UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

Management of *Colletotrichum gloeosporioides* on papaya fruits using biological control agents and *Moringa oleifera* leaf extract

By

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DISSERTATION SUMMARY

Colletotrichum gloeosporioides (Penzig) Penzig & Saccardo is one of the major pathogens causing anthracnose on several plants. This fungal pathogen causes infection both in the field and during postharvest, leading to reduced production and severe economic losses. There is a need to develop eco-friendly strategies to reduce the number of losses in production and the use of synthetic chemicals and their negative impact on human health and the environment. The aim of the current study was to control *C. gloeosporioides* of papaya using biological control agents and *Moringa oleifera* leaf extract.

A total of 45 yeast and 55 *Bacillus* isolates were obtained from the fruit surfaces of guava (*Psidium guajava* L.), papaya (*Carica papaya* L.), cassava (*Manihot esculenta Crantz*), aloe (*Aloe vera* L.) and syringa (*Syringa vulgaris*) and screened against *C. gloeosporioides*. Twenty-seven yeast and fifty-one *Bacillus* isolates successfully inhibited the mycelial growth of *C. gloeosporioides* by greater than 50% during the *in vitro* preliminary trial. In the secondary screening, 21 yeasts and 47 *Bacillus* isolates managed to control the mycelial growth of the pathogen, and the reduction was significantly different ($P<0.05$) compared to the control treatment. Yeast isolates YCR3, YSY1 and *Bacillus* isolates BMR2 and B16 provided the best control of *C. gloeosporioides* both in primary and secondary *in vitro* trials. The scanning electron microscopy studies indicated that yeasts and *Bacillus* isolates inhibited the mycelial growth and spore production of *C. gloeosporioides*. Preventive application of the *Bacillus* isolate B16 resulted in a reduced incidence of anthracnose on papaya fruits, with a percentage of 88.67% at day 10 post-inoculation. The water control treatment had the lowest incidence of anthracnose (16.67%) and it was significantly different ($P\leq0.001$) from fruits inoculated with pathogen-only control (100%).

Five concentrations of moringa leaf extract were prepared (MLE1%, MLE1.5%, MLE2%, MLE2.5% and MLE3%) and these concentrations showed better performance during the primary *in vitro* trial with a significant difference of ($P<0.05$) when compared to the untreated control. On the secondary screening, MLE2.5% and MLE3% were further screened against *C. gloeosporioides* and inhibited mycelial growth by 96.9% and 97.5%, respectively. The preventive application of MLE2.5% (88.67%) at day 7 post inoculation, provided a better control and delayed anthracnose incidence when compared to MLE3% (100%) and water control (16.67%) *in vivo*.

MLE2.5% treated fruits developed anthracnose incidence of 66%, compared to *Bacillus megatarium* treated fruit with 55% at day 7 after inoculation. However, the anthracnose incidence increased with each successive day of the trial leading to an incidence of 33% for water control. The integrated control of MLE2.5% with *B. megatarium* was the most effective treatment for the control of anthracnose and delayed anthracnose incidence when compared to MLE3% integrated with *B. megatarium* at day 3, 5, 7 and 10 days post-inoculation. Anthracnose incidence on papaya fruits treated with MLE2.5%+B16 (B16 being the label used to represent *B. megatarium* isolate) was 55%, and 100% on the fruits treated with MLE3%+B16 compared to 100% anthracnose incidence on pathogen-treated fruits at day 10 after inoculation. The physicochemical properties of papaya fruits, weight loss and total soluble solids, were not negatively affected by MLE2.5% + *B. megatarium* treatment. *Bacillus megatarium* can be integrated with moringa leaf extracts to manage anthracnose of papaya.

DECLARATION

I, NOMUSA MARIA SITHOLE, declare that

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

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

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

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

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…… [redacted] ……

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(MSc Candidate)

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Dr Nokwazi Carol Mbili (Supervisor)

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DEDICATION

To my family, more especially my mother Thembekile Sithole, my father Julius Sithole and my sister Sinethemba Sithole for the love, encouragements, and support.

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Chapter 1 Introduction

1.1. Background

Papaya (*Carica papaya L*.) is one of the most popular fruits and its production varies worldwide (Silva *et al*., 2007). It is cultivated in more than 60 countries, including India, Indonesia, Brazil, Mexico, Nigeria, Dominican Republic, United States, and Australia (Olubode, 2012). This crop is grown under tropical and sub-tropical climates, where the growing cycle is fast and occurs throughout the year (Murayama *et al*., 2006). It has been reported that about 185.95 million tonnes of *C. papaya* fruits were produced between 2000 and 2017 with India being the largest producer with about 5,699,000 million tons per year while Brazil was the second-largest producer with 1.60 million tons per year (Alara *et al.*, 2020).

In addition, papaya seeds, leaves, and fruits have pharmacological activities for humans such as anti-protozoan, anti-fertility, and anthelmintic. They also contain bioactive compounds that are beneficial for preventing diseases such as cancer (Ikram *et al*., 2015; Alara *et al*., 2020). The digestive enzyme-papain which effectively treats causes of allergies trauma and sports injuries is found in raw papaya fruits (Olivier, 2015). This fruit consists mostly of water, carbohydrates, calories, and is rich in natural vitamins and minerals, particularly vitamins A and C, ascorbic acid, and potassium. Papaya is a good source of fiber and it lowers high cholesterol levels (Begum, 2014) but as a climacteric fruit, its ripening process continues after harvest (Rodrigues *et al*., 2021) causing fruit quality loss due to immature harvest, injuries caused by improper handling and storage, postharvest diseases, damage caused by cold or high temperatures, pests, and physiological disorders (Tan *et al*., 2022).

Colletotrichum gloeosporioides (Penzig) Penzig & Saccardo, *Lasiodiploidia theobromae* (Patouillard) Griffon & Maublanc., *Fusarium solani* (von Martius) Saccardo, *Phomopsis caricae-papayae* (Petrak & Ciferri) Rossman & Udayanaga and *Rhizopus stolonifer* (Ehrenberg) *Vuillemin* (Soma, 2020; Rodrigues *et al*., 2021) are postharvest pathogens that have been confirmed to be a threat to papaya fruits and cause an irreversible effect on the fruit by creating various symptoms that indicate inedibility, including the formation of water-soaked lesions, secretion of toxic byproducts and pungent smell (Zang *et al*.,

2021). Most crops grown worldwide are susceptible to at least one or more *Colletotrichum* species which cause more than 50% of losses of fresh fruits and vegetables (Awang *et al*., 2011).

Colletotrichum is associated with economically significant diseases of grasses, cereals, vegetables, legumes, perennial crops, and fruits (Latunde-Dada, 2001). *Colletotrichum* has been nominated as the most important genus of phytopathogenic fungi throughout the world, based on its scientific and economic significance (Dean *et al.*, 2012). This pathogen has the delayed onset of disease symptoms in a crop and is often devastating, leading to severe postharvest losses (Ali *et al*., 2011). The highly perishable nature of this fruit highlights the urgent need to develop biological controls that can reduce their deterioration and extend shelf life to impart value to consumers (Hassan *et al*., 2021).

1.2. Problem statement

Perishable fruits with short nematode diseases (Mirza and Ismael, 2021) and papaya is one of the fruits affected by postharvest diseases. Several reports indicated that papaya fruits are high in water content and susceptible to postharvest shriveling which negatively impacts their appearance and market potential (Kader and Yahia, 2011; Workneh *et al*., 2012; Sivakumar and Wall, 2013). Kader and Yahia (2011) reported that all papaya fruits are characterized by high water content and potential for water loss resulting in shriveling, susceptibility to mechanical injury and susceptibility to attacks by microorganisms and insects. Several important postharvest fungi affecting and causing losses to papaya fruit include *C. gloeosporioides* which causes anthracnose, and is the most common and widespread papaya pathogen worldwide (Bautista-Baños *et al*., 2013).

Anthracnose is classified as a postharvest disease caused by the fungus *C. gloeosporioides* which is a devastating and economically important pathogen causing approximately 40-100% of papaya fruit losses in developing countries (Madani *et al*., 2014). *C. gloeosporioides* produces enzymes which degrades the plant cell wall and cause high economic losses (Rodríguez-López *et al*., 2009). The disease symptoms on infected fruits are characterized by small subcircular or angular small water-soaked lesions with translucent light brown margins (Jain *et al*., 2023.). This can be managed by post-harvest hot water treatment and synthetic fungicides. However, hot water treatment

causes damage to fruits, and the pathogen has become resistant to some of the fungicides currently in use (Aguayo *et al*., 2008; Djioua *et al*., 2010).

Synthetic fungicides such as prochloraz, benomyl, carbendazim, propineb and difenoconazole have been used traditionally in the pre- and postharvest control of anthracnose (Sundravadana *et al*., 2007; Ali *et al*., 2016). These synthetic fungicides have severe health problems in humans, causing eye defects and birth-related problems by disrupting the process of cell division making their use unacceptable and dangerous (Bordoh *et al*., 2020). The long-term use of these fungicides has negative impacts on the environment, causing water and soil pollution (Mahmood *et al*., 2016; Bordoh *et al*., 2020).

The increasing health concerns expressed by consumers to regulate fungicide use and their residues in fresh produce have necessitated the development of non-toxic and nonfungicidal management approaches for achieving more papaya production and profits (Droby, 2005; Claeys *et al*., 2011). Therefore, interest in using environmentally-friendly approaches has gradually increased to achieve more papaya production and profits as well as improved fruit quality.

1.3. Research Aim and Objectives

The aim of this project was to examine the effect of biological control agents (bacteria and yeast) and moringa leaf extract in maintaining post-harvest quality of papaya

The specific objectives were to:

- Isolate antagonistic yeasts and *Bacillus* from different medicinal plants to evaluate their efficacy for the control of *C. gloeosporioides*.
- Investigate the *in vitro* and *in vivo* effect of yeast and *Bacillus* isolates on *C. gloeosporioides*.
- Investigate the effect of different concentrations of moringa leaf extract on *C. gloeosporioides in vitro* and *in vivo*.
- To determine independent and combined effects of *Bacillus* or yeast isolates with moringa leaf extracts for the control of *C. gloeosporioides* of papaya.

1.4. Motivation of study

More than 50% of losses of fruits and vegetables are caused by *Colletotrichum* species (Awang *et al*., 2011). This pathogen is managed by hot water treatment (HWT) (Li *et al*., 2013). However, HWT causes decolorization and scalding of fruit skin and softens the fruit pulp since the temperature must be higher than the thermal death point of the pathogen (Barrera-Necha *et al*., 2008). Ozone is also used as one of the control approaches, fruits treated with a high concentration of ozone (4ppm) have a high decay rate and result in surface browning of fruits due to surface oxidation (Tzortzakis and Chrysargyris, 2017).

Several chemical fungicides including propiconazole, prochloraz, benomyl, and thiabendazole are commonly used to control anthracnose disease both on pre- and postharvest, even though they reduce the inoculum they are harmful to humans (Ali *et al*., 2015). Olisah *et al*. (2020) reported that some pesticides have been banned due to concerns related to their carcinogenic potential. Some of the synthetic fungicides cause severe health problems, causing birth-related problems and eye defects to human (Ali *et al*., 2016). Additionally, long-term use of synthetic fungicides negatively impacts the environment causing water and soil pollution (Northover and Zhou, 2002; Szczygiel *et al*., 2024). These negative effects are coupled with concerns of using non-toxic alternative approaches for controlling postharvest diseases without health implications to humans.

1.5. Justification

The use of synthetic fungicides to control postharvest deterioration is now losing popularity due to their long degradation period, high and acute residual toxicity, environmental pollution and their effects on food, side-effects on humans, and also their cost (Nath *et al*., 2002). The effects of synthetic fungicides suggest the need for alternative strategies to be developed for reducing papaya fruit losses due to postharvest decay that are perceived as safe and pose no risk to human health and the environment (Rodrigues *et al*., 2021).

Microbial antagonists have been reported to protect most of harvested perishable commodities against several postharvest pathogens (Singh *et al*., 2023) and these antagonists include the use of biological control agents and moringa leaf extracts. Biological control agents have antifungal activity against plant pathogens by the secretion of antifungal metabolites and enzymes or induction of disease resistance in fruits, which makes them an environmentally friendly strategy (Cheon *et al*., 2016). The antagonists

induce systemic resistance response in a host/fruit and have a long shelf-life effect with low manufacturing costs.

Plant extracts have antioxidant activity and exhibit antibacterial and antifungal potential against several pathogens (Hernández-Albiter *et al*., 2007). They contain a profile of important minerals, proteins, vitamins, and phytochemical compounds with biological activity and can potentially be used to retard the growth of microorganisms (Gurjar *et al*., 2012; Kavitha and Satish, 2013; Arora and Onsare, 2014).

Moringa oleifera is one of the plant extracts that has gained much importance, due to its multiple uses and benefits in agriculture and fruit industry (Ashfaq *et al*., 2012). Moringa leaves are popularly used as a dietary supplement and herbal medicine, and therefore, have nutritional benefits for humans. The leaf extracts of *M. oleifera* have been reported to exhibit antioxidant activity both *in vitro* and *in vivo* based on their abundance of flavonoids and phenolic acids (El Khateeb and El-Bahrawy, 2013; Vongsak *et al*., 2013). This requires an appropriate extraction method and solvent that promote the extract with high biological activities and high yield of active compounds for nutraceutical production (Vongsak *et al*., 2013).

1.6. References

Aguayo, E., Escalona, V.H. and Artés, F., 2008. Effect of hot water treatment and various calcium salts on quality of fresh cut 'Amarillo' melon. *Postharvest Biology and Technology*. 47, 397-406.

Alara, O.R., Abdurahman, N.H. and Olalere, O.A., 2020. Ethanolic extraction of flavonoids, phenolics and antioxidants from *Vernonia amygdalina* leaf using twolevel factorial design. *Journal King Saud University-Science. 32*, 7-16.

Ali, A., Bordoh, P.K., Singh, A., Siddiqui, Y. and Droby, S., 2016. Post-harvest development of anthracnose in pepper (Capsicum spp): Etiology and management strategies. *Crop Protection*. 90,132-141.

Ali, A., Muhammad, M.T.M., Sijam, K., Siddiqui, Y., 2011. Effect of chitosan coatings on the physicochemical characteristics of Eksotika II papaya (*Carica papaya L.*) fruit during cold storage. *Food Chemistry*. 124, 620–626.

Ali, M.E., Yong, C.K., Ching, Y.C., Chuah, C.H. and Liou, N.S., 2015. Effect of single and double stage chemically treated kenaf fibers on mechanical properties of polyvinyl alcohol film. *Bio Resource*. 10, 822-838.

Arora, D.S. and Onsare, J.G., 2014. *In vitro* antimicrobial evaluation and phytoconstituents of Moringa oleifera pod husks. *Industrial Crop Production*. 52, 125-135.

Ashfaq, M., Basra, S.M. and Ashfaq, U., 2012. Moringa: a miracle plant for agro- forestry. *Journal in Agriculture and Social Science. 8*, 115-122.

Awang, Y., Ghani, M.A.A., Sijam, K. and Mohamad, R.B., 2011. Effect of calcium chloride on anthracnose disease and postharvest quality of red-flesh dragon fruit (*Hylocereus polyrhizus*). *African Journal in Microbiological Research*. 5, 52505259.

Barrera-Necha, L.L., Bautista-Baños, S., Flores-Moctezuma, H.E. and Estudillo, A.R., 2008. Efficacy of essential oils on the conidial germination, growth of *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc and control of postharvest diseases in papaya (*Carica papaya* L.). *Plant Pathology Journal.* 7, pp.174-178.

Bautista;Banos, S., Sivakumar, D., Bello-Perez, A., Villanueva-Arce, R. and HernandezLopez, M. 2013. A Rev. of the management alternatives for controlling fungi on papaya fruit during postharvest supply chain. *Crop Protection.* 1, 8-20.

Begum, M., 2014. Phytochemical and pharmacological investigation of Carica papaya leaf Doctoral dissertation, East West University.

Bordoh, P.K., Ali, A., Dickinson, M. and Siddiqui, Y., 2020. Antimicrobial effect of rhizome and medicinal herb extract in controlling postharvest anthracnose of dragon fruit and their possible phytotoxicity. *Sciencia Horticulturae. 265*, 109249.

Cheon, W., Kim, Y.S., Balaraju, K., Kim, B.S., Lee, B.H. and Jeon, Y., 2016. Postharvest disease control of *Colletotrichum gloeosporioides* and *Penicillium expansum* on stored apples by gamma irradiation combined with fumigation. *The Plant Pathology Journal*. 32, 460.

Claeys, W.L., Schmit, J.F., Bragard, C., Maghuin-Rogister, G., Pussemier, L. and Schiffers, B., 2011. Exposure of several Belgian consumer groups to pesticide residues through fresh fruit and vegetable consumption. *Food control*. 22, 508516.

