed into ice for 5 minutes for cooling and the absorbance was read at 736 nm using the UV-1800 spectrophotometer (Shimadzu, UV-Vis). The total phenolic content was calculated using the gallic acid calibration curve and expressed as milligram gallic acid equivalent (GAE) per gram sample dry mass (mg GAE/ g DW).

5.2.5.3 Determination of ascorbic acid content

Ascorbic acid was quantitatively determined following 2,6-Dichlorophenolindophenol (DCPIP) method developed by Boonkasem *et al* (2015) with slight modifications. A mass of 0.5 g of freeze-dried potato powder was weighed and transferred into centrifuge tubes. A volume of 20 ml of 3% metaphosphoric acid was added into each tube, then tubes were centrifuged (Avanti J-HC, Beckman Coulter) at 4000 rpm for 10 minutes. The supernatant was collected and used for further analysis. A volume of 1 ml of the supernatant and 3 ml of 0.2 mM DCPIP was simultaneously added into test tubes. Immediately after mixing for 15 seconds, the absorbance was measured at 515 nm using a UV-1800 spectrophotometer (Shimadzu, UV-Vis). The ascorbic acid concentration was calculated and expressed in milligram ascorbic acid equivalent (AAE) per 100 g sample dry mass (mg AAE / 100 g DM).

5.2.5.4 Determination of antioxidant activity [DPPH (2, 2-Diphenyl-1-picrylhydrazyl)]

Free radical scavenging ability was calculated according to an antioxidant (DPPH) assay described by Rocchetti *et al* (2019) with slight modifications. Freeze-dried potato powder (0.5 g) was weighed and transferred into test tubes, and a volume 10ml of 95 % ethanol (v/v) was added

simultaneously, followed by mixing using a vortex (Scientific Industries, Vortex-Genie 2). The supernatant was transferred to clean test tubes and incubated at 25°C for 10 minutes. After obtaining clear supernatant, 100 µl of the sample and 300 µl of 0.1 mM DPPH (0.1 mM in 95% methanol) reagent were added into test tubes. The tubes were then incubated in the dark for 30 minutes, thereafter, absorbance was read at 517 nm using a UV-1800 spectrophotometer (Shimadzu, UV-Vis). The scavenging ability of DPPH was calculated and expressed in milligram ascorbic acid equivalent (AAE) per gram sample dry mass (mg AAE/ g DM).

5.2.5.5 Determination of protein content

The determination of protein content was carried out following the procedure developed by Bradford (1976). A mass of 0.5 g of freeze-dried potato powder was weighed and transferred into centrifuge tubes; then, 30 ml 100 mM TRIS buffer was added and mixed using a vortex (Scientific Industries, Vortex-Genie 2). The samples were then centrifuged (Avanti J-HC, Beckman Coulter) at 10 000 rpm for 15 minutes at 2°C. A supernatant of 0.1 ml was transferred into test tubes, and 5 ml of Bradford reagent was added to each test tube. The absorbance of each sample was measured at 590 nm using a UV-1800 spectrophotometer (Shimadzu, UV-Vis). The concentration of each sample was calculated and expressed in milligrams per gram sample dry mass (mg/g DM).

5.2.6 Statistical analysis

The data collected was subjected to GenStat 23rd edition to analyse variance (ANOVA). Mean separation was done according to Duncan's multiple range test (DMRT) at a 5% significance level.

5.3 Results

5.3.5 In vitro screening of melatonin integrated with *Burkholderia cenocepacia* and *Bacillus amyloliquefaciens* against *F. oxysporum*

Integrating *B. cenocepacia* (*Bcen*) with different melatonin concentrations has significantly different effects on the growth of *F. oxysporum* under i*n vitro* conditions (P-value = 0,001). Treatment *Bcen* + MEL100 had the highest mycelial growth inhibition percentage, with a value of 52%, 9 days post-inoculation. *Bcen* + MEL50 and *Bcen* + MEL15 treatments had mycelial growth inhibition percentages of 51% and 48%, respectively (Figure 5.1 and Table 5.1).