Dean, R., Van Kan, J.A., Pretorius, Z.A., Hammond- Kosack, K.E., Di Pietro, A., Spanu, P.D., Rudd, J.J., Dickman, M., Kahmann, R., Ellis, J. and Foster, G.D., 2012. The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*. 13, 414- 430.

Djioua, T., Charles, F., Freire Jr, M., Filgueiras, H., Ducamp-Collin, M.N. and Sallanon, H., 2010. Combined effects of postharvest heat treatment and chitosan coating on quality of fresh-cut mangoes (*Mangifera indica L*.). *International Journal of Food Science and Technology*. 45, 849- 855.

Droby, S., 2005, March. Improving quality and safety of fresh fruits and vegetables after harvest by the use of biocontrol agents and natural materials. *In International Symposium on Natural Preservatives in Food Systems. 709*, 45-52.

El-Khateeb, M.A. and El-Bahrawy, A.Z., 2013. Expensive post treatment using constructed wetland. Life Science Journal. 10, 560-568.

Gurjar, M.S., Ali, S., Akhtar, M. and Singh, K.S., 2012. Efficacy of plant extracts in plant disease management. *Agricultural Science*. 3, 425-433.

Hassan, N., M El-Deeb, A. and Ahmed, E.F., 2021. Improving the quality properties and extension the shelf life of soy cheese. *International Journal in Family Studies and Food Science in Nutrition. Heal*. 2,120-142.

Hernández-Albíter, R.C., Barrera-Necha, L.L., Bautista-Baños, S. and Bravo-Luna, L., 2007. Antifungal Potential of Crude Plant Extracts on Conidial Germination of Two Isolates of *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. *Revista Mexicana de Fitopatología,* 25, 180-185.

Ikram, E.H.K., Stanley, R., Netzel, M. and Fanning, K., 2015. Phytochemicals of papaya and its traditional health and culinary uses. *A Review- Journal of Food Composition and Analysis.* 41, 201-211.

Jain, K., Marwal, A., Sharma, K. and Desai, N., 2023. Identification of etiological agent of Telya disease of pomegranate, its pathogenesis and control using integrated management approach. *Research Journal of Biotechnology*. 18, 1.

Kader, A.A. and Yahia, E.M., 2011. Postharvest biology of tropical and subtropical fruits. In Postharvest biology and technology of tropical and subtropical fruits. *Woodhead Publishing*. 8, 79-111.

Kavitha, K.S. and Satish, S., 2013. Evaluation of antimicrobial and antioxidant activities from Toona ciliata Roemer. *Journal of Analytical Science and Technology*. 4, 1-7.

Latunde- Dada, A.O., 2001. Colletotrichum: tales of forcible entry, stealth, transient confinement and breakout. *Molecular Plant Pathology*. 2,187-198.

Li, X., Zhu, X., Zhao, N., Fu, D., Li, J., Chen, W. and Chen, W., 2013. Effects of hot water treatment on anthracnose disease in papaya fruit and its possible mechanism. *Postharvest Biological Technology*. 86, 437-446.

Madani, B., Mohamed, M.T.M., Biggs, A.R., Kadir, J., Awang, Y., Tayebimeigooni, A. and Shojaei, T.R., 2014. Effect of pre-harvest calcium chloride applications on fruit calcium level and post-harvest anthracnose disease of papaya. *Crop Protection*. 55, 55-60.

Mahmood, I., Imadi, S.R., Shazadi, K., Gul, A. and Hakeem, K.R., 2016. Effects of pesticides on environment. *Plant, soil and microbes Implications in Crop Science*. 1, 253-269.

Mirza, A.N. and Ismael, J.H., 2021. Tikrit Journal for Agricultural Sciences. *Academic Science Journal*. 21, 112-129.

Murayama, H., Katsumata, T., Endou, H., Fukushima, T. and Sakurai, N., 2006. Effect of storage period on the molecular-mass distribution profile of pectic and hemicellulosic polysaccharides in pears. *Postharvest Biological Technology*. 40, 141-148.

Nath, B.S., Sarwar, S., Usha, M.A. and Unnikrishnan, V., 2002. Organochlorine pesticide residues in market milk from Karnataka. *Indian Journal Diary in Biological Science*. 2, 98-101.

Northover, J. and Zhou, T., 2002. Control of rhizopus rot of peaches with postharvest treatments of Tebuconazole, Fludioxonil, and Pseudomonas syringae. *Canadian Journal in Plant Pathology*. 24, 144-153.

Olibode, O.O., Aiyelaagbe, I.O. and Bodunde, J.G., 2012. Effect of stage of introduction on performance of okra as an intercrop in pawpaw orchards. *Biological Agriculture and Horticulture*. 28, 61-70.

Olisah, C., Okoh, O.O. and Okoh, A.I., 2020. Occurrence of organochlorine pesticide residues in biological and environmental matrices in Africa: *A two-decade Review Heliyon*. 6, 3518.

Oliver, R., 2015. An update on the use of proteolytic enzymes as a means to reduce inflammation and pain. *Original Internist.* 22, 300-304.

Rodrigues, J.P., de Souza Coelho, C.C., Soares, A.G. and Freitas-Silva, O., 2021. Current technologies to control fungal diseases in postharvest papaya (Carica papaya L.). *Biocatalysis and Agricultural Biotechnology*. 36, 102128.

Rodríguez-López, E.S., González-Prieto, J.M., Mayek-Pérez, N., 2009. La infección de Colletotrichum gloeosporioides Penz. Penz. and Sacc. en aguacatero (Persea americana Mill.): Aspectos bioquímicos y genéticos." *Revista mexicana de fitopatología* 27, 53-63.

Szczygiel, T., Kozirog, A. and Otlewska, A., 2024.Synthetic and natural antifungal substances in cereal grain protection. *A Review of Bright and Dark sides. Molecular*. 29, 1

Singh, J., Mirza, A., Kaur, S., Arya, S.K. and Singh, G., 2023. Application of microbial antagonists for the preservation of fruits: An effective strategy to inhibit the postharvest disease. *In Relationship between Microbes Environmental Sustainable Ecosystem Services*. 261- 288.

Sivakumar, D. and Wall, M.M., 2013. Papaya fruit quality management during the postharvest supply chain. *Food Review International*. 29, 24-48.

Silva, J.D., Rashid, Z., Nhut, D.T., Sivakumar, D., Gera, A., Souza, M.T. and Tennant, P., 2007. Papaya (Carica papaya L.) biology and biotechnology. *Tree and Forest Science and biotechnology*. 1, 47-73.

Soma, A.B., 2020. Postharvest diseases of some selected fruits, their causes and management. Doctoral Dissertation. *Department of Plant Pathology*.

Sundravadana, S., Alice, D., Kuttalam, S. and Samiyappan, R., 2007. Efficacy of azoxystrobin on Colletotrichum gloeosporioides penz growth and on controlling mango anthracnose. *Journal in Agricultural Biological Science*. 2, 10-15.

Tan, G.H., Ali, A. and Siddiqui, Y., 2022. Current strategies, perspectives and challenges in management and control of postharvest diseases of papaya. *Scientia Horticulturae*. 301, 111139.

Tzortzakis, N. and Chrysargyris, A., 2017. Postharvest ozone application for the preservation of fruits and vegetables. *Food Review International*. 33, 270-315.

Vongsak, B., Sithisarn, P., Mangmool, S., Thongpraditchote, S., Wongkrajang, Y. and Gritsanapan, W., 2013. Maximizing total phenolics, total flavonoids contents and antioxidant activity of Moringa oleifera leaf extract by the appropriate extraction method. *Indian Crops Production*. 44, 566-571.

Workneh, T.S., Azene, M. and Tesfay, S.Z., 2012. A review on the integrated agro-technology of papaya fruit. *African Journal in Biotechnology*. 11, 15098-15110.

Zang, L.S., Wang, S., Zhang, F. and Desneux, N., 2021. Biological control with Trichogramma in China: History, present status, and perspectives. *Annual Review in Entomology.* 66,463-484.

Chapter 2 Control of *Colletotrichum gloeosporioides* causing anthracnose on papaya fruits: A literature review

2.1. Introduction

Fruits are an essential part of human diet as they supply essential nutrients such as vitamins and minerals (Keatinge et al., 2010). Farming is an important method that avails fruits in the society. It provides a variety of new ways of providing food that improve the quantity and quality of the nutrients that are available to many families. About 4.8 million tons of fruits are produced in South Africa, 63% are exported, 26% are processed, and the remaining 11% are destined for the local markets (Sibaluli, 2018) To reduce hunger, food production has to increase and reduce post-harvest losses such as weight loss due to spoilage, nutritional loss, and quality loss (Boxall, 2001). However, post-harvest decay of fruits is a challenge faced by farmers worldwide which reduces the availability of fruits. For increased fruit production, fruits must not have physiological disorders, mechanical injuries, and must be free from diseases (Ferguson and Boyd, 2002).

Papaya (*Carica papaya*) is a fruit consumed either raw or in processed forms such as jelly, jam, candy and pickles (Tan *et al*., 2020). As a climacteric fruit, it matures quickly and vulnerable to numerous post-harvest challenges leading to difficult maintenance during storage. (Ong *et al*., 2013; Zhu *et al*., 2013). Softening and senescence associated with post-harvest ripening make papaya fruit vulnerable to several post-harvest diseases.

Post-harvest diseases during storage and transportation significantly lowers the quality and value of this commodity. The incidence of post-harvest diseases, especially anthracnose caused by *Colletotrichum gloeosporioides*, becomes a problem when fruits have 25% or more skin yellowing (Fernandes *et al*., 2024). This has been the main concern for fruit growers, distributors, and exporters in extending the storage life of papaya fruit while keeping the fruits at an acceptable quality. This review will focus on *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc, which highly affects and causes losses on both tropical and subtropical fruits worldwide.

2.1.1. Origin and cultivation of papaya

Papaya (*Carica papaya L*.) is placed in *Caricaceae* family and is the most economically important species of the genus *Carica* (Carvalho and Renner, 2012), characterized as the third most cultivated tropical crop worldwide (Ong *et al*., 2013). The center of origin and domestication of this crop is the Mesoamerican region. Papaya seeds were distributed to the Caribbean and South-East Asia during the Spanish exploration in the 16th Century, and spread rapidly to the Pacific, India and Africa (OECD, 2003; Daagema *et al*., 2020). Currently it has become the most cultivated and consumed in tropical and subtropical areas in the whole world (dos Santos et al., 2014; Vij and Prashar, 2015).

This is a climacteric fruit that undergoes a variety of physical and chemical changes after harvest (Kebede, 2020). However is a fast-growing, short-lived crop cultivated for its fruit, papain, pectin, and antibacterial substances, producing mature fruits within 9-12 months after planting (Niklas and Marler, 2007). The extreme cold or heat can injure the tree and its fruits. *C. papaya* needs irrigation of around 0.80 m, mainly in the summer and spring. As a result, papaya fruits can grow more effectively at temperatures between 21°C and 32°C. India has been reported as the largest producer of *C. papaya* followed by Brazil, Mexico, Indonesia, Dominican Republic and Nigeria (Figure 1.1)
(Tridge.com, 2019). India and Brazil produced about 57% of the world's global papaya in the year 2019 (Fitch, 2020).

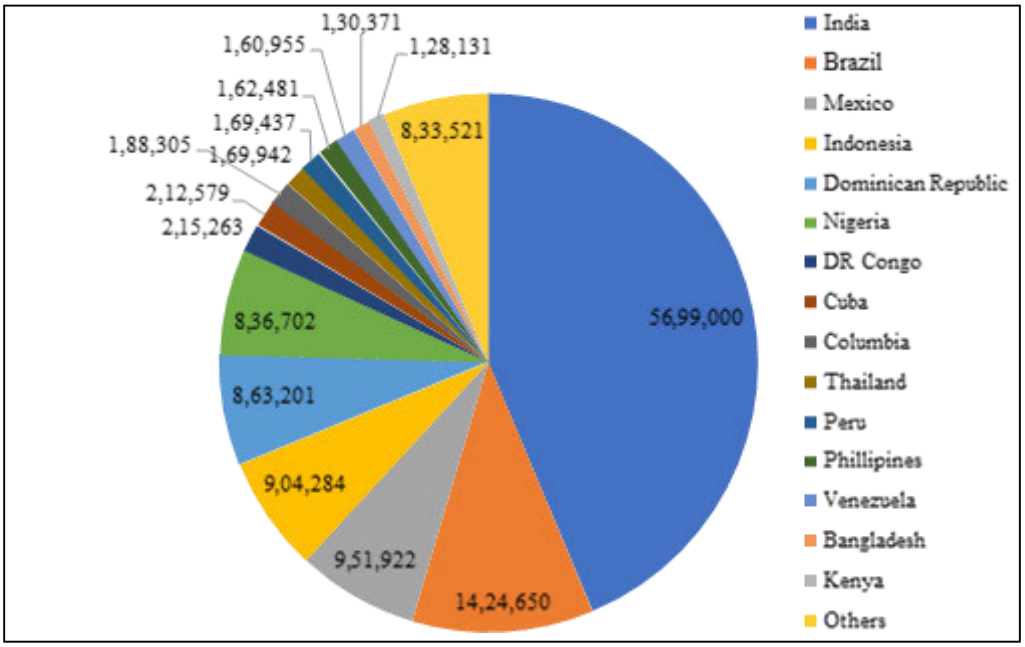

Figure 1.1: The top producer countries of *C. papaya L.* (Tridge.com, 2019).

2.1.2. Economic importance and quality of papaya

C. papaya has been famous for its medicinal and nutritional benefits for ages and it was the first fruit discovered to be genetically modified for human consumption due to its medicinal and nutritional values (Yadav *et al*., 2017). Besides its geographical origin and importance as food or traditional medicine, it is now used as herbal tea and also for the industrial production of beverages. It is also used in the production of chewing gum, gives shrinking resistance to wool, and helps to degum natural silk (Mahomud *et al*., 2008). Papaya also contains more carotenoids than other fruits such as apples, guavas and plantains, which play an important role in preventing age related cardiovascular, macular and heart disease (Krishna *et al*., 2008). These fruits have a pleasant taste and their pulp has sensory, and nutritional characteristics that make it healthy for all ages. Its chemical composition varies according to climate, cultivar, and crop practices, cultivate period, location and maturity stage (Souza *et al*., 2009).

2.2. Anthracnose of papaya

Anthracnose is one of the most common plant diseases that is caused by a fungal pathogen *Colletotrichum gloeosporioides*, which is characterized by the development of dark, sunken lesions, often with a raised rim, on affected fruits, foliage and stems (Waller, 1992; Tripathi, 2024). It is considered one of the most problematic and economically harmful plant diseases that occur in a variety of hosts from trees to grasses (Freeman *et al*., 1998; Ajay Kumar, 2014). Along with cereals and pulses the disease affects many medicinally and economically important plants such as citrus, papaya, banana, yam, coffee, eggplant, avocado, sweet pepper, mango and tomato. Although all anthracnose pathogens on these crops are classified as the same pathogen species, in the case of papaya and mango the pathogen is highly host specific (Ploetz, 1999; Zakaria, 2021). The pathogen produces a substantial amount of pre- and postharvest loss in these crops worldwide and acts as a secondary invader of injured tissue but can also survive as a saprophyte (Kamle *et al*., 2013).

2.2.1. Causal organism

Colletotrichum gloeosporioides is a fungal plant pathogen that cause anthracnose it has a white cottony appearance, which turns light grey with age on potato dextrose agar (PDA) (Muntala *et al*., 2020). The pathogen belongs to the family Phyllachoraceae of the division Ascomycota and is an asexual facultative parasite and comprises *C. gloeosporioides* as anamorph imperfect or asexual state (Cannon *et al*., 2012). It was later determined that its perfect or sexual stage was *Glomerella cingulata* (Stoneman) Spaulding & von Schrenk but since this stage is rarely seen, the name of the imperfect stage is so commonly used. Therefore, *C. gloeosporioides* is regarded as the correct name to describe this pathogen (Maeda and Nelson, 2014). The perfect stage, *G. cingulata,* and the imperfect stage, *C. gloeosporioides*, may be present on the stem of the papaya fruit. However, *G. cingulata* does not produce either the symptoms on the fruit or the pinkish spores on agar typical of *C. gloeosporioides* (Maeda and Nelson, 2014).

2.2.2. Taxonomy and morphology of *C. gloeosporioides*

The taxonomic and phylogenetic studies on the genus *Colletotrichum* are in expansive phase following the epitypification of *C. gloeosporioides* (Penz.) Penz. & Sacc. (Cannon *et al*., 2008). The species is exceedingly inconstant in its morphology; however, previous reports have discussed its differentiation and classification in several groups based on conidial size and sexual state morphology (Weir *et al*., 2012).

C. gloeosporioides was first identified in 1884 and its perfect state (telomorph) was given the generic name *Glomerella cingulata* (Stonem) in 1903. More than 600 synonyms and at least seven *formae speciales* of the fungus were described and are recognized as a heterogeneous group with great variation in morphophysiological characteristics (Von Arx, 1957; Sutton, 1992). The fungus produces hyaline, one-celled, ovoid to oblong, slightly curved or dumbbell- shaped conidia with obtuse ends (Figure 1.2) (Xie *et al*., 2010).

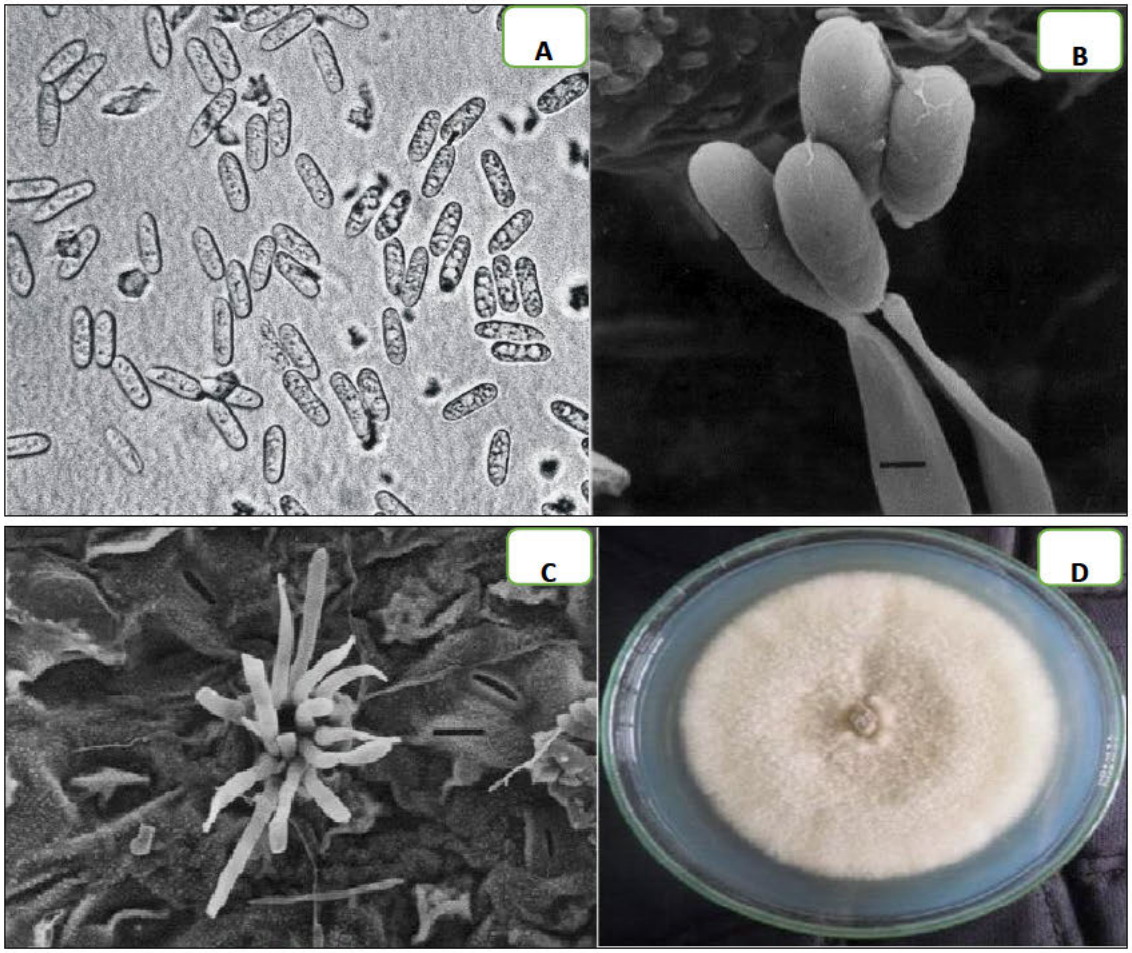

Figure 1.2: *Colletotrichum gloeosporioides* conidiophores (A and B), (c) setae viewed under the SEM and mycelial growth of C. gloeosporioides on PDA medium (D) (Benbouazza and Douira, 2013; Chowdhury *et al*., 2021).

2.2.3. Host range of *C. gloeosporioides*

The fungus is known to infect a wide variety of important host plants including banana, citrus, papaya, yam, coffee, eggplant, avocado, sweet pepper, mango, passion fruit tomato, and other tropical fruits (Sharma and Kulshrestha, 2015). In India, *C. gloeosporioides* is one of the most important plant pathogens causing anthracnose disease in a wide range of hosts including cereals and grasses, legumes, fruits, vegetables, perennial crops and trees (Siddiqui and Ali, 2014). In South Africa, there are small climatic zones such as Mpumalanga, Limpopo and KwaZulu Natal suited for the cultivation of subtropical crops such as avocado, papaya and mango which are susceptible to *C. gloeosporioides* (Sanders and Korsten, 2003).

2.2.4. Symptoms

C. gloeosporioides causes the development of sunken dark lesions, with a raised rim on the infected fruit (Tengku and Mohamed, 2017). These lesions contain conidial masses on the surface of infected fruits, but the immature fruits do not show any symptoms until they ripens. The first symptoms are small well defined dried pinkish- orange, or salmoncoloured "cordial masses" spots on the surface of ripening fruit (Zhang, 2021).

As the fruit continues to ripen, these spots quickly enlarge, forming circular (20 mm in diameter), sunken lesions (from 3 to 5 mm deep) and changes from brown to black in colour (Figure 1.3A and 1.3B). The lesions can be water-soaked or dried and hard. In the center of the lesions, the fungus produces dark acervuli, frequently in a concentric pattern and an orange to a pink gelatinous mass of conidia can be observed (Peres et al., 2005). The reason why the same pathogen causes two different symptoms is unknown (Sarkar, 2016).

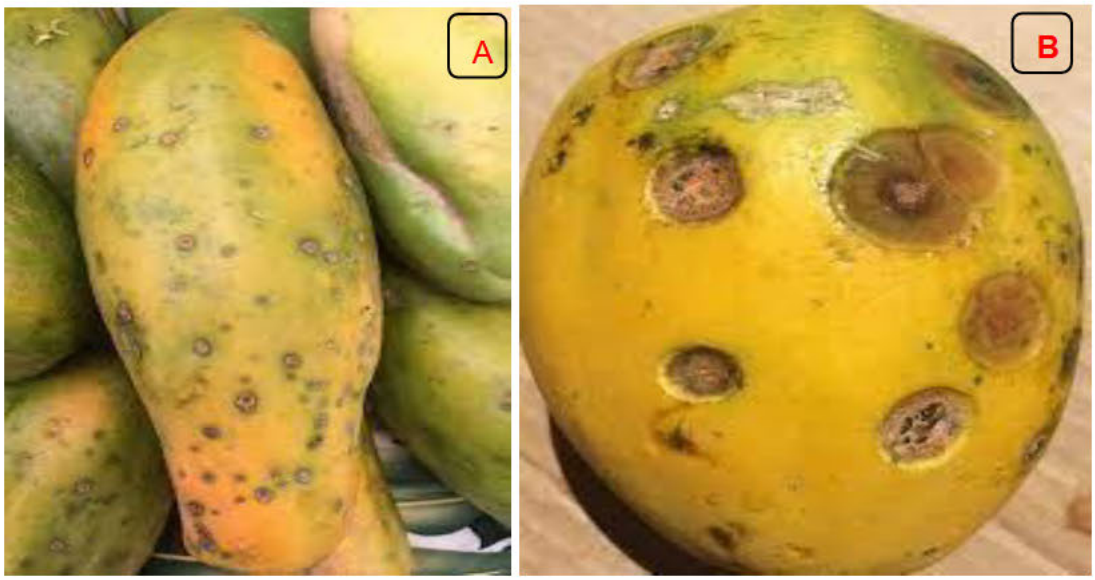

Figure 1.3: Anthracnose disease symptoms on the ripened papaya fruits (A and B) (Maeda and Nelson, 2014).

2.2.5. Economic importance and distribution

C. gloeosporioides can significantly affect fruits that are refrigerated and exported and is recognized globally as a major post-harvest disease. *C. gloeosporioides* is indicated as a common anthracnose pathogen of papaya in several regions and countries, including the Yucatan peninsula, Mexico, Malaysia, the Miyako Islands, Okinawa, Japan, South Florida and Trinidad Island (Maharaj and Rampersad, 2012). To date several species belonging to *Colletotrichum* occur on papaya fruits in Australia, South Africa and China (Cannon *et al.*, 2012).

2.2.6. Disease cycle and epidemiology

The life cycle of *C. gloeosporioides* is illustrated in Figure 1.4 (Zakaria, 2021). The anthracnose infection begins with conidial germination followed by the formation of appressoria and penetration pegs, which are classified as fungal structures that assist in the penetration into host tissues (Wharton and Dieguez- Uribeondo, 2004; Talhinhas *et al*., 2011). Pathogen spread and infection in fruits and plants depend on moisture provided by rainfall. Anthracnose reaches its highest disease incidence and severity in areas where relative rainfall and relative humidity are high and the air temperature is warm, allowing fungal development. Optimum air temperatures for the pathogen occur between 18°C and 25°C.

Anthracnose symptoms often develop after harvest, during storage, transportation, and marketing (Saxena, 2016). Spores produced on fungal structures called acervuli are dispersed through rain droplets, splashing water or irrigation, and wind-blown rain, thus becoming the primary inoculum for fruit infection at the preharvest stage (Sarkar, 2016). These spores can germinate and form germ tubes if deposited on immature green fruit with the availability of free water and a minimum relative humidity of at least 97% (Sarkar, 2016). Appressoria then forms at the end of the germ tubes on the fruit surface, which penetrates the host cuticle and epidermal layer with a “penetration peg” to establish infection. The infection remains latent until the post- climacteric stage of the fruit and when the host physiology and environmental conditions are favourable for their reactivation and further development (Kamle and Kumar, 2016).

As the fruit starts to ripen, the pathogen continues growing and the typical anthracnose symptoms appear, more often the symptoms develop after harvest, during storage, transportation, and marketing (Kamle and Kumar, 2016; Sarkar, 2016). The fungus absorbs nutrients from the fruit as it colonizes it, and again forms acervuli and spores to complete the life cycle (Nelson, 2008a). In the absence of its host plant, C. gloeosporioides can survive as a saprophyte in dead infected banana tissue or other organic matter.

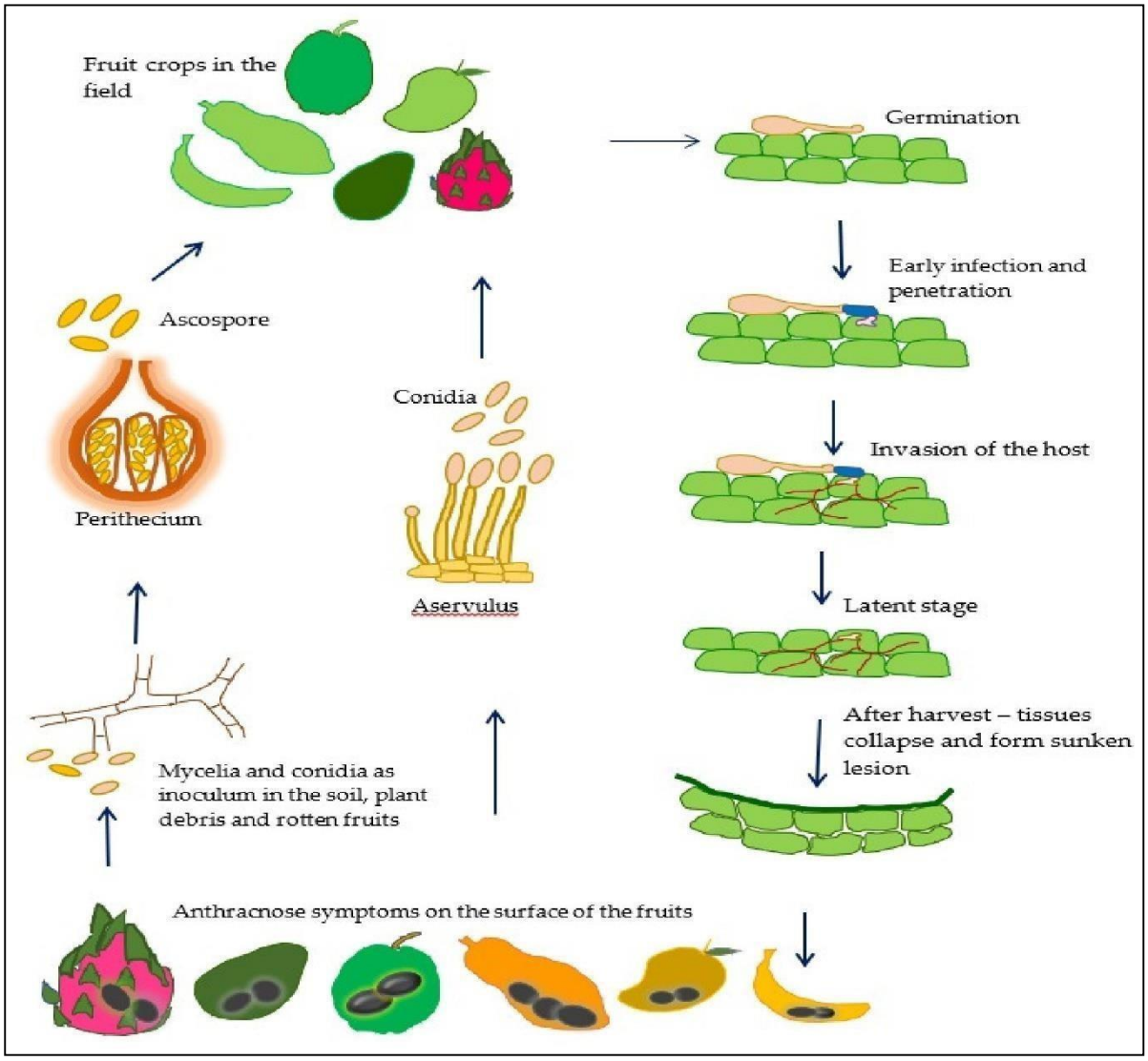

Figure 1.4 Anthracnose disease cycle of tropical fruit crops. (Drawn by Latiffah Zakaria) (Zakaria, 2021).

2.3. Control of *C. gloeosporioides* of papaya using biological control agents

Cook and Baker (1983) defined biological control as the process of reducing the amount of a pathogen's inoculum carried out by one or more species other than man. The biological control of pathogens in crops using microbial antagonists has been an ecofriendly alternative to the user rather than the use of chemical pesticides (Moenne Loccoz *et al.* 2001) and is nowadays being studied extensively with many different plant diseases using a variety of microbial antagonists as part of integrated disease management programs. Microbial antagonists such as bacteria and yeast play an important role in naturally controlling the numerous postharvest pathogens of fruits (Heydari and Pessarakli, 2010).

2.3.1. Characteristics of successful biological control

Biological control agents are most effective when they exhibit the following characteristics:

(a) Narrow host range contributing to specificity of a biological control to a targeted enemy and also influences its effectiveness; (b) synchrony with host life cycle; (c) non- pathogenic to the host so as to establish itself successfully without causing significant damage to that host; (d) Climatic adaptability to avoid the targeted pathogen to multiply faster than the biological control itself; (e) effective at low concentrations to prevent the potential outbreaks of the targeted pathogen; (f) stable for the consistent control of the available pathogen; (g) High reproductive potential to keep up with the capacity to reproduce for multiple generations throughout the hosts life cycle (Hoddle, 2006).

2.3.2. Mode of action of biological control agents

The biocontrol agents produced the antifungal activity via secretion of antifungal metabolites and enzymes or induction of disease resistance in fruits, this makes biological control agents the acceptable control strategy (Kim *et al*., 2018). These control agents interact with plants by inducing resistance or priming plants without any direct interaction with the targeted pathogen and antagonistically act through hyperparasitism and antibiosis, while directly interfering with the pathogen (Heimpel and Mills, 2017) and suppress the activity of post-harvest pathogens on fruits and vegetables (El-Ghaouth *et al*., 2004).

2.3.3. *Bacillus* as biological control agents

Bacillus has a variation in nutrient utilization, motility, and physiochemical growth allowing these bacteria to inhabit diverse niches in agro-ecosystems (Mahapatra *et al*., 2022). They are classified as aerobic or facultative anaerobic, endospore- forming grampositive flagellated rods that appear singly, in pairs or chains (Logan and Vos, 2015). *Bacillus* species have numerous advantages over other bacteria due to their long shelf life resulting from their ability to form endospores and the broad-spectrum activity of their antibiotics (Han *et al*., 2021). They have been mostly used in agriculture both for plant growth promotion and as biocontrol agents (Niranjan *et al*., 2003; Dobbelaere *et al*., 2003; Vessey, 2003) and successfully control plant pathogens.