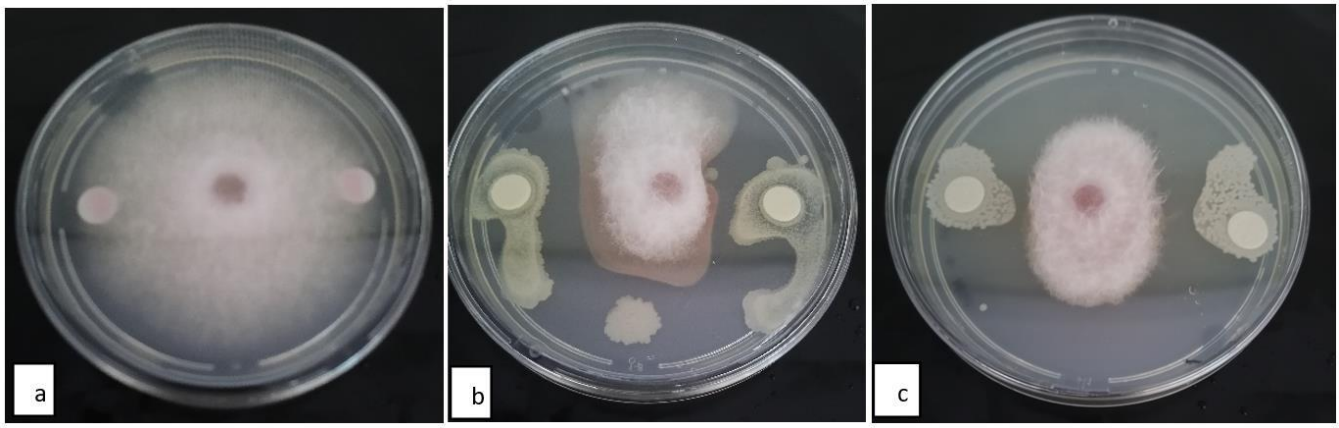

Figure 5.1: Mycelial growth inhibition of *F. oxysporum* by best two treatments nine days post-inoculation at 25℃. **a**: *F. oxysporum* only control. **b**: Interaction between *Bcen* + 100 and *F. oxysporum* on PDA. **c**: Interaction between *Bcen* + 50 and *F. oxysporum* on PDA.

Integrating *B. amyloliquefaciens* (*Bamy*) with different melatonin concentrations showed significantly different effects on the growth of *F. oxysporum* under i*n vitro* conditions (P-value = 0,001). Treatment *Bamy* + MEL100 had the highest mycelial growth inhibition percentage, with a value of 60%, 9 days post-inoculation. Treatments *Bamy* + MEL15 and *Bamy* + MEL50 exhibited mycelial growth inhibition percentages of 56% and 55%, respectively (Table 5.2). Treatment *Bamy* + MEL0 had the lowest mycelial growth inhibition percentage with an inhibition rate of 13% (Figure 5.2). Figure 5.3 shows the difference between the combined effects of *B. cenocepacia* + melatonin and *B. amyloliquefaciens* + melatonin on the mycelial growth of *F. oxysporum* after incubation for 9 days at 25℃. Treatments with *B.*

amyloliquefaciens yielded higher mycelial growth inhibition rates than those treated with *B. cenocepacia*.

Table 5.1: Mean mycelial growth inhibition percentage (MGI) and mean mycelial growth (MMG) of *F. oxysporum* treated with *B. cenocepacia* integrated with different melatonin concentrations at 25℃ 5,7, and 9 days post inoculation (DPI).

Treatments	5 DPI		7 DPI		9 DPI	
	MMG (mm)	**MGI (%)**	**MMG (mm)**	**MGI (%)**	**MMG (mm)**	**MGI (%)**
Bcen + MEL0	49,00^{c}	5,77	62,67^{b}	10,48	73,00^{bc}	7,59
Bcen + MEL1	43,33^{b}	16,67	59,33^{b}	15,24	69,67^{b}	11,81
Bcen + MEL10	35,33^{a}	32,05	40,00^{a}	42,86	44,33^{a}	43,88
Bcen + MEL15	35,00^{a}	32,69	39,67^{a}	43,33	41,33^{a}	47,68
Bcen + MEL50	34,33^{a}	33,97	37,33^{a}	46,67	38,67^{a}	51,05
Bcen + MEL100	34,00^{a}	34,62	36,67^{a}	47,62	37,67^{a}	52,32
Control	52,00^{c}	0,00	70,00^{c}	0,00	79,00^{c}	0,00
P-value	<0,001	-	<0,001	-	<0,001	-
LSD	5,127	-	6,054	-	6,508	-
CV (%)	7,2	-	7,0	-	6,8	-

Means with the same letters within a column are not significantly different at 5% level of significance DMRT.

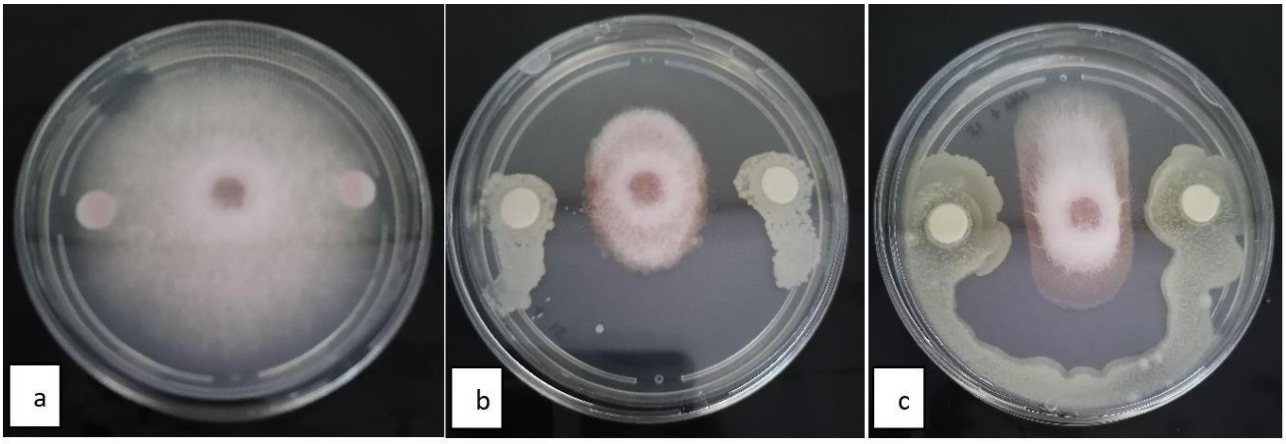

Figure 5.2: Mycelial growth inhibition of *F. oxysporum* by best two treatments nine days post-inoculation at 25℃. **a**: *F. oxysporum* only control. **b**: Interaction between *Bamy* + MEL100 and *F. oxysporum* on PDA. **c**: Interaction between *Bamy* + MEL15 and *F. oxysporum* on PDA.