2.3.4. Mode of action of Bacillus as biological control agents

Bacillus can reproduce actively and resistant to unfavorable environmental conditions (Shafi et al., 2017). They also have the potential to produce metabolites with antibiotic properties (Dunlap et al., 2011), and activation of plant defense mechanisms by triggering induced systemic resistance (ISR) in plants (Compant et al., 2005). Bacillus spp. also directly antagonizes fungal pathogens by competing and depriving them of essential nutrients, by producing fungitoxic compounds, and by inducing systemic acquired resistance in plants (Cawoy et al., 2015; Radhakrishnan et al., 2017).

2.3.5. Yeast as biological control agents

Yeasts stand out due to the specific characteristics that make them effective as biological control agents, especially in commercial fruits such as papaya, because they generally do not produce allergenic or toxic substances, also, they can grow rapidly on inexpensive substrates and are easy to produce in large quantities (Spadaro and Droby, 2016; Droby 2016). Yeasts generally have simple nutritional requirements and can colonize dry surfaces for longer periods, as well as withstanding many pesticides used in the postharvest environment (El-Tarabily and Sivasithamparam, 2006). Most yeasts are unicellular, although some species may become multicellular through the formation of a string of connected budding cells (Gakkhar and Nair, 2013) and they can grow rapidly on inexpensive substrates in fermenters and are therefore easy to produce in large quantities (Druvefors, 2004).

2.3.6. Mode of action of yeasts as biological control agents

Yeast has antagonistic action against pathogens, due to different mechanisms such as colonization, competing for nutrients, producing antifungal compounds, induced systemic resistance, and mycoparasitism process (Morath *et al*. 2012). They can reduce spore germination, decrease germ tube length, and inhibition of mycelial growth by volatile and diffusible metabolites (Nally *et al*. 2015).

2.4. Control of *C. gloeosporioides* of papaya using plant extracts as edible coatings

Edible coatings are mainly used to improve food appearance and preservation of the fruit since they can provide selective barriers against respiration, moisture loss and decay (Ali and Mahmud, 2007; Ali *et al*., 2013). Plant extracts are classified as one of the edible coatings controlling techniques and it has been reported as being effective against pathogens and also environmentally friendly (Hernández-Albiter *et al*., 2007). These have high antioxidant activity and exhibit antibacterial potential against several organisms (John *et al*., 2013).

Moringa leaf extract is best used as a plant growth enhancer, insect repellent, and fungicide (Phiri and Mbewe, 2010). These are good prospects for food application as they can preserve food by inhibiting lipid oxidation and controlling a wide range of pathogenic microorganisms, such as bacteria and fungi (Shah *et al*., 2015).

2.4.1. Mode of action of plant extracts and edible coatings

Edible coatings are classified as the thin layer of material that can be consumed and applied to provide a barrier to microbes of external sources, moisture, oxygen, and solute movement for food (Senturk Parreidt *et al*., 2018). These coatings also extend the fruit's shelf life by decreasing moisture and solute migration, gas exchange, oxidative reaction rates, and respiration as well as reducing physiological disorders in fresh-cut fruits (Adhikary *et al*., 2022). Plant extracts possess several phytochemicals such as flavonoids, tannins, alkaloids, and terpenoids, having antimicrobial and antioxidant properties (Talib and Mahasneh, 2010). Moringa leaf extract plays an important role in enhancing crop growth and yield under stress (Yasmeen *et al*., 2013). Moringa leaves are potential sources of vitamins A and C, iron, calcium, riboflavin, b-carotene, and phenolics (Nambiar *et al*. 2005).

2.4.2. Characteristics of successful edible coatings

The successive edible coatings possess the following characteristics (Han, 2014): (a) good barrier properties to water, moisture, carbon dioxide, oxygen and ethylene; (b) improve the appearance and mechanical handling to maintain structure and colour of coated fruits or vegetables; (c) they contain active components such as antioxidants, vitamins, etc., and also enhances the nutritional composition of fruits and vegetables without affecting the quality; (d) these coatings provide a protective covering on fruits and vegetables and enhance their shelf life.

2.5. Integration of biological control agents and plant extracts in controlling *C. gloeosporioides* of papaya

The biological control agents are mostly active against fungal pathogens and operates through a range of mechanisms such as the competition for space and nutrients, parasitism, induced resistance. Whereas plant extracts can act via the induced responses or directly on pathogen. The antifungal performance of the integrated plant extracts together with biocontrol agents can successively control the growth and development of the fungal pathogen and these plant volatiles are both eco-friendly and safe for human health (Moenne-Loccoz *et al.* 2001).

The association of bio control and plant extracts enhance the control of phytopathogens and also assists the biological agents in their antagonistic action in diseases associated with fruits (Pimenta *et al*., 2010). The combination use has the multifactorial mode of action, with a good efficacy and the reduction in chance of resistance of a pathogen (Rahman *et al*., 2016). Several studies have reported some bacteria and plant extracts has the ability to control most pathogens (MercadoBlanco *et al*., 2004; Uppal e*t al*., 2008).

2.6. References

Adhikary, T., Gill, P.P.S., Jawandha, S.K. and Sinha, A. 2022. Chitosan coating modulates cell wall degrading enzymes and preserved postharvest quality in cold-stored pear fruit. *Journal of Food Measurement and Characterization*. 16, 1395-1403.

Ajay Kumar, G., 2014. *Colletotrichum gloeosporioides*: biology, pathogenicity and management in India. *Journal of Plant Physiology and Pathology*. 2, 2-11.

Ali, A. and Mahmud, T.M.M., 2007. The potential use of locally prepared chitosan to control in vitro growth of *Colletotrichum gloeosporioides* isolated from papaya fruits. *In Europe-Asia Symposium on Quality Management in Postharvest Systems-Eurasia* 2007. 804, 177182.

Ali, A., Maqbool, M., Aldelson, P.G. and Zahid, N., 2013. Effect of gum arabic as an edible coating on antioxidant capacity of tomato (*Solanum lycopersicum L*.) fruit during storage. *Postharvest Biology and Technology*. 76, 119-124.

Benbouazza, A. and Douira, A., 2013. First report of olive anthracnose, caused by *Colletotrichum gloeosporioides*, in Morocco. *Atlas Journal of Biology* 2, 171-174.

Boxall, R.A., 2001. Post-harvest losses to insects—a world overview. *International Biodeterioration and Biodegradation.* 48,137-152.

Cannon, P.F., Damm, U., Johnston, P.R. and Weir, B.S., 2012. *Colletotrichum*— Current status and future directions. *Studies in Mycology*. 73, 181–213.

Cannon, T.D., Cadenhead, K., Cornblatt, B., Woods, S.W., Addington, J., Walker, E., Seidman, L.J., Perkins, D., Tsuang, M., McGlashan, T. and Heinssen, R., 2008. Prediction of psychosis in youth at high clinical risk: a multisite longitudinal study in North America. *Archives of General Psychiatry.* 65, 28-37.

Carvalho, F.A. and Renner, S.S., 2013. The phylogeny of the *Caricaceae*. In Genetics and genomics of *papaya*. *New York, Springer New York.* 81-92.

Cawoy, H., Debois, D., Franzil, L., De Pauw, E., Thonart, P. and Ongena, M., 2015. Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus* stibilis. *Microbial Biotechnology*. 8, 281-295.

Chowdhury, S., Rashid, H., Ahmed, R., Azad, A.K., Raihan, T. and Haque, M.M.U., 2021. First report of *Colletotrichum gloeosporioides* causing Leaf Blight of Dalbergia sissoo in Bangladesh. *J. Mycopathology Research.* 59, 327-329.

Compant, S., Duffy, B., Nowak, J., Clément, C. and Barka, E.A., 2005. Use of plant growthpromoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied Environmental Microbiology.71,* 4951-4959.

Cook R.J. and Baker K.F., 1983. The Nature and Practice of Biological Control of Plant Pathogens. *Amer. Phytopathol. Soc*. St. Paul, Minnesota, United State.SA.

Dobbelaere, S., Vanderleyden, J. and Okon, Y., 2003. Plant growth-promoting effects of diazotrophs in the rhizosphere. *Critical Reviews in Plant Science*. 22, 107-149.

dos Santos, C.M., de Abreu, C.M.P., Freire, J.M., Queiroz, E.R. and Mendonça, M.M., 2014. Chemical characterization of the flour of peel and seed from two papaya cultivars. *Food Science and Technology.* 34,353–357.

Droby, S., Wisniewski, M., Teixidó, N., Spadaro, D. and Jijakli, M.H., 2016. The science, development, and commercialization of postharvest biocontrol products. *Postharvest Biology and Technology.* 122, 22-29.

Druvefors, U., 2004. Yeast biocontrol of grain spoilage moulds. Mode of action of Pichia anomala, Swedish University of Agricultural Sciences Uppsala, Sweden, 10.

Dunlap, C.A., Schisler, D.A., Price, N.P. and Vaughn, S.F., 2011. Cyclic lipopeptide profile of three *Bacillus subtilis* strains; antagonists of *Fusarium* head blight. *The Journal in Microbiology. 49*, 603-609.

El-Ghaouth, A., Wilson, C.L. and Wisniewski, M.E., 2004. Biologically based alternatives to synthetic fungicides for the postharvest diseases of fruit and vegetables. *In, Dis. Fruit and Veg.* Naqvi, S.A.M.H., Ed. *Kluwer Academic Publishers*. The Netherlands, 2, 511-535.

El-Tarabily, K.A. and Sivasithamparam, K., 2006. Potential of yeasts as biocontrol agents of soil borne fungal plant pathogens and as plant growth promoters. *Mycoscience*. 47, 25-35.

Ferguson, I.B. and Boyd, L.M., 2002. Inorganic nutrients and fruit quality. *Fruit Quality and Biological Basis.* 17-45.

Fernandes, K.F.D., Queiroga, T.S., Lima, M.D.CD., de Oliveira, K.A.R. and de Souza, E.L., 2024. Interventions based on alternative and sustainable strategies for postharvest control of anthracnose and maintain quality in tropical fruits. *Comprehensive Reviews in Food Science and Food Safety*. 23, 13427.

Fitch, M.M., 2020. *Carica papaya*. *Biotechnology of fruit and nut crops*. 3, 35-36.

Freeman, S., Katan, T. and Shabi, E., 1998. Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. *Plant Disease*, 82, 596- 605.

Gakkhar, N. and Nair, R., 2013. Production of Alcohol through fermentation in saccharum officinarum (Sugarcane) and jaggery (Gur). *International Journal of Recent Research and Review.* 6.

Han, J.H., 2014. Edible films and coatings: A Review. *Innovations in Food Packaging*. 2, 213255.

Han, X., Shen, D., Xiong, Q., Bao, B., Zhang, W., Dai, T., Zhao, Y., Borriss, R., and Fan, B., 2021. The plant beneficial *rhizobacterium Bacillus velezensis* FZB42 controls the sobean pathogen *Phytophthora sojae* due to bacilysin production. *Applied and Environmental Microbiology*. 87, 1601-1621.

Heimpel, G. E. and Mills, N., 2017. Biological Control - Ecology and Applications, Cambridge: *Cambridge University Press,* Cambridge.

Hernandez-Albiter, R.C., Barrera-Necha, L.L., Bautista-Banos, S. and Bravo-Luna, L., 2007. Antifungal potential of crude plant extracts on conidial germination of two isolates *Colletotrichum gloeosporioides* (Penz) Penz and Sacc. Revista *Mexicana de Fitopatologia*. 25, 180-185.

Heydari, A and Pessarakli, M., 2010. A review on biological control of fungal plant pathogens using microbial antagonists. *Journal of biological sciences*. 10, 273-290.

Hoddle, M.S., 2006. Historical review of control programs for *Levuana iridescens* (*Lepidoptera: Zygaenidae*) in Fiji and examination of possible extinction of this moth by Bessa remota (Diptera: Tachinidae). *Pacific Science.* 60,439–453

John, R.N., Gala, V.C. and Sawan, C.S., 2013. Inhibitory effects of plant extracts of multi species dental biofilm formation *in vitro*. *International Journal of Pharmacy BioScience*. 4, 487–495.

Kabede, T.A.G., 2020. Facts of packing materials and storage environment on postharvest quality of papaya (*Carica papaya* L.) *Journal of Biology Agriculture and Healthcare.* 10, 21-32.

Kamle, M. and Kumar, P., 2016. *Colletotrichum gloeosporioides:* pathogen of anthracnose disease in mango (Mangifera indica L.). *Current trends in plant disease diagnostics and management practices*. 2, 207-219.

Kamle, M., Kumar, P., Gupta, K. V., Tiwari, K. A., Misra, K. A., Pandey, K. Brijesh., 2013. Identification and phylogenetic correlation among *Colletotrichum gloeosporioides* pathogen of anthracnose for mango. *Biocatalysis in Agriiculture and Biotechnology.* 2, 285-287.

Keatinge, J.D., Waliyar, F., Jamnadas, R.H., Moustafa, A., Andrade, M., Drechsel, P., Hughes, J.D.A., Kadirvel, P. and Luther, K., 2010. Relearning old lessons for the future of food by bread alone no longer: diversifying diets with fruit and vegetables. *Crop Science.* 50, 51.

Kim, C., Hassan, O., Lee, D. and Chang, T., 2018. First report of anthracnose of apple caused by Colletotrichum fructicola in Korea. *Plant Disease*. 102, 2653-2653.

Krishna, K.L., Paridhavi, M. and Patel, J.A., 2008. Review on nutritional, medicinal and pharmacological properties of Papaya (*Carica papaya Linn.). Journal in Pharmacy and Science*. 28, 735- 744.

Logan, N.A. and Vos, P.D., 2015. Bacillus. Bergey's manual of systemics of archaea and bacteria. *Research*. 1, 1-163.

Maeda, C. and Nelson, S. 2014. Anthracnose of papaya in Hawai'i. Mãnoa (US): College of Tropical Agriculture and Human Resources, University of Hawai'i.

Maeda, C. and Nelson, S., 2014. Anthracnose of papaya in Hawaii. Manoa (US): College of Tropical Agriculture and Human Resources, University of Hawaii.

Mahapatra, S., Yadav, R. and Ramakrishna, W., 2022. Bacillus substilis impact on plant growth, soil health and environment: Dr Jekyll and Mr. Hyde. *Journal of applied Microbiology*. 132, 3543-3562.

Maharaj, A., Rampersad, S.N., 2012. Genetic Differentiation of *Colletotrichum gloeosporioide*s and *C. truncatum* associated with anthracnose disease of papaya (*Carica papaya L*.) and bell pepper (*Capsicum annuum L.)* based on ITS PCR-RFLP fingerprinting. *Molecular Biotechnology.* 50, 237–249.

Mahomud, M.S., Pervin, K.M. and ea Gupta, N., 2008. Processing and preservation of papaya jam. *Journal of Science and Technology.* 6, 37-41.

Mercado-Blanco, J., Rodriguez-Jurado, D., Hervas, A. and Jimenez-Diaz, R. M., 2004. Suppression of verticillium wilt in olive planting stocks by root associated fluorescent *Pseudomonas* spp. *Biological Control*. 30, 474-486.

Moenne-Loccoz, Y., Tichy, H., O'Donnel, A., Simon, R. and O'Gara, F., 2001. Impact of 2,4Diacetylphloroglucinol-producing biocontrol strain *Pseudomonas fluorescens* F113 on intraspecific diversity of resident culturable fluorescent Pseudomonads associated with the roots of field-grown sugar beet seedlings. *Applied and Environmental Microbiology.* 67**,** 3418-3425.

Morath, S.U., Hung, R. and Bennett, J.W., 2012. Fungal volatile organic compounds: a review with emphasis on their biotechnological potential. *Fungal Biology Review. 26,* 73-83.

Muntal, S., Doval, E., Badenes, D., Casas-Hernanz, L., Cerulla, N., Calzado, N. and Aguilar, M., 2020. New normative data from the Spanish-language version of the Repeatable Battery for the Assessment of Neuropsychological Status (RBANS), form A. Neurología (English Edition), 35, 303-310.

Muntala, A., Norshie, P.M., Santo, K.G. and Saba, C.K.S., 2020. Colletotrichum gloeosporioides species complex: Pathogen causing anthracnose, gummosis and die- back diseases of cashew (Anacardium occidentale L.) in Ghana. *European Journal of Agricultural Food Science*. 2, 2.

Nally, M.C., Pesce, V.M., Maturano, Y.P., Assaf, L.R., Toro, M.E., De Figueroa, L.C. and Vazquez, F., 2015. Antifungal modes of action of Saccharomyces and other biocontrol yeasts against fungi isolated from sour and grey rots. *International Journal in Food Microbiology*. 204, 91-100.

Nambiar, V.S., Mehta, R. and Daniel M., 2005. Polyphenols content of three Indian green leafy vegetables. *Journal in Food Science and Technology*. 42, 312–315.

Nelson, S., 2008a. Mango anthracnose (*Colletotrichum gloeosporioides*). CTAHR *Department of Plant and Environmental Protection Science*. 2, 48.

Niklas, K.J. and Marler, T.E., 2007. Carica papaya (Caricaceae): a case study into the effects of domestication on plant vegetative growth and reproduction. *American Journal in Botany*, 94,999-1002.

Niranjan, S.R., Deepak, P., Basavaraju, H.S., Shetty, M.S., Reddy, M. and Kloepper, J.W. 2003. Comparative performance of formulations of plant growth promoting rhizobacteria in growth promotion and suppression of downy mildew in peal millet. *Crop Protection*. 22, 579-588.

OECD (Organization for Economic Cooperation and Development)., 2003. Draft Consensus Document on the Biology of *Carica papaya (L.)* (Papaya) OECD, France.

Ong, M.K., Kazi, F.K., Forney, C.F. and Ali, A., 2013. Effect of gaseous ozone on papaya anthracnose. *Food Bioprotection and Technology. 6*, 2996-3005.

Peres, N. A., Timmer, L.W., Adaskaveg, J.E. and Correll, J.C., 2005. Lifestyles *Colletotrichum acutatum*. *Plant Disease.* 89, 784-796.

Phiri, C. and Mbewe, D.N., 2010. Influence of *Moringa oleifera* leaf extracts on germination and seedling survival of three common legumes. *International Journal of Agriculture and Biology*. 12, 315- 317.

Pimenta, R.S., Silva, J. F.M., Coelho, C.M. and Morais, P. B., 2010. Integrated control of *Penicillium digitatum* by the predacious yeast *Saccharomycopsis crataegensis* and sodium bicarbonate on oranges. *Brazilian Journal Food Microbiology.* 41, 404-410.

Radhakrishnan, R., Hashem, A. and Abd_Allah, E.F., 2017. *Bacillus*: A biological tool for crop improvement through bio-molecular changes in adverse environments. *Frontiers Physiology. 8*, 667.

Rahman, S., Biswas, S.K., Barman, N.C. and Ferdous, T., 2016. Plant extract as selective pesticide for integrated pest management. *Biotechnology Research*. 2, 6-10.

Sanders, G.M. and Korsten, L., 2003. Comparison of cross inoculation potential of South African avocado and mango isolates of *Colletotrichum gloeosporioides*. *Microbiology Research. 158,*143-150.

Sarkar, A.K., 2016. Anthracnose diseases of some common medicinally important fruit plants. *Journal in Medicinal Plants Studies. 4,* 233-236.

Sddiqui, Y. and Ali, A., 2014. *Colletotrichum gloeosporioides* (Anthracnose). In Postharvest Decay. *Academic Press*. 1, 337-371.

Senturk Parreidt, T., Muller, K. and Schmid, M., 2018. Alginate based edible films and coatings for food packaging applications. *Foods*. 7, 170.

Senturk Parreidt, T., Muller, K. and Schmid, M., 2018. Aligninate- based edible films and coatings for food packaging applications. *Foods*. 7, 170.

Shafi, J., Tian, H. and Ji, M., 2017. Bacillus species as versatile weapons for plant pathogens: a review. *Biotechnology and Biotechnological Equipments. 31,* 446-459.

Sharma, M. and Kulshrestha, S., 2015. *Colletotrichum gloeosporioides:* an anthracnose causing pathogen of fruits and vegetables. *Biosciences Biotechnology Research Asia*. 12. 12331246.

Souza, T., Coelho, E.F., Paz, V.P.S. and Ledo, C.A.S., 2009. Physical and chemical evaluation of papaya cv. Tainung n.1 fruits fertirrigated with different combinations of nitrogen sources. *Brazilian Journal in Agriculture and Science*. 4, 179-184.

Spadaro Padaro, D. and Droby Roby, S., 2016. Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists. *Trends Food Science and Technology.* Norwich, 47, 39-49.

Talhinhas, P., Mota-Capitao, C., Martins, S., Ramos, A.P., Neves-Martins, J., Guerra- Guimaraes, L., Varzea, V., Silva, M.C., Sreenivasaprasad, S. and Oliveira, H., 2011. Epidemiology, histopathology and etiology of olive anthracnose caused by *Colletotrichum acutatum* and *C. gloeosporioides* in Portugal. *Plant Pathology.* 60, 483- 495.

Talib, W. H., and Mahasneh, A. M., 2010. Antimicrobial, cytotoxicity and phytochemical screening of Jordanian plants used in traditional medicine. *Molecular.* 15, 1811-1824.

Tan, C.X., Tan, S.T. and Tan, S.S., 2020. An overview of papaya seed oil extraction methods. *International Journal of Food Science and Technology. 55,* 1506-1514.

Tengku, M, and Mohamed, M. 2017. An Unsung Solution fo Food Security. *Earth Environmental Science.* 591, 012088.

Tridge.com (2019). Papaya.

Uppal, A.K., El-Hadrami, L.R., Adam, M., Tenuta. and Daayf, F., 2008. Biological control of potato Verticillium wilt under controlled and field conditions using selected bacterial antagonists and plant extracts. *Biological Control*. 44, 90-100.

Vessey, J.K., 2003. Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil.* 255, 57

Vij, T. and Prashar Y., 2015. A review on medicinal properties of *Carica papaya* Linn. *Asian Pacific Journal in Tropical Disease.* 5,1-6.

Von Arx, W.S., 1957. An experimental approach to problems in physical oceanography. *Physics and Chemistry of the Earth*. 2, 1-29.

Waller, J. M., 1992. *Colletotrichum* diseases of perennial and other cash crops. *Colletotrichum Biology and Pathology. Contr*. 1, 167-185.

Weir, B.S., Johnston, P.R. and Damm, U., 2012. The *Colletotrichum gloeosporioides* species complex. *Studies in Mycology*. 73, 115-180.

Wharton, P.S. and Dieguez-Uribendo, J., 2004. The biology of Colletotrichum acutatum. *In Anales del jardin botanico de Madrid*. 1, 3-22.

Xie, L., Zhang, J.Z., Cai, L. and Hyde, K.D., 2010. Biology of *Colletotrichum horii,* the causal agent of persimmon anthracnose. *Mycology*. 1, 242-253.

Yadav, A.N., Kumar, R., Kumar, S., Kumar, V., Sugitha, T.C.K., Singh, B., Chauahan, V.S., Dhaliwal, H.S. and Saxena, A.K., 2017. Beneficial microbiomes: biodiversity and potential biotechnological applications for sustainable agriculture and human health. *Journal in Applied Biology and Biotechnology.* 5, 45-57.

Yadav, J., Yadav, S. and Mishra, S., 2017. Experimental evidences showing nutritional and medicinal property of *Carica papaya* plant. *International Journal of Scientific Research.* 6, 21-25.

Yasmeen, A., Basra, S.M.A., Farooq, M., Rehman, H.U., Hussain, N. and Athar, H.U.R., 2013. Exogenous application of moringa leaf extract modulates the antioxidant enzyme system

to improve wheat performance under saline conditions. *Plant Growth Regulators. 69*, 225233.

Zakaria, L., 2021. Diversity *Colletotrichum* species associated with anthracnose disease in tropical fruit crops-A Review. *Agriculture*. 11, 297.

Zakaria, L., 2021. Diversity of *Colletotrichum* species associated with anthracnose disease in tropical fruit crops—*A Review in Agriculture. 11,* 297.

Zhang, Y., Sun, W., Ning, P., Guo, T., Huang, S., Tang, L., Li, Q. and Mo, J., 2021. First report of anthracnose of papaya (*Carica papaya*) caused by *Colletotrichum siamense* in China. *Plant Disease.* 105, 2252.

Zhu, X., Li, X., Chen, W., Lu, W., Mao, J. and Liu, T., 2013. Molecular cloning, characterization and expression analysis of CpCBF2 gene in harvested papaya fruit under temperature stresses. *Electronic Journal of Biotechnology. 16,* 1.

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Chapter 3 Investigating the *in vitro* and *in vivo* effects of antagonistic microorganisms on *Colletotrichum gloeosporioides* of papaya

Abstract

The use of biological control agents as pathogen control agents has gained significance due to being non-hazardous to humans, biodegradable and environmentally friendly. The aim of the study was to evaluate *in vitro* and *in vivo* antagonistic activity of yeasts and *Bacillus* spp. as biological control agents against *Colletotrichum gloeosporioides* of *Carica papaya* L. Isolation and *in vitro* bioassays of forty-five yeasts and fifty-five *Bacillus* spp. against *C. gloeosporioides* were conducted on potato dextrose agar and incubated at 25°C for 7 days. The mycelial growth was measured in mm, and the percentage inhibition was calculated. Out of one hundred biological control agents isolated from different plant parts of different hosts, 51 *Bacillus* isolates and 27 yeast isolates inhibited mycelial growth of *C. gloeosporioides* by greater than 50% during the *in vitro* primary screening. *Bacillus* spp. had a greater number of isolates that exhibited the strongest antagonistic activity and effectively inhibited the mycelial growth of *C. gloeosporioides* compared to yeast isolates. For *in vitro* secondary screening, twenty-one yeast isolates and forty-seven *Bacillus* spp. inhibited mycelial growth of *C. gloeosporioides* by more than 50%. The reduction of pathogen growth was significantly different in comparison to the untreated control. The SEM showed a successive control of mycelial growth and conidia formation of *C. gloeosporioides* by BMR2 and YCR3. There was no significant difference ($P \leq 0.001$) in anthracnose incidence between the fruit treated with BMR2 and B16 isolates from day 3-10 post inoculation. The control fruit exhibited 100% anthracnose incidence compared to 16.67% in the water control. Yeast isolates (YCR3 and YSY1) and *Bacillus* isolates (BMR2 and B16) provided the best control of *C. gloeosporioides in vitro*, whereas fruits treated with B16 at a concentration of 1×10^8 spore $m\ell^{-1}$ had the lowest anthracnose incidence of anthracnose (88.67%), compared to control fruits (100%) *in vivo*. B16, identified as *Bacillus megaterium*, was highly antagonistic against *C. gloeosporioides* both *in vitro* and *in vivo*.

Keywords: *C. gloeosporioides,* anthracnose, papaya*, Bacillus* and yeast isolates.

3.1. Introduction

Papaya (*Carica papaya* L.) is a climacteric fruit that produces more ethylene and has a respiration burst after being detached from a mother plant. However, ripening is a complex process that involves many factors, including physiological changes that can lead to post harvest diseases. Post-harvest diseases cause severe economic losses and reduce fruit quality due to decay leading to blemished and unmarketable fruits (Cannon *et al*., 2000). More than 50% of losses of fresh fruits and vegetables are caused by postharvest pathogens (Awang *et al*., 2011), resulting in severe economic damage of fruits for local consumption and exportation (Kitinoja and Kader, 2015).

Colletotrichum gloeosporioides (Penzig) Penzig & Saccardo, the causal agent of anthracnose, is an important postharvest pathogen worldwide, infecting several crops (Pomper *et al*., 2010). Anthracnose attacks all parts of the plant at any stage of growth (Torres-Calzada *et al*., 2013), exhibiting symptoms such as small, brownish spots with light-coloured centers on the leaves and twigs (Kitinoja and Kader, 2015). Factors such as humidity, temperature, fruit texture, and inoculum concentration, trigger anthracnose infection on different fruits (do Nascimento Nunes, 2008).

Synthetic fungicides (propiconazole, prochloraz, benomyl, and thiabendazole) are commonly used to control anthracnose disease on various crops (El- Ghaouth *et al*., 2003). However, the long-term use of these fungicides results in residues harmful to consumers and the emergence of fungicide-resistant strains of the fungus (Hoa, 2008). The hot-water-dip treatment of 43 - 49°C for 20 minutes is also used but affects the ripening process of papaya (Hewajulige and Wijeratnam, 2010). Control strategies based on antagonistic activity have become an attractive alternative approach. Biological control agents, especially in commercial fruits such as papaya, have the potential to control post-harvest pathogens because they are environmentally friendly, do not produce toxic substances, can grow rapidly on inexpensive substrates, and are easy to produce in large quantities (Spadaro and Droby, 2016).

The objective of this study was to isolate yeasts and *Bacillus* isolates and investigate their effect on *C. gloeosporioides* in both *in vitro* and *in vivo* trials and to investigate the mode of action of the best isolates using scanning electron microscopy.

3.2. Materials and methods

3.2.1. Isolation of *Colletotrichum gloeosporioides*

C. gloeosporioides was isolated from infected papaya fruit obtained from local supermarkets in Scottsville, Pietermaritzburg, South Africa. The infected fruit parts with spores were then directly plated onto potato dextrose agar (PDA) and incubated at 28°C for seven days. Pure cultures were obtained by sub culturing on fresh PDA plates and maintained in McCartney bottles three-quarter with PDA at 4°C. The inoculum was prepared from 14-day-old culture dishes incubated at 28°C, and spores were viewed under the light microscope (Carl Zeiss, Geschäftsbereich, Germany).

3.2.2. Pathogenicity test

Mature papaya fruits were dipped into 70% ethanol for 1 minutes, rinsed three times in sterilized distilled water and then air dried before wounding. There were three fruits per replicate and three replicates per treatment. The fruits were uniformly wounded, approximately 3mm deep and 3mm wide, with a sterile pipette tip at the equatorial side and four wounds were made in each fruit. The wounds were inoculated with 20 µl of the conidial suspension of *C. gloeosporioides* at concentrations of $1x10^5$ conidia ml^{-1} and controls inoculated with 20 µl of sterilized distilled water. Inoculated fruits were stored at 25°C, relative humidity of about 90%. Wounds were examined at day 3, 5, 7 and 10 postinoculation, and the disease incidence (DI) was calculated using the following formula:

$$DI\ (\%) = (Number\ of\ infected\ fruits \div Total\ number\ of\ inoculated\ fruits)\ \mathrm{X}\ 100$$

3.2.3. Isolation of yeast and *Bacillus* isolate

Bacillus spp. and yeasts were isolated from the medicinal plants guava, papaya, cassava, aloe and syringa collected at Botanical Garden and Ceru, University of KwaZulu Natal, Scottsville, South Africa. Plant materials were disinfected with 70% ethanol, and then washed with distilled water to remove excess ethanol. The plant material was allowed to air dry on a paper towel. 80 g of each plant material was cut into small pieces using the sterile scalpel and transferred in a 250 mL Erlenmeyer flask containing 100 mL sterile distilled water.

The mixture was placed in a water bath at 130 revolutions per minutes (rpm) for one hour at 80˚C, this was to allow only species belonging to the *Bacillus* genus to survive as they can resist temperatures above 80˚C. For the isolation of yeasts, Erlenmeyer flasks were

placed in a water bath at 28˚C for 1 hour. After one hour the solutions were cooled, filtered and the aliquot was used to make serial dilutions of 10^{-1}, 10^{-2} and 10^{-3}. An aliquot of 20 µL of each dilution was spread into PDA plates for *Bacillus* isolation, and yeast peptone dextrose agar (YPDA) for isolation of yeast, using a hockey stick.

The inoculated petri plates were incubated at 28˚C for three days. To obtain pure cultures, sub-culturing was done by transferring single colonies of each isolate of *Bacillus spp.* and yeasts into fresh PDA plates and incubated at 28˚C for 3 days. The pure cultures were stored in 70% (v/v) double sterilized glycerol at -80°C for long-term storage.

3.2.4. *In vitro* effect of yeasts and *Bacillus* isolates on *Colletotrichum gloeosporioides*

To determine the effect of yeast and *Bacillus* isolates on *C. gloeosporioides*, 45 yeasts and 55 *Bacillus* isolates were streaked on either side of the Petri plate using the flamed inoculating loop and thereafter placing a 5-day old pathogen plug at the centre of the plate using the sterile scalpel and inoculating needle and incubated at 28°C. There were three replicates per treatment. Measurements of mycelial growth were taken at day three, five and seven after inoculation. Control plates were centrally seeded with the pathogen and distilled water on either side of the plate. The percentage inhibition

(PI) was measured as follows:

$$\text{PI (\%)} = (mycelialgrowthofcontrol - mycelialgrowthwithtreatment) \div mycelialgrowthofcontrol) \text{X } 100$$

The secondary screening of yeasts and *Bacillus* isolates was conducted using the same methodology, 21 yeasts and 47 *Bacillus* isolates succeeded in controlling *C. gloeosporioides.*

3.2.5. Molecular identification of bacterial isolate

The molecular identification of the isolated biocontrol agent B16 was done according to Stephen et al., (1997) and the genetic DNA was extracted from the pathogen cultures using the Quick-DNATM Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The targeted region 16S was amplified on PCR using both the reverse and forward primers (Table 3.1), OneTaq@ Quick-load@ 2X Master mix (NEB, Catalogue No M0486). The PCR products were then 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 ABI 3500x1 Genetic Analyser (Applied Biosystems, ThermoFisher Scientific) was used to purify fragment that was analysed for each reaction. CLC Bio Main Workbench v7.6 was used to analyse a file with the sample generated by the ABI 3500XL Genetic Analyser and results were obtained by a BLAST search (NCBI).