Table 5.2: Mean mycelial growth inhibition percentage (MGI) and mean mycelial growth (MMG) of *F. oxysporum* treated with *B. cenocepacia* integrated with different melatonin concentrations at 25°C.

Treatments	5 DPI		7 DPI		9 DPI	
	MMG (mm)	MGI (%)	MMG (mm)	MGI (%)	MMG (mm)	MGI (%)
Bamy + MEL0	45,00^{d}	13,46	60,67^{c}	13,33	68,67^{c}	13,08
Bamy + MEL1	36,67^{c}	29,49	41,67^{b}	40,48	45,67^{b}	42,19
Bamy + MEL10	35,00^{bc}	32,69	39,33^{b}	43,81	43,33^{b}	45,15
Bamy + MEL15	31,67^{ab}	39,10	34,33^{a}	50,95	34,67^{a}	56,12
Bamy + MEL50	31,67^{ab}	39,10	34,00^{a}	51,43	35,33^{a}	55,27
Bamy + MEL100	30,00^{a}	42,31	31,00^{a}	55,71	31,67^{a}	59,92
Control	52,00^{e}	0,00	70,00^{d}	0,00	79,00^{d}	0,00
P-value	<0,001	-	<0,001	-	<0,001	-
LSD	4,586	-	4,407	-	5,170	-
CV (%)	7,0	-	5,7	-	6,1	-

Means with the same letters within a column are not significantlydifferent at 5% level of significance DMRT.

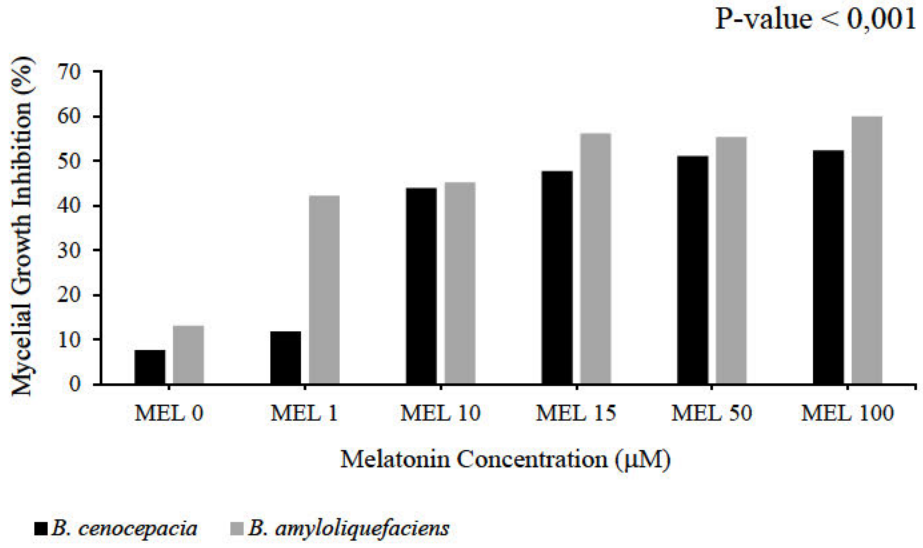

Figure 5.3: Comparison between the effects of *B. cenocepacia* and *B. amlyloliquefaciens* integrated with different melatonin concentrations 9 days post-inoculation at 25°C.

5.3.6 *In vivo* screening of melatonin integrated with *Bacillus amyloliquefaciens* and *Burkholderia cenocepacia* against *F. oxysporum*

The melatonin concentrations with the highest mycelial growth inhibition percentages under the *in vitro* screening trial were integrated with BCAs, *B. cenocepacia* and *B. amyloliquefaciens* for *in vivo* screening. The integrated treatments had significantly different effects on the severity of FDR on potato tubers inoculated with *F. oxysporum* 21 days post-inoculation. Potato tubers treated with treatment *Bamy* + MEL100 had the lowest severity of FDR with a disease severity percentage of 50,61% and a pathogen penetration value of 6,39 mm. Treatments *Bcen* + MEL100 and *Bamy* + MEL50 showed disease severity percentages of 52,63% and 59,72%, respectively (Table 5.3).

Table 5.3: Severity of *Fusarium* dry rot on potato tubers treated with *B. amyloliquefaciens* and *B. cenocepacia* combined with melatonin 21 days post-inoculation.

Treatment		Pathogen Penetration (mm)	Mean lesion diameter (mm)	Disease severity (%)
BCA	**Melatonin (μM)**			
B. cenocepacia	100	6,84[a]	14,62[a]	47,13%
	50	8,45[b]	22,69[c]	73,15%
	15	8,23[ab]	19,88[b]	64,09%
B. amyloliquefaciens	100	6,39[a]	14,06[a]	50,61T%
	50	8,20[ab]	16,59[b]	59,72%
	15	9,23[b]	21,66[c]	77,97%
Control	-	9,70[b]	31,02[d]	
P-value	-	0,019	<0,001	-
CV	-	12,70%	7,90%	-
LSD	-	1,653	2,762	-

Means with the same letters are not significantly different at a 5% level of significance in DMRT.