Table 3.1: 16S Primers sequences

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

3.2.6. The effect of biological controls on *C. gloeosporioides* under the scanning electron microscopy (SEM)

The effect of biological control agents on the hyphal development of *C. gloeosporioides* was studied using the method described by Capdeville *et al*. (2007), with few amendments. Samples were cut into 3 mm x 3 mm blocks of the pathogen from the 4 yeast and 4 *Bacillus* treated and 1 non-treated PDA plated. The treatments used were, BMR2, B16, BMR3 and Bql for the *Bacillus sp*., YCR3, YSY1, Yzl and YS3 for yeast isolates. The samples were primarily fixed in 3% (v/v) glutaraldehyde for 3 hours and then washed in 0.05M sodium cacodylate buffer for 5 minutes and dehydrated in different percentages (30%, 50%, 70%, 80%, 90%) of ethanol, 10 min each and 3 × 10 min in 100% ethanol in a fume hood. The samples were then transferred into a critical point dryer basket under 100% ethanol and dried using a Quorum K850 critical point dryer. All the samples were mounted onto the SEM stubs and coated with gold in a gold palladium sputter coater (Quorum Q150R ES). The dried coated samples were examined using a

ZEISS, EVO LS 15 scanning electron microscope (Carl Zeiss SMT Ltd., Cambridge) operating at 5 kV.

3.2.7. *In vivo* effects of yeasts and *Bacillus* isolates on *Colletotrichum gloeosporioides*

To determine the effect of the best *Bacillus* and yeast isolates on the growth and development of *C. gloeosporioides*, papaya fruits were similarly treated as described in Section 2.2.2. The wounded fruits were inoculated with a suspension of 20 µℓ at a concentration of 1×10^5 conidia $m\ell^{-1}$. After 24 hrs, each wound was inoculated with 20 µℓ of the best 4 biocontrol agents (BMR2, B16, YCR3 and YSY1) of 1×10^8 spore $m\ell^{-1}$. The control was inoculated with 20 µℓ of *C. gloeosporioides* only. Inoculated fruit was then stored in enclosed plastic trays to maintain a high relative humidity (RH) of about 90% at 25°C. There were 10 fruits per replicate and 3 replicates per treatment. The wounds were examined, and the disease incidence was calculated at 3, 5, 7 and 10 days postinoculation.

3.2.8. Statistical analysis

All data were subjected to analysis of variance (ANOVA) using GenStat 18th edition. Differences between treatment means were separated using Duncan's multiple range test at $P<0.05$.

3.3. Results

3.3.1. Isolation and pathogenicity of *Colletotrichum gloeosporioides*

The light microscope showed ovoid and slightly curved conidial spores of *C. gloeosporioides* (Figure 3.1 A and B) after 10 days of pathogen growth at 28°C. The spores of the pathogen were concentrated together.

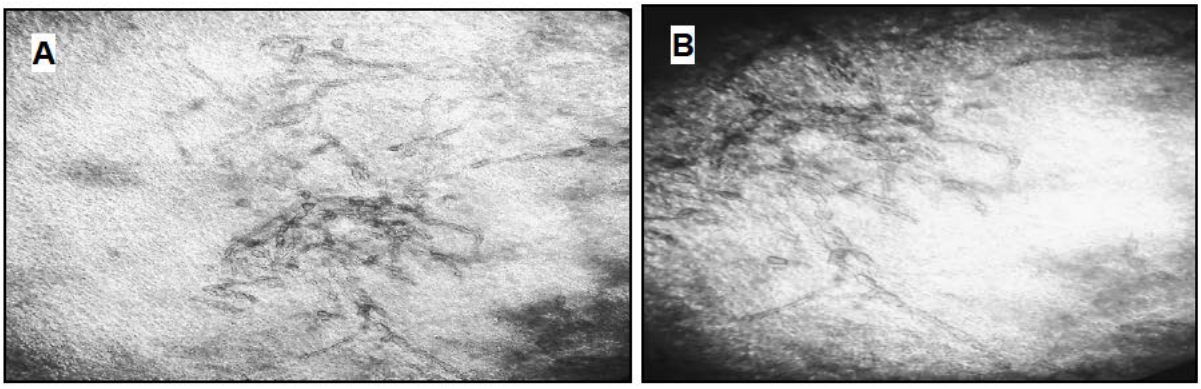

Figure 3.1: Light electron micrograph showing the spores of *Colletotrichum gloeosporioides* at 40x magnification (A and B).

The effect of the pathogen on papaya fruit was significantly different at different stages of papaya fruit and days after post-inoculation (Figures 3.2 and 3.3), with fruits being less affected at the turning stage and mostly affected when ripen. At day 5, 7 and 10 postinoculation, papaya fruit had 66.7%, 100% and 100% anthracnose incidence, respectively, compared to control treatment with only 10% infection at day 10 post-inoculation.

Figure 3.2 Untreated fruit inoculated with sterilized distilled water (A) showing no symptoms of *C. gloeosporioides*; treated papaya fruit with *C. gloeosporioides* at day five

of inoculation (B); treated papaya fruit at day seven (C) and day ten (D) showing severe symptoms of the fungal pathogen.

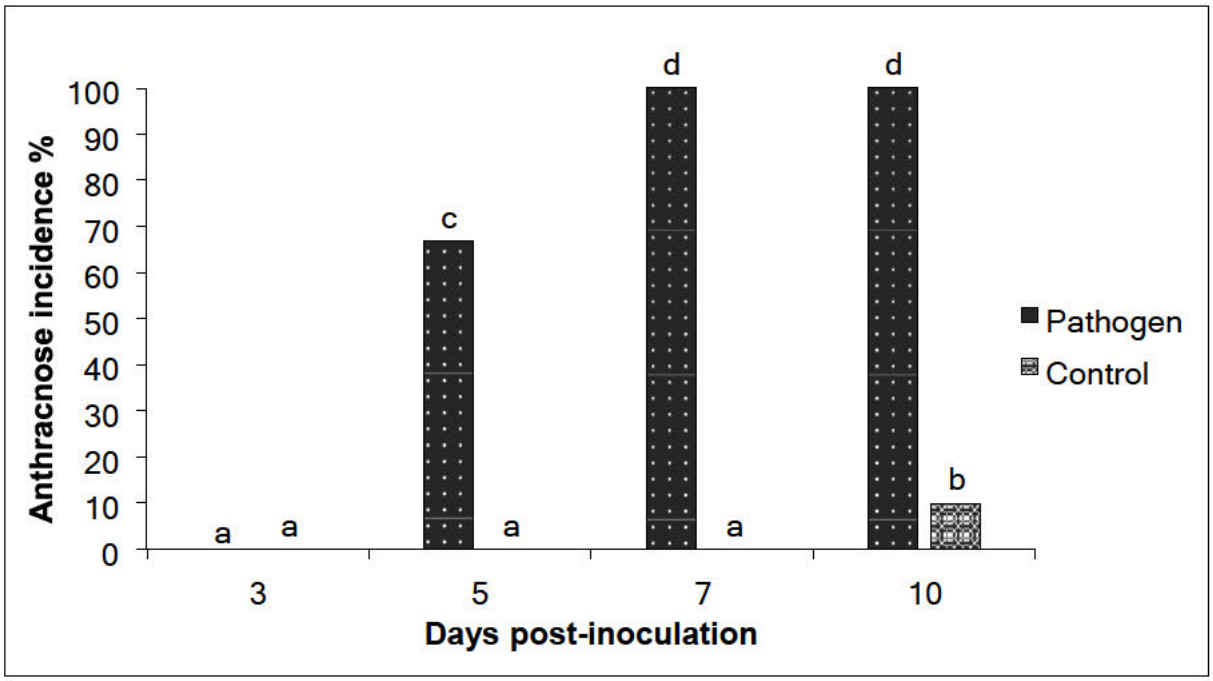

Figure 3.3 Anthracnose incidence on papaya fruit inoculated with *Colletotrichum gloeosporioides*. Bars with the same letters are not significantly different according to Duncan's multiple range test ($P<0.05$).

3.3.2. *In vitro* effect of yeast and *Bacillus* isolates on *Colletotrichum gloeosporioides*

From a total of 45 yeast isolates that were used in the preliminary screening trial, only 27 yeast isolates inhibited *C. gloeosporioides* by > 50% and 10 of these controls inhibited by >60% when compared to the control (Figure 3.5). Out of 55 *Bacillus* isolates that were preliminary screened, 51 of the isolates successfully inhibited *C. gloeosporioides* by > 50% (Figure 3.6). The promising yeast and *Bacillus* isolates were further tested to confirm their antagonistic activity against *C. gloeosporioides.* For secondary screening, 21 yeasts and 47 *Bacillus* isolates succeeded in controlling *C. gloeosporioides* when compared to the untreated control. Yeast isolates YCR3 provided the best control and successfully inhibited *C. gloeosporioides* (Figure 3.4 B) compared to no inhibition on control plates (Figure 3.4 D). For *Bacillus* isolates, BMR2 performed better in controlling the pathogen (Figure 3.4 A), with the worst control with the MeRo2y (Figure 3.4 C).

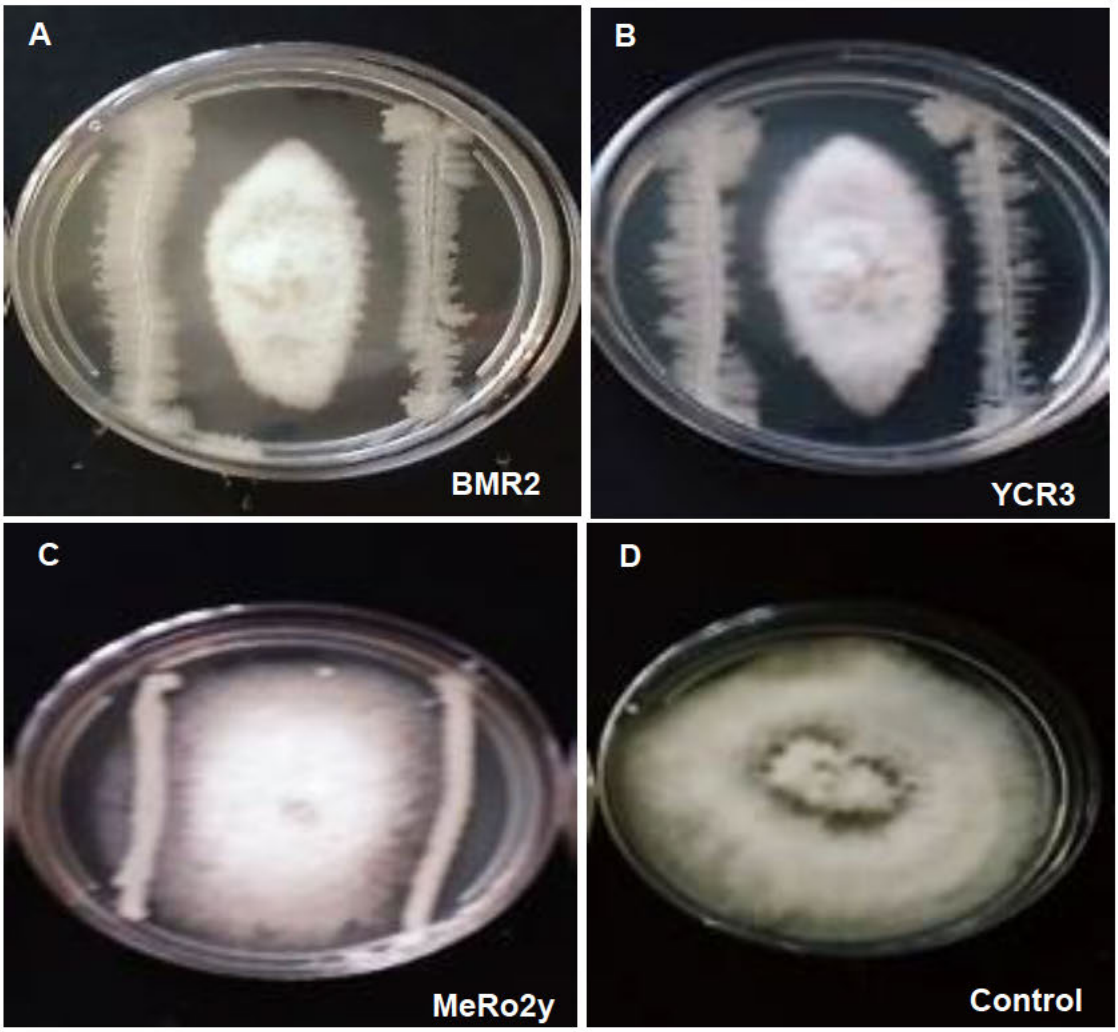

Figure 3.4 Antagonistic effect of the best yeast and *Bacillus* isolates against *C. gloeosporioides* after seven days of incubation at 25°C. The performance of a pathogen on a plate treated with BMR2 (A) and treated with YCR3 (B) showed clear inhibition zones when compared with MeRo2y (C) and untreated control (D) where the fungal pathogen spreads throughout the plate with no restrictions.

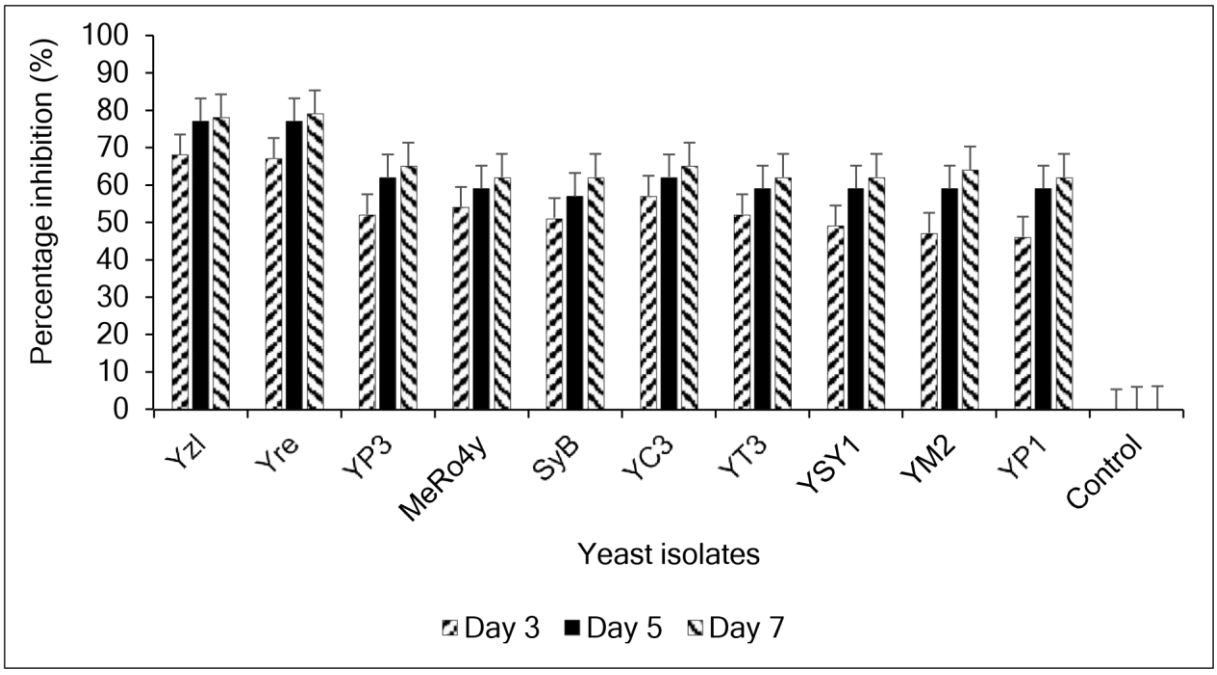

Figure 3.5 Mycelial growth inhibition of *Colletotrichum gloeosporioides* by best 10 yeast isolates at day 3, 5 and 7 at 25℃. All of these isolates managed to inhibit the pathogen by more than 50%.