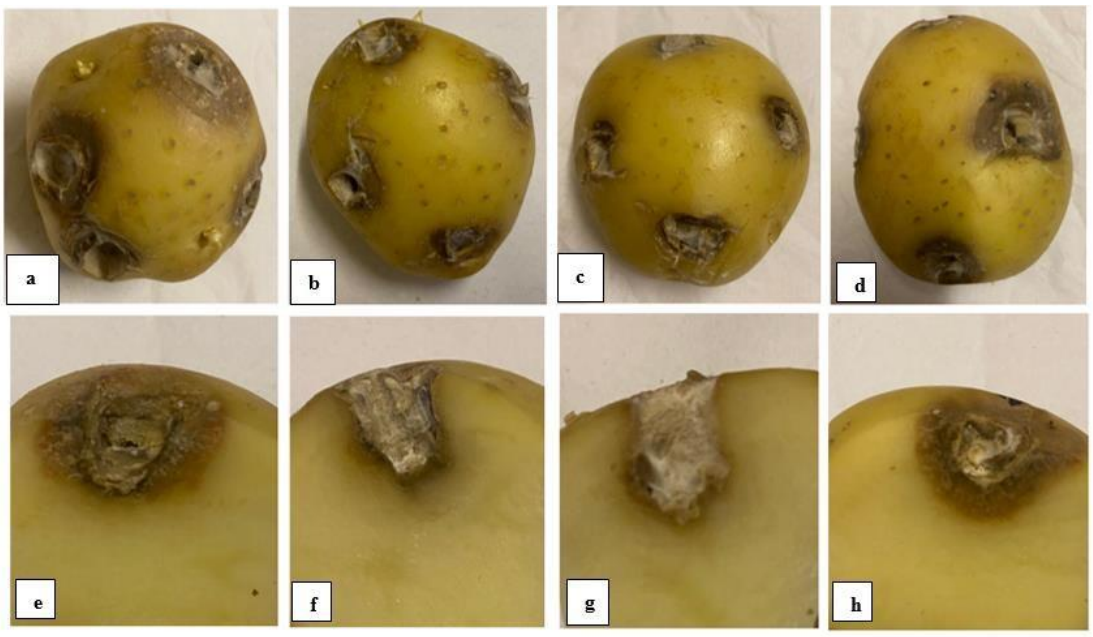

Figure 5.4: Effects of the integration of *B. cenocepacia* and different melatonin concentrations on tubers inoculated with *F. oxysporum* 21 days post-inoculation. **a** and **e**: Untreated control. **b** and **f**: Tuber treated with *Bcen* + MEL100. **c** and **g**: Tuber treated *Bcen* + MEL50. **d** and **h**: Tuber treated with *Bcen* + MEL15.

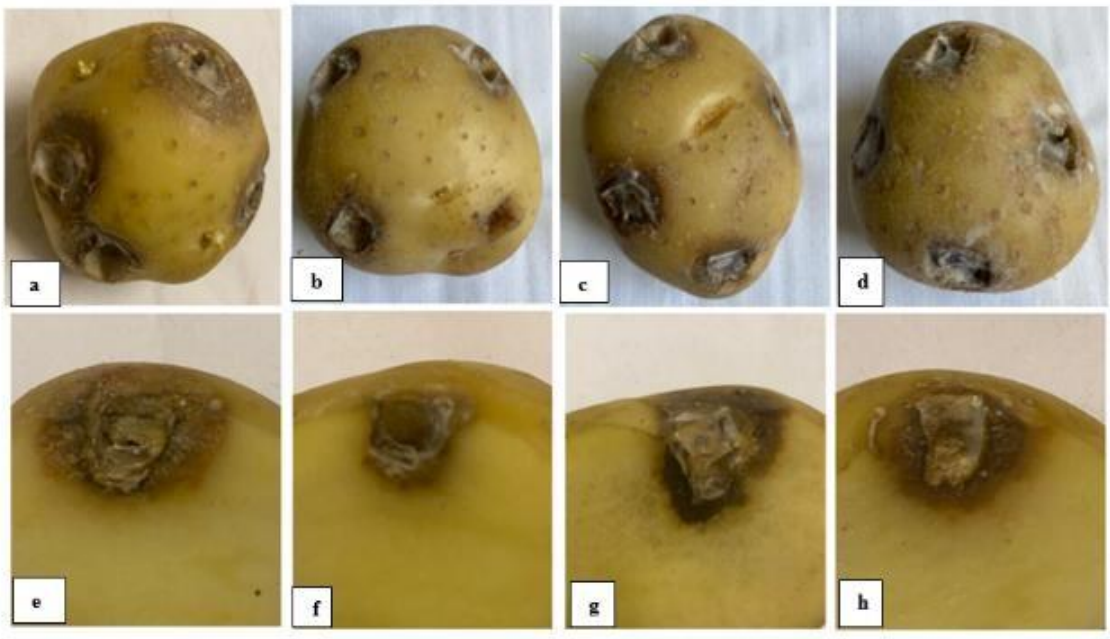

Figure 5.5: Effects of the integration of *B. amyloliquefaciens* and different melatonin concentrations on tubers inoculated with *F. oxysporum* 21 days post-inoculation. **a** and **e**: Untreated control. **b** and **f**: Tuber treated with *Bamy* + MEL100. **c** and **g**: Tuber treated *Bamy* + MEL50. **d** and **h**: Tuber treated with *Bamy* + MEL15.

5.3.7 Scanning electron microscopy studies of the interactions of melatonin integrated with *B. cenocepacia* and *B. amyloliquefaciens* against *F. oxysporum*

The *in vitro* effects of treatments *Bcen* + MEL100 and *Bamy* + MEL100 on *F. oxysporum* growing on PDA plates was viewed under the SEM. The untreated control showed normal growth of *F. oxysporum* hyphae and microconidia with no shrinking or deformation (Figure 5.6a and b). Samples treated with *Bcen* + MEL100, and *Bamy* + MEL100 presented effects of hyphae busting, deformation, and shrinking in various locations. There were no microconidia and fewer hyphae on the treated samples.

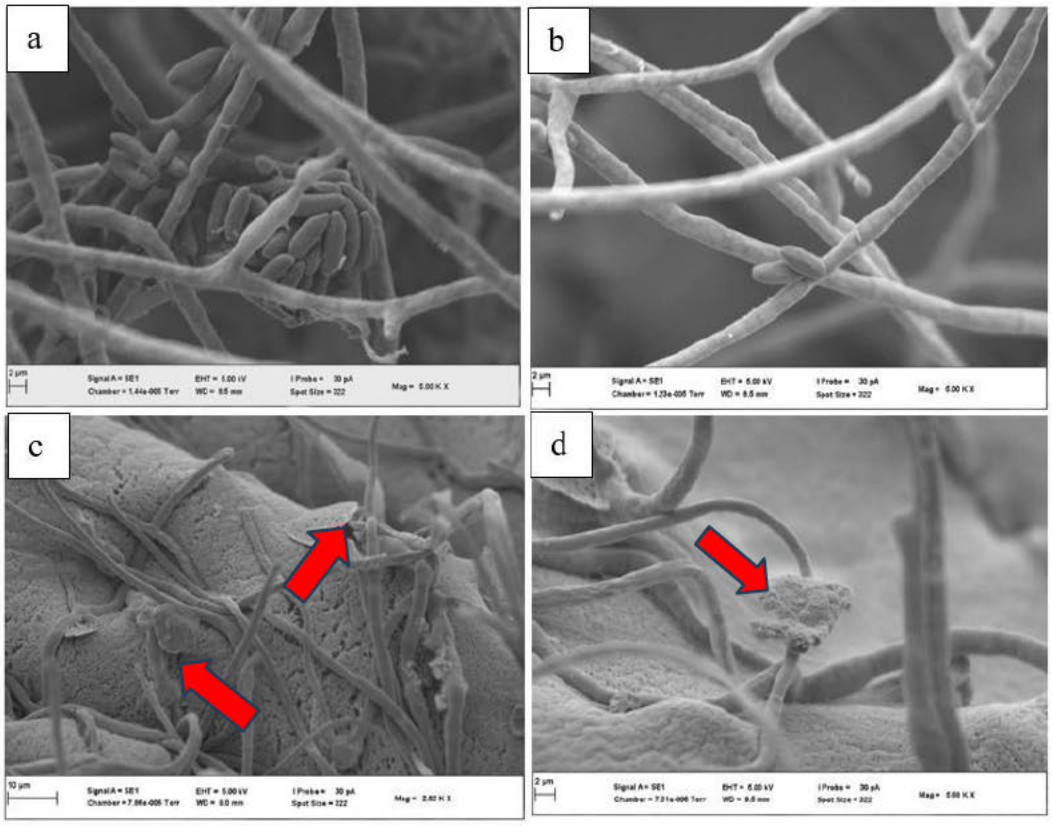

Figure 5.6: Effects of BCAs and melatonin on *F. oxysporum* under the SEM (5000x). **a** and **b**: Untreated *F. oxysporum* mycelia branching at 90° angle. **c**: Effect of treatment *Bamy* + MEL100 on *F. oxysporum* mycelia shown with arrows. **d**: Effect of treatment *Bcen* + MEL100 on *F. oxysporum* mycelia shown with an arrow.

5.3.8 Determination of phenolic content

The treatments had varying effects on the phenolic content of treated 'Sifra' potato tubers after 14 days at ambient temperature (Table 5.4). There were no significant differences between the effects of the treatments on the phenolic content days 0 and 7 post-exogenous application (P = 0,135 ; P = 0,079). However, on day 14, there were significant differences in the effects of the different treatments on phenolic content (P = 0,02). On day 14, potato tubers treated with *B. amyloliquefaciens* (BCA) had the lowest phenolic content with a value of 87,3 mg GAE/g followed by those treated with melatonin 100 μM with a phenolic content value of 91,1 mg GAE/g. Potato tubers treated with the integrated treatment of melatonin and *B. amyloliquefaciens* had the highest phenolic content of 144,1 mg GAE/g.

Table 5.4: The phenolic content of 'Sifra' potato tubers treated with *B. amyloliquefaciens* and melatonin independently and in combination after 14 days at ±25°C.

Treatment	**Mean phenolic content (mg GAE/g DW)**		
	Day 0	**Day 7**	**Day 14**
Bamy	81,28^{ab}	119,0^{ab}	87,3^{a}
MEL100	75,16^{a}	83,3^{a}	91,1^{a}
Bamy + MEL100	88,42^{b}	119,8^{ab}	144,1^{b}
Control	81,74^{ab}	135,6^{b}	104,4^{a}
P-value	0,135	0,079	0,02
LSD	11,2	39,73	34,77
CV (%)	7,30	18,4	17,3

Means with the same letters within a column are not significantly different at 5% level of significance DMRT.