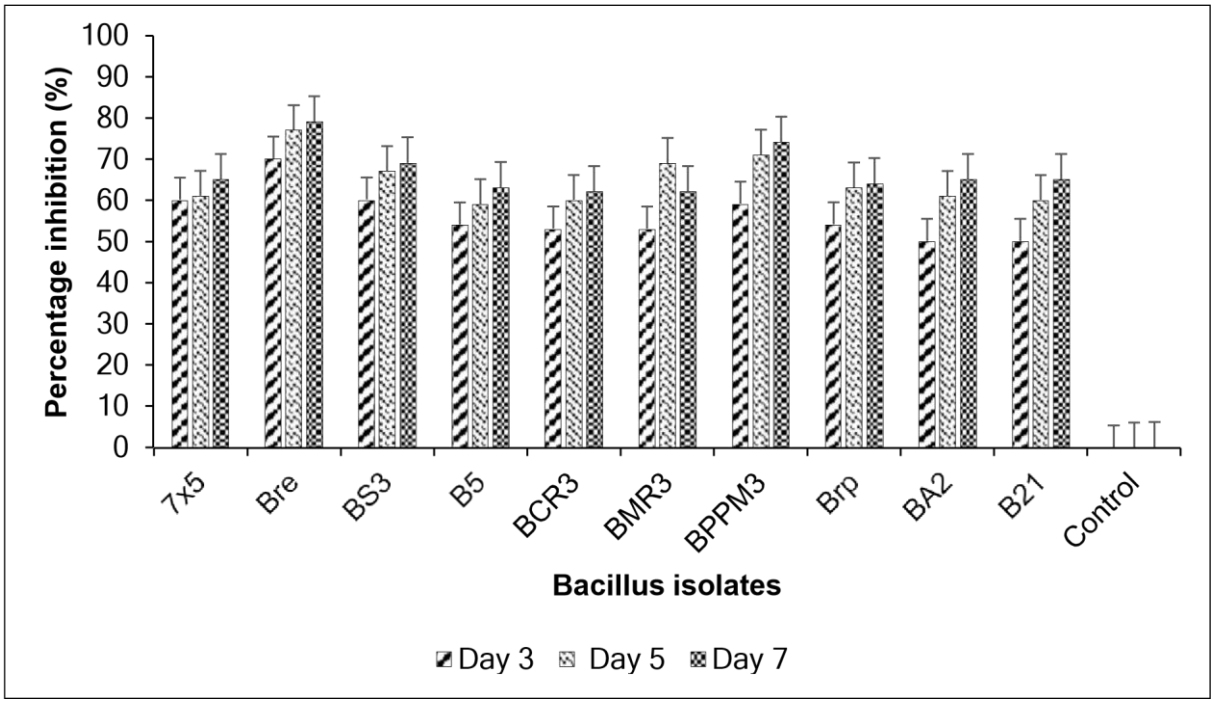

Figure 3.6 Mycelial growth inhibition of *Colletotrichum gloeosporioides* by best 10 *Bacillus* isolates after day 3, 5 and 7 at 25°C. The percentage of the isolates at day 7 of evaluation were above 50%.

Mycelial growth of *C. gloeosporioides* managed to increase for all the evaluated yeasts and Bacillus isolates from days 3 to 7. However, YCR3 (73%) and YSY1 (71%) isolates

had a highest inhibition percentage of *C. gloeosporioides* (Table 3.1). The selected *Bacillus* isolates progressively inhibited the mycelial growth of *C. gloeosporioides* with BMR2 (81%) and B16 (76%) being the best treating isolates (Table 3.2).

Table 3.2. Grouping of bacterial and yeast isolates based on the average percentage inhibition

Groups	Ranges of average % inhibition
Group 1	0-25%
Group 2	26-50%
Group 3	51-75%
Group 4	76-100%

Table 3.3. Effect of yeast isolates against *C. gloeosporioides* at day 7 at 25°C *in vitro.*

Treatments	Mycelial growth (mm)	Inhibition (%)	Group
YCR3	23.00 a	73	3
YSY1	24.33 a	71	3
Yzl	25.33 a	70	3
YS3	25.67 a	70	3
YSY3	25.67 a	70	3
YM1	26.00 a	69	3
YP3	26.00 a	69	3
YSY2	26.00 a	69	3
YGT3	26.33 a	69	3
YM2	26.67 a	69	3
YP2	26.67 a	69	3
MeRo4y	27.00 a	68	3
SyB	27.33 a	68	3
YC2	29.33 a	65	3
YC3	30.33 a	64	3
Control	84.67 b		
P value	<0.001		
LSD	7.4		
%CV	14.7		

Table 3.4. Effect of *Bacillus* isolates against *C. gloeosporioides* incubated at 25°C at day 7 after inoculation *in vitro.*

Treatments	Mycelial growth (mm)	Inhibition (%)	Group
BMR2	16.00 a	81	4
B16	20.00 ab	76	4
BMR3	26.00 bc	69	3
Bqi	26.33 bc	69	3
BG1	26.67 bc	69	3
BS2	27.33 c	68	3
BCr3	27.67 c	67	3
BP3	27.67 c	67	3
Bzl	27.67 c	67	3
BPPM1	28.00 c	67	3
BS3	29.00 c	66	3
BSY1	29.00 c	66	3
BP2	29.67 c	65	3
BC1	30.00 c	65	3
BMR1	30.00 c	65	3
BPPM3	30.00 c	65	3
BSP2	30.00 c	65	3
BT1	30.00 c	65	3
BT3	30.33 c	64	3
Control	84.67 d		
P value	<0.001		
LSD	3.7		
%CV	7.4		

3.3.3. Molecular identification of bacterial isolate

According to the molecular identification, the *Bacillus* isolate B16 was classified as *Bacillus megaterium* (Table 3.4), which successfully inhibited the mycelial growth of *C. gloeosporioides* growth and reduced anthracnose incidence on papaya.

Table 3.5: The identification of *Bacillus* isolate B16

Isolate name	Species name	Primer	Accession no.
B16	*Bacillus megaterium*	16S rDNA	MN509795.1

3.3.4. The effect of biological controls on *C. gloeosporioides* under scanning electron microscopy (SEM)

The SEM shows that the pathogen spores were reduced on plates treated with BMR2 and YRC3 (Figure 3.7 B and D, respectively) but some were still present in treatment MeRo2y (Figure 3.7 C). The yeast YCR3 and *Bacillus* BMR2 managed to suppress the mycelial of *C. gloeosporioides* by breaking and inhibiting its growth, whereas the pathogen's mycelia progressively increased on plates with yeast control MeRo2y and the conidia was observed but not as much as in the control plates (Figure 3.7 A).

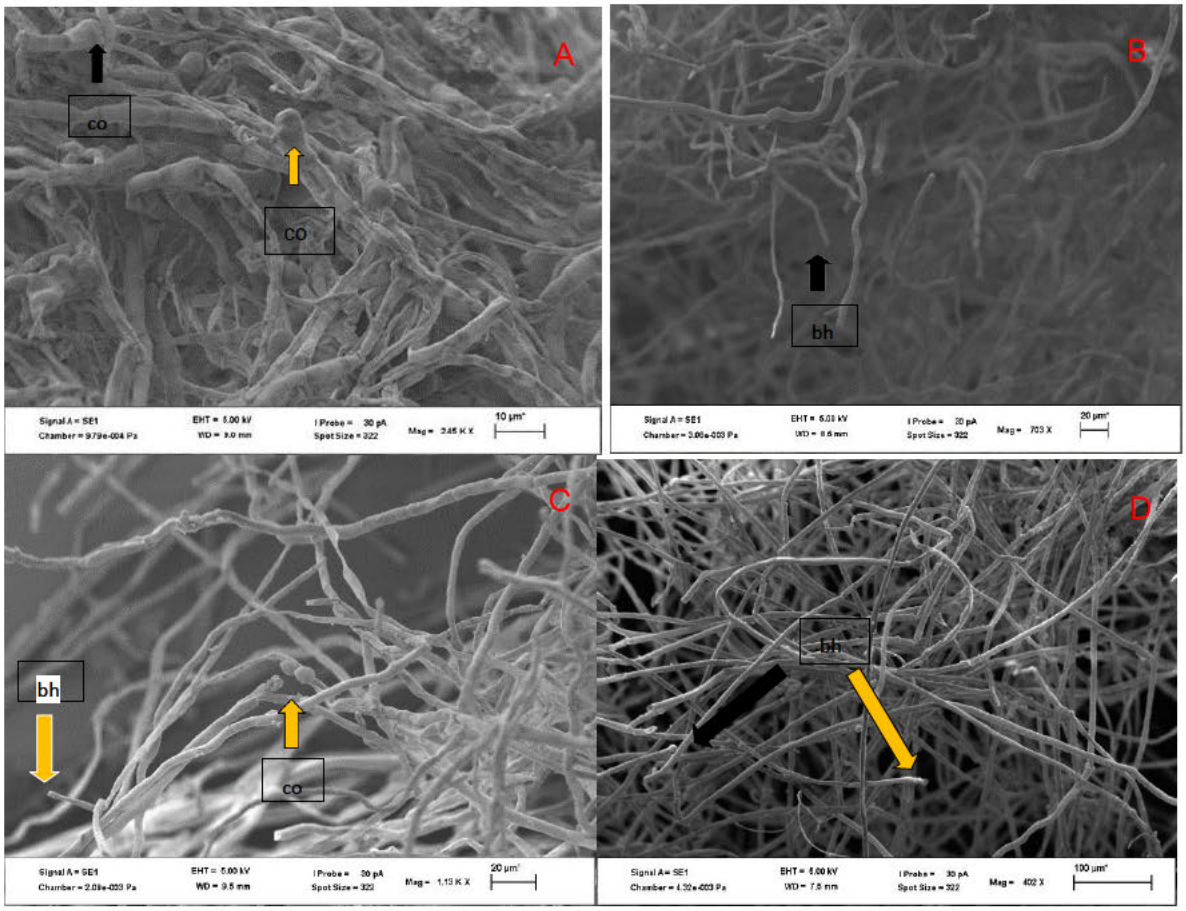

Figure 3.7 Scanning electron micrographs of *C. gloeosporioides* on potato dextrose agar after 10 days at 25°C (A) showing a successful mycelial growth and conidia (co) (B). Interaction between the pathogen and BMR2, MeRo2y (C) and YCR3 (D) showing the broken hyphae (bh).

3.3.5. *In vivo* effects of yeasts and *Bacillus* isolates on *C. gloeosporioides* There was a significant difference (P≤0.001) between fruits inoculated with pathogen only and fruits treated with yeast isolate YCR3 and *Bacillus* isolate B16 (*B. megaterium*) at day 3, and no significant differences from day 5 to day 10 post inoculation. The water control treatment had the lowest incidence of *C. gloeosporioides* (16.67%) and it was

significantly different (P≤0.001) from fruits inoculated with 1 10^5 conidia $m\ell^{-1}$ pathogen suspension (Figure 3.8).

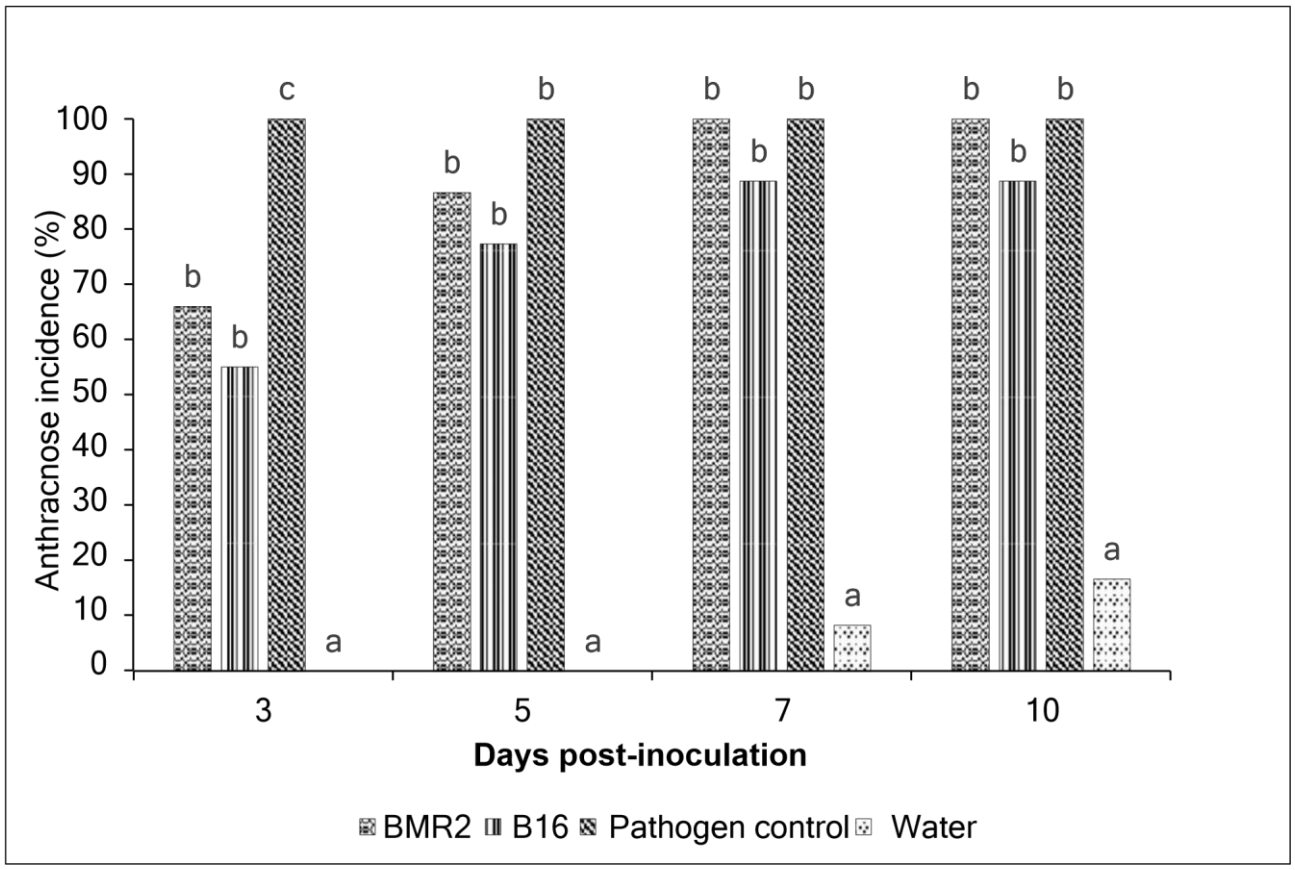

Figure 3.8 Sensitivity of *C. gloeosporioides* to *Bacillus* isolates, BMR2 and B16, treatments on papaya fruits after 10 days post-inoculation at 25°C inoculation. The means followed by the same letter are not significantly different according to Fisher's Least Significant Difference Test (P = 0.05).

There was no significant difference (P≤0.001) in anthracnose incidence between the fruit treated with YCR3 and YSY1 suspensions from day 3 up to day 10 (Figure 3.9). Control fruit exhibited 100% pathogen incidence compared to 16.67% in the water control.

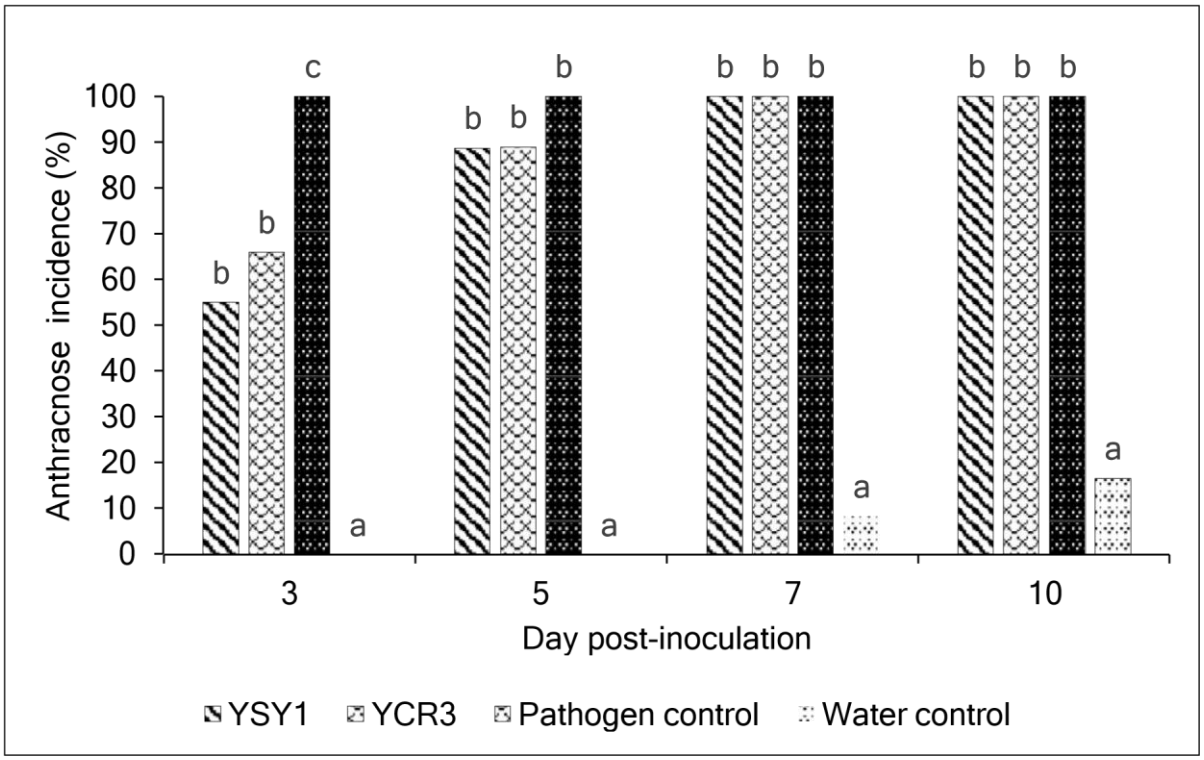

Figure 3.9 Anthracnose incidence on papaya fruits treated with yeast isolates after 10 days postinoculation at 25°C. Means followed by the same letter are not significantly different according to Fisher's Least Significant Difference Test (P = 0.05). Therefore, there was no significant difference amongst the yeast controls.