5.3.9 Determination of ascorbic acid

The treatments had varying effects on the phenolic content of treated potato tubers (*cv*. 'Sifra') after 14 days at ambient temperature (Table 5.5). There were significant differences between the effects of the treatments on the ascorbic acid content days 0 and 7 post-exogenous application (P = 0,009; P = 0,046). However, on day 14, there were no significant differences in the effects of the different treatments on ascorbic content (P = 0,060). On day 14, potato tubers treated with *B. amyloliquefaciens* (BCA) had the lowest ascorbic acid content with a value of 2,70 mg AAE/ 100g DM. *Bamy* + MEL100 treated tubers showed the ascorbic acid content value of 3,62 mg AAE/ 100g DM. Potato tubers treated with MEL100 had the highest content, valued at 5,48 mg AAE/ 100g DM.

Table 5.5: The Ascorbic aid content of tubers treated with *B. amyloliquefaciens* and Melatonin independently and in combination after 14 days stored in ambient temperature (±25°C).

Treatment	**Mean Ascorbic Acid (mg AAE/100g DM)**		
	Day 0	**Day 7**	**Day 14**
Bamy	3,49^{a}	2,14^{a}	2,70^{a}
MEL100	4,29^{b}	5,28^{b}	5,48^{b}
Bamy + MEL100	4,62^{b}	5,83^{b}	3,62^{ab}
Control	4,13^{b}	4,89^{b}	4,09^{ab}
P-value	0,009	0,046	0,060
LSD	4,116	2,601	1,954
CV (%)	7,0	30,5	26,1

Means with the same letters within a column are not significantly different at 5% level of significance DMRT.

5.3.10 Determination of antioxidant activity [DPPH (2, 2-Diphenyl-1-picrylhydrazyl)]

All the treatments did not have a significant effect on the antioxidant activity of treated potato tubers (*cv*. 'Sifra') at day 0, 7, and 14 stored at ambient temperature (Table 5.6). All the P-values were greater than 0,005 at a 5% significance level (DMRT).

Table 5.6: The antioxidant activity (DPPH) of tubers treated with *B. amyloliquefaciens* and Melatonin independently and in combination after 14 days stored in ambient temperature (±25℃).

Treatment	Antioxidant activity (mg/g DM)		
	Day 0	**Day 7**	**Day 14**
Bamy	0,5863[a]	0,5863[a]	0,5859[a]
MEL100	0,5863[a]	0,5863[a]	0,5863[a]
Bamy + MEL100	0,5863[a]	0,5863[a]	0,5863[a]
Control	0,5863[a]	0,5863[a]	0,5863[a]
P-value	0,798	0,862	0,405
LSD	-	-	-
CV (%)	0	0	0,1

Means with the same letters within a column are not significantly different at 5% level of significance DMRT.

5.3.11 Determination of protein content

The treatments had varying effects on the phenolic content of treated potato tubers (*cv*. "Sifra") after 14 days at ambient temperature (Table 5.7). There were no significant differences between the effects of the treatments on the protein content on days 7 and 14 after the exogenous application of the treatments (P = 0,978; P = 0,579). However, on day 0, there were significant differences in the effects of the different treatments on protein content (P = 0,016). On day 14, potato tubers treated with *Bamy* + MEL100 had the highest protein content, valued at 64,81 mg/g DM whereas, the protein content of the potato tubers treated with *Bamy* and MEL100 only, had protein content concentrations of 50,88 mg/g DM and 51,69 mg/g DM, respectively.

Table 5.7: The protein content of tubers treated with *B. amyloliquefaciens* and Melatonin independently and in combination after 14 days stored in ambient temperature (±25℃).

Treatment	Mean Protein Content (mg/g DM)		
	Day 0	**Day 7**	**Day 14**
Bamy	55,78[b]	56,73[a]	50,88[a]
MEL100	56,10[b]	52,56[a]	51,69[a]
Bamy + MEL100	53,97[a]	51,88[a]	64,81[a]
Control	55,28[b]	54,73[a]	50,06[a]
P-value	0,016	0,978	0,579
LSD	1,210	28,52	27,32
CV (%)	1,2	28,1	26,7

Means with the same letters within a column are not significantly different at 5% level of significance DMRT.

5.4 Discussion

Fusarium dry rot is a devastating postharvest disease of potatoes which is caused by *Fusarium* species including *F. oxsyporum*. Biological control agents, natural plant extracts and GRAS substances have been widely studied for their potential as alternative control agents that can be used as alternatives to synthetic chemical fungicides. Numerous bacterial species have proven to have antimicrobial effects against *F. oxysporum* causing diseases in potato (Nguvo and Gao, 2019). GRAS substances, such as edible coatings, organic and inorganic salts, organic and inorganic acids, and phytohormones, can be used to control *Fusarium* diseases (Xue *et al.*, 2023).

The primary aim of the study was to evaluate the integrated effects of potential BCA species, *B. cenocepacia* and *B. amyloliquefaciens*, together with different concentrations of melatonin on the growth and development of FDR caused by *F. oxsporum*. The results indicated the compatibility of melatonin with the two BCAs tested as the combined treatments significantly reduced the mycelial growth of *F. oxysporum* on PDA *in vitro* and reduced the severity of FDR on potato tubers in the *in vivo* trials. Tubers treated with a combination of melatonin and *B. amyloliquefaciens* showed less severe symptoms of FDR across all concentrations compared to those treated with *B. cenocepacia*. Melatonin concentration 100 μM showed the best results when combined with both *B. cenocepacia* and *B. amyloliquefaciens*.

The individual efficacy of *B. amyloliquefaciens* and melatonin concentration 100 μM against the growth of *F. oxysporum* was established in previous studies (Chapter 3 and 4). In this study, the combination of these treatments did not improve the efficacy of *B. amyloliquefaciens* in inhibiting the growth of *F. oxysporum in vitro* and *in vivo*. However, the integration of the two treatments did improve the effectiveness of melatonin in inhibiting the growth of *F. oxysporum in vivo*. Zang *et al* (2022), demonstrated that melatonin at a concentration of 10 μmol/l integrated with selenium, significantly reduced the occurrence of *Botrytis cinerea* on tomato fruits. Melatonin at 100 μM have been proved to have efficacy against *F. oxyporum* causing *Fusarium* wilt on banana, *B. cinerea*, *Rhizoctonia solani*, and *Phytophthora capsici* (Aghdam and Fard, 2017; Wei *et al.*, 2017; Mandal *et al.*, 2018).

Melatonin plays a significant role in promoting plant response against abiotic and biotic stress including controlling plant diseases (Wang *et al.*, 2020; Zhao *et al.*, 2021). Melatonin inhibits the growth of fungal pathogens by enhancing the activity of defence-related enzymes which suppress pathogen development and deforming the cellular structure of the pathogen (Li *et al*., 2023). Mandal *et al* (2018) demonstrated how the exogenous application of melatonin on watermelon upregulated the expression of defence genes involved in effector immunity mediated defences. *B. amyloliquefaciens* is a member of the *Bacillus* genus which has been extensively studied and used as BCAs (Khedher *et al.*, 2021; Bonaterra *et al.*, 2022). The SEM micrographs (Figure 5.6c and d) show adverse deformations such as shrinking and bursting of the mycelia of *F. oxysporum*. The deformations are from the suspected effects of the secondary metabolites released by *B. amyloliquefaciens* that act against the development and growth of *F. oxysporum*. *Bacillus* species produce inhibitory secondary metabolites such as volatile organic compounds as their mode of action (Anckaert *et al.*, 2021; Soliman *et al.*, 2022).

Various studies have demonstrated the ability of *B. amyloliquefaciens* to inhibit spore germination and mycelial growth of different *F. oxysporum* strains by releasing volatile organic compounds (Minerdi *et al.*, 2009; Yuan *et al.*, 2012). The secondary aim of the study was to determine the effects of the exogenous application of biological control agents, *B. amyloliquefaciens* and melatonin on the nutritional quality of potato tubers (*cv*. 'Sifra'). The findings of this study indicated that the application of melatonin at a concentration of 100 μM had the highest amount of ascorbic acid after being stored at ambient temperature for 14 days.

Exogenous application of melatonin at 100 μM increased the ascorbic acid content of potato tubers by 34% compared to the untreated tubers. The ascorbic acid content of potato tubers treated with melatonin combined with *B. amyloliquefaciens* decreased by 11,5% compared to the untreated control tubers. Potato tubers treated with *B. amyloliquefaciens* had the lowest amount of ascorbic acid, with a non-significant percentage decrease of 34% compared to the untreated tuber control. In contrast to the findings of this study, Yu *et al* (2023) demonstrated the application of *Bacillus cereus, B. subtilis,* and *Serratia* spp. XY21 increased the ascorbic content of sweet potato tubers.

Potato tubers treated with melatonin combined with *B. amyloliquefaciens* had the highest phenolic content compared to the untreated tubers. The combined treatment increased the phenolic content by 38%. In a study by Saleh *et al* (2019), the content of endogenous melatonin in legumes increased as the total phenol and antioxidant activity increased. This indicated a positive correlation between melatonin and phenolic content. The exogenous application of melatonin and *B. amyloliquefaciens,* individually and in combination, significantly increased the protein content on the treated tubers. These results agree with the results obtained by Zarzecka *et al* (2019), where the exogenous application of herbicides combined with growth regulators (Harrier 295ZC, Sencor 70WG, and Kelpak SL) increased the phenolic and protein content on potato tubers.

5.5 Conclusion

The primary aim of the study was to evaluate the combined effects of melatonin and *B. amyloliquefaciens* and *B. cenocepacia* against *F. oxysporum*. The secondary aim of the study was to evaluate the effects of the treatments on the postharvest quality of potato tubers. Based on the findings of this study, it can be concluded that the integration of melatonin at a concentration of 100 μM and *B. amyloliquefaciens* strain MPA 1034, significantly inhibits the growth of *F. oxysporum*. It can be further concluded that the exogenous application of melatonin and *B. amyloliquefaciens* does not have negative effects on the quality of treated ‘Sifra’ potato tubers. The combined treatments can be recommended for use as an alternative control strategy for the control of FDR disease caused by *F. oxysporum* on potatoes. The findings of this study lay a foundation for further research on melatonin and *B. amyloliquefaciens* as postharvest quality enhancers on potatoes.

5.6 References

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Chapter 6

Dissertation overview and recommendations

6.1 Introduction

Potatoes significantly improve food and nutritional security in developing countries in Africa, Latin America, and Asia (Devaux *et al.*, 2020). However, the production of potatoes is hindered by postharvest fungal diseases, such as *Fusarium* dry rot (FDR), that result in great yield and subsequent economic losses. Biological control agents and GRAS substances are widely studied for their potential as sustainable and safer alternatives to synthetic chemical fungicides used in the agricultural industry. Melatonin (N-acetyl-5-methoxytryptamine) is a low molecular weight organic compound found across many different kingdoms (Zang *et al.*, 2022). Melatonin has valuable characteristics that contribute to improving the physiological functions of the plant, such as abiotic and biotic stress tolerance, promoting propagation, and growth and development (Nawaz *et al.*, 2016). The *Bacillus* spp. has been extensively studied and used as BCAs against a plethora of fungal pathogens, including *F. oxysporum* (Khedher *et al.*, 2021; Bonaterra *et al.*, 2022). These bacterial species use antibiosis and the production of inhibitory secondary metabolites as their mode of action (Yuan *et al.*, 2012; Anckaert *et al.*, 2021). There is limited information on the use of melatonin combined with bacterial antagonistic microorganisms against *F. oxysporum*.

6.2 Research objectives and major findings

The aim of the study was to evaluate the efficacy of biological control agents (yeast and bacteria) and melatonin, individually and in combination, against *F. oxysporum in vitro* and *in vivo*. The research objectives were as follows:

1. Evaluate the *in vitro* and *in vivo* effects of BCAs against dry rot disease caused by *F. oxysporum* - Chapter 3
2. Evaluate the *in vitro* and *in vivo* effects of melatonin against dry rot disease caused by *F. oxysporum* – Chapter 4

3. Evaluate the integrated effects of BCAs and melatonin against dry rot disease caused by *F. oxysporum* and the postharvest quality of 'Sifra' potatoes tubers – Chapter 5

6.2.1 Chapter 3: Effects of biological control agents against *F. oxysporum* infecting potatoes

- The best-performing isolates, NG1 and NG2, were obtained from the Ganoderma mushroom (*Ganoderma austroafricanum*)
- Isolates NG1 and NG2 were identified using BLAST as bacterial species, *Burkaholderia cenocepacia* strain LMG 16656 and *Bacillus amyloliquefaciens* strain MPA 1034.
- SEM images showed antibiosis interaction between the BCAs and *F. oxysporum*, which inhibited the growth of the fungus by shrinking and deforming the mycelium.
- *B. cenocepacia* strain LMG 16656 and *B. amyloliquefaciens* strain MPA 1034 significantly reduced the spread of FDR symptoms on potato tubers (*cv*. "Sifra").

6.2.2 Chapter 4: Effects of melatonin against *F. oxysporum* infecting potatoes

- Melatonin had antifungal effects against *F. oxysporum* at 100 μM, 50 μM, and 15 μM concentrations.
- The inhibitory effects were more prominent *in vivo* than *in vitro* due to the mode of action of melatonin, which includes the upregulation of host's defence-related enzymes.
- Melatonin inhibited the growth of *F. oxysporum* by suppressing microconidia formation and shrinking the mycelium.
- Melatonin concentration of 100 μM significantly suppressed FDR symptoms on potato tubers (*cv*. "Sifra")

6.2.3 Chapter 5: Integrated effects of biological control agents and melatonin against *F. oxysporum* infecting potatoes

- Melatonin can be used in combination with *B. cenocepacia* strain LMG 16656 and *B. amyloliquefaciens* strain MPA 1034 to inhibit the growth of *F. oxysporum*.
- *B. amyloliquefaciens* strain MPA 1034 combined with melatonin 100 μM significantly inhibited the growth of *F. oxysporum* both *in vitro* and *in vivo*.

- Integrating *B. amyloliquefaciens* strain MPA 1034 with melatonin improved the efficacy of melatonin against *F. oxysporum*.
- SEM micrographs demonstrated the aggravated effects of integrating *B. amyloliquefaciens* and 100 μM melatonin on *F. oxysporum*.
- Exogenous application of BCA, *B. amyloliquefaciens* strain MPA 1034, and melatonin 100 μM had varying effects on the content of phenolics, Vitamin C, and protein on potato tubers (*cv*. 'Sifra').
- Exogenous application of melatonin 100 μM increased protein and ascorbic acid content but did not significantly affect phenolic content.
- Exogenous application of *B. amyloliquefaciens* did not have significant effects on the phenolic, ascorbic acid, and protein content of potato tubers.
- The application of melatonin integrated with *B. amyloliquefaciens* significantly improved the content of phenolics, proteins, and ascorbic acid.
- The treatments do not have deteriorating effects on the quality of potato tubers (*cv*. "Sifra").

6.3 Recommendations and future research

This study demonstrated the efficacy of *B. amyloliquefaciens* and melatonin as biological control agents that can be used to control *Fusarium* dry rot caused by *F. oxysporum* on potatoes. Based on the findings of this study, further studies may cover the following recommendations:

- The exploration of the effects of *B. amyloliquefaciens* and melatonin on the expression of defence-related enzymes and other secondary metabolites should be evaluated to determine the mode of action of the treatments.
- Evaluation of the effects of *B. amyloliquefaciens* and melatonin on other quality parameters of potato tuber such as chip colour, specific gravity, and dry matter.
- Evaluation of the effects of *B. amyloliquefaciens* and melatonin on the production of *Fusarium*-related mycotoxins.
- *B. amyloliquefaciens* and melatonin should be screened against other problematic potato pathogens, including preharvest and postharvest diseases caused by bacteria and pests.

- The efficacy of *B. amyloliquefaciens* and melatonin as potato seed treatment should be evaluated to determine the preharvest effects of the treatments.

6.4 References

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