3.4. Discussion

The findings presented in this study demonstrate the potential of biological control agents to control anthracnose caused by *C. gloeosporioides*. *Colletotrichum gloeosporioides* isolated from papaya fruit was pathogenic and caused sunken lesions on papaya fruits during the pathogenicity trial (Figures 3.2 and 3.3). As the days were increasing, the spots on papaya fruits were also enlarging, becoming sunken and water-soaked (Figure 3.2 C and D). Jeffries (1990) stated that the most visible anthracnose symptoms are sunken lesions containing conidial masses on the surface of infected fruits, which concurs with the results from this study.

The *Bacillus* spp. and yeast isolates showed better inhibition performance against *C. gloeosporioides* (Figure 3.4), the isolation of yeast and *Bacillus* with different level of biocontrol efficiency against pathogens has been reported in several studies (Viňas *et al*.,1998). The effectiveness of some isolates was lower probably because some isolates have a high production of antimicrobials with different modes of action and perform a better competition for nutrients and space (Spadaro and Droby, 2016). The

best 10 yeasts and 10 *Bacillus* isolates showed a better inhibition at day 3, 5 and 7 of screening, with the control having 0% inhihibion (Figure 3.5 and 3.6). For further screening yeast Isolates YCR3, YSY1, Yzl managed to successfully control the pathogen by 73%, 71% and 70% (Table 3.1), respectively, and *Bacillus* isolate BMR2 and B16 provided the best control and reduced the incidence of *C. gloeosporioides* 81% and 76% (Table 3.2), respectively, when compared to the untreated control.

The *in vitro* antagonist application on either sides of the testing plate and the pathogen mycelial block application at the center, provided a better the control of *C. gloeosporioides* which is in agreement with the results by Liu *at al* (2018). The yeast Isolates YCR3, YSY1 and *Bacillus* BMR2 and B16 showed a greater control of the fungal pathogen. As Figure 3.7 shows the mechanism used by the biological control agents in limiting the growth of the fungal pathogen, by degrading or cutting off the tip of a hypha (Del Frari *et al*., 2019). Postharvest biocontrol agents which include *Bacillus* spp. and yeast have been described to work by inhibiting target pathogens through antibiosis, production of lytic enzymes, parasitism, induced resistance and competition (Janisiewicz and Korsten, 2002), but few organisms antagonize by a single mechanism (Kefialew and Ayalew, 2008).

During the *in vivo* trial, the disease incidence of 16.67% was recorded on the papaya fruits treated with water and there was a significant difference when compared to the pathogen-controlled fruits with anthracnose incidence of 100% (Figure 3.8 and 3.9). This is caused by pre-existing infection of the pathogen on the fruit, since the pathogen is latent, and the pathogen occurs during preharvest activities, but the symptoms appear during post-harvest handling (Talhinhas and Baroncelli, 2023). There was a better control in fruits treated with B16 (88.67%), even though the infection was delayed but successfully controlled when compared to BMR2, YSY1 and YCR3.

B16 isolate was the best isolate against *C. gloeosporioides* both *in vitro* and *in vivo*. It was identified as *Bacillus megatarium* with an accession number of MN509795.1.
These results are similar to a study by Farungsang *et al*., 2012, which showed that *Bacillus megaterium* was the most effective antagonist among a large number of isolates tested *in vitro* against *C. gloeosporioides*. This isolate managed to reduce the amount of anthracnose incidence of this pathogen on the mango leaves which proved it as an aggressive antagonist towards *C. gloeosporioides* (Farungsang *et al*., 2012).

B. megaterium strains produce antifungal and antibacterial compounds differing in their spectra of action which contribute to the control of plant bacterial and fungal diseases (Malanicheva *et al.,* 2012). The antagonistic properties of B16 might be through the enzyme-mediated lytic mechanism, which has been proven to be effective in controlling fungal pathogens (Thakur *et al*., 2014).

3.5. Conclusion

This study has indicated that yeast antagonists and *Bacillus* spp. may be used as a non-chemical alternative treatment to control postharvest anthracnose of papaya. *Bacillus megatarium* (B16) exhibited better biocontrol activity on *C. gloeosporioides* than yeast isolates both *in vitro* and *in vivo*.

3.6. References

Awang, Y., Ghani, M. A.A., Sijam, K., Mohamad, R.B., 2011. Effect of calcium chloride on anthracnose disease and postharvest quality of re-flesh dragon fruit (*Hylocereus polyrhizus*). *African Journal of Microbiology Research*. *5*, 5250-5259.

Cannon, P. F., Bridge, P. D., and Monte, E., 2000. Linking the past, present, and future of *Colletotrichum* systematics. In *Colletotrichum* host specificity, pathology and host–pathogen interaction. *The American Phytopathology Society.* 3*,* 1-20.

Del Frari, G., Cabral, A., Nascimento, T., Boavida Ferreira, R. and Oliveira, H., 2019. Epicoccum layuense a potential biological control agent of esca-associated fungi in grapevine. *Plant Science.* 14,213-273.

do Nascimento Nunes, M. C., 2008. Impact of environmental conditions on fruit and vegetable quality. *Stewart Postharvest Review*. 4, 1-14.

El Ghaouth, A., Wilson, C.L and Wisniewski, M., 2003. Control of postharvest decay of apple fruit with *Candida saitoana* and induction of defense responses. *Phytopathology*. *93*, 344348.

Farungsang, U., Sinlapasunthorn, S., Rattanakreetakul, C., Phavaphutanon, L. and Farungsang, N., 2012. *Bacillus megaterium* isolate 3103: Antagonistic spectrum on *Colletotrichum gloeosporioides* diversity and impact of field application on postharvest incidence of mango fruit anthracnose. *In International Conference Postharvest Pest and Disease Management Expo Horticultural Crops.* 973, 81-88.

Hewajulige, I.G. and Wijeratnam, S.W., 2010. Alternative postharvest treatments to control anthracnose disease in papaya during storage. *Fresh Produce*. 4, 15-20.

Hoa, N. V., 2008. Current research activities and the development of good agricultural practice (GAP) for pitaya in Vietnam. Pitaya Production. *Market Export Challenges Prospects*, Putrajaya, Malaysia.

Janisiewicz, W.J. and Korsten, L., 2002. Biological control of postharvest diseases of fruits. *Annual Review in Phytopathology*. *40*, 411-441.

Kefialew, Y. and Ayalew, A., 2008. Postharvest biological control of anthracnose (*Colletotrichum gloeosporioides)* on mango (*Mangifera indica*). *Postharvest Biology and Technology*. 50, 8-11.

Kitinoja, L. and Kader, A.A., 2015. Measuring postharvest losses of fresh fruits and vegetables in developing countries. *PEF white paper*. 15, 26.

Liu, Z., Du, S., Ren, Y. and Liu, Y., 2018. Biocontrol ability of killer yeasts (Saccharomyces cerevisiae) isolated from wine against *Colletotrichum gloeosporioides* on grape. *Journal in Basic Microbiology*. *58*, 60-67.

Malanicheva, I.A., Kozlov, D.G., Sumarukova, I.G., Efremenkova, O.V., Zenkova, V.A., Katrukha, G.S., Reznikova, M.I., Tarasova, O.D., Sineokii, S.P. and El'-Registan, G.I., 2012. Antimicrobial activity of Bacillus megaterium strains. *Microbiology Journal*. 81, 178- 185.

Pomper, K.W., Lowe, J.D., Lu, L., Crabtree, S.B., Dutta, S., Schneider, K. and Tidwell, J., 2010. Characterization and identification of papaya cultivars and advanced selections by simple sequence repeat markers. *Journal of American Society in Horticultural Science. 135*, 143- 149.

Prusky, D., 1996. Pathogen quiescence in postharvest diseases. *Annual Review in Phytopathology*. *34*, 413-434.

Sepiah, M., 1993. Efficacy of propiconazole against fungi causing postharvest diseases on Eksotika papaya. Paper presented at: *International Postharvest Conference in Handling Tropical Fruits* (Chiangmai, Thailand), 53.

Spadaro, D and Droby, S., 2016. Development of biocontrol products for postharvest diseases of fruit: The importance of elucidating the mechanisms of action of yeast antagonists. *Trends Food Science and Technology*. *47*, 39-49.

Talhinhas, P. and Baroncelli, R., 2023. Hosts of *Colletotrichum*. *Microsphere*. 14, 158261.

Thakur, A., Thakur, K., Kumar, A., Warghat, A.R., Kumar, D. and Pandey, S.S., 2024. Endophyte-based fungal elicitors for enhanced production of valepotriates and sesquiterpenoids in leaf cell suspension cultures of Valeriana jatamansi Jones. *Journal of Applied Microbiology*. 135, 242.

Torres-Calzada, C., Tapia-Tussell, R., Higuera-Ciapara, I and Perez-Brito, D., 2012. Morphological, pathological and genetic diversity of *Colletotrichum* species responsible for anthracnose in papaya (*Carica papaya* L). *European Journal in Plant Pathology. 135*, 67-79.

Viñas, I., Usall, J., Teixidó, N. and Sanchis, V., 1998. Biological control of major postharvest pathogens on apple with Candida sake. *International Journal Food Microbiology.* 40, 9-16.

Chapter 4

In vitro and *in vivo* antifungal activity of *Moringa oleifera* leaf extract against *C. gloeosporioides*

Abstract

Anthracnose caused by *Colletotrichum gloeosporioides*, is one of the major pathogens causing negative effects in the papaya industry. Thus, this study evaluated the antifungal activity of ethanolic moringa leaf extract against *C. gloeosporioides*. Five concentrations (MLE1%, MLE1.5%, MLE2%, MLE2.5% and MLE3%) of moringa leaf extracts (MLE) were prepared from the moringa leaf powder. Out of five MLE prepared and screened in the *in vitro* trial, only MLE2%, MLE2.5%, and MLE3% preliminary controlled the pathogen mycelial growth with an inhibition percentage greater than 50%. In *in vitro* secondary screening, MLE2.5% and MLE3% inhibited *C. gloeosporioides* by 96.9% and 97.5%, respectively. For the *in vivo* screening, papaya fruits were wounded and inoculated with 2 µℓ of 1×10^5 conidia ml^{-1} pathogen suspension; after 24 hours the fruits were inoculated with MLE2.5% and MLE3%and stored at 25°C in enclosed boxes covered with black plastic bags to maintain a high relative humidity of 90%. There was a significant difference in fruits treated with MLE2.5% moringa leaf extract, and water control compared to the pathogen treated control at day 3 and day 5 after inoculation. At day 7 and day 10 post-inoculation, there was no significant difference between MLE2.5% and MLE3% treated fruits and the pathogen control. Scanning electron microscopy trial showed that MLE2.5% disrupted the mycelial growth of *C. gloeosporioides* by inhibiting the spore germination and shrinkage in mycelia compared to the control treatment. MLE2.5% screened in this study was more effective in controlling *C. gloeosporioides* when compared to other concentrations both *in vitro* and *in vivo* at day 3 to day 10 at 25^{o}C and may be used as a non-chemical control against anthracnose.

Keywords: *C. gloeosporioides*, *Moringa oleifera*, papaya

4.1. Introduction

There is a high demand for papaya (*Carica papaya L*.) fruits in both National and International markets. However, postharvest rots portray the biggest problems in commercializing high-quality fruits and reducing the storage life of papaya. Anthracnose caused by *Colletotrichum gloeosporioides* [(Penz.) Penz. and Sacc.] is one of the most widespread fungal diseases, a cosmopolitan infection for many tropical and subtropical fruits (Capdeville *et al*., 2007). However, there is a need to explore alternative methods to control this important disease as pathogens develop resistance to fungicides, and also a continuous loss of chemicals has occurred through regulatory actions (Bautista Baños *et al*., 2013). Several studies demonstrate the fungicidal potential of plant extracts against postharvest fungi (Tlibi *et al*., 2012; Šernaitė, 2017; El Khetabi *et al*., 2022).

Most of the plant extracts are rich in antifungal properties and successfully suppress the growth of most diseases (Djioua *et al*., 2010; Sitara *et al*., 2011). The use of plant extracts as a control method for postharvest diseases of fruits and vegetables is well advancing and can be attributed to the negative consequences attached to the usage of fungicides to the environment and human health (Palou *et al*., 2015). Studies on the inhibitory effects of a diversity of plant extracts to control various fungi such as *C. gloeosporioides*, *Lasiodiplodia theobromae*, *Botrytis cinerea*, *Glomerella cingulata*, *Penicillium expansum*, have proved the fungicidal potential (Bommarito *et al*., 1998; Bong *et al*., 1997; Mohamed *et al*., 1996 Bautista-Banos *et al*., 2000).

One of the most utilized plant extracts is *Moringa oleifera*, which is primarily indigenous to Africa and India and is regarded as one of the most beneficial trees in the world. Every part of the moringa tree can be used for medication, food and industrial intentions (Moyo *et al*., 2011). The leaves are consumed as salad, cooked leaves, or stored as dried powder for months without loss of nutritional value. Apart from treating malnutrition, leaf extracts of *M. oleifera* have also been reported to exhibit antioxidant activity both *in vitro* and *in vivo* due to its abundance of flavonoids and phenolic acids (Vongsak *et al*., 2013). The objective of this study was to investigate the effect of different concentrations of moringa leaf extracts on *C. gloeosporioides* papaya *in vitro* and *in vivo*.

4.2. Materials and methods

4.2.1. Isolation of *Colletotrichum gloeosporioides* from diseased papaya fruit

C. gloeosporioides was isolated from infected papaya fruit obtained from local supermarkets in Scottsville, Pietermaritzburg, South Africa. The infected fruit parts with spores were then directly plated onto potato dextrose agar (PDA) and incubated at 28°C for seven days. Pure cultures were obtained by subculturing on fresh PDA plates and maintained in McCartney bottles three-quarter with PDA at 4°C. The inoculum was prepared from 14-day-old culture dishes incubated at 28°C, and spores were viewed under the light microscope and confirmed to be pathogenic on papaya and mango (Carl Zeiss, Geschäftsbereich, Germany).

4.2.2. Preparation of ethanolic moringa leaf extracts

Moringa powder was extracted using 70% (v/v) ethanol as a solvent. Five concentrations of moringa were prepared by dissolving 10, 15, 20, 25 and 30 grams of leaf powder in 1000mL of ethanol, separately in flasks, and allowed to stand in a shaker for 24 hours. A cheesecloth was used to sieve extracts and moringa leaf extracts (MLE) 10, 15, 20, 25 and 30% were used against *C. gloeosporioides* both *in vitro* and *in vivo*. The solvents were then evaporated using the rotary evaporator to get crude extracts. The concentration was calculated using the formula: C = (g/100) x 100. Where g is the gram. Thus MLE10%, MLE15%, MLE20%, MLE25%, and MLE30% concentration, respectively, were used for the inhibition of mycelia growth.

4.2.3. Effect of moringa leaf extracts on *C. gloeosporioides in vitro*

The effect of moringa leaf extract on the mycelial growth of *C. gloeosporioides* was evaluated using the food poisoning technique (Songoyomi, 2004). A volume of 1mL of MLE10%, MLE15%, MLE20%, MLE25% and MLE30% was mixed with 9mL of the media (PDA), poured into Petri dishes, and carefully spread evenly over the plate. This resulted to PDA-extract mixture with corresponding MLE1.0%, MLE1.5%, MLE2.0%, MLE2.5% and MLE3.0% extract concentrations. The plates were then gently rotated to ensure the even dispersion of the PDA media on the plates. The agar extract mixture was allowed to solidify for 24 hours and then inoculated at the center with a 4mm diameter mycelial block of a 7-day old *C. gloeosporioides* culture. There were three replicates for each treatment. The inoculated plates were incubated at 25°C for 7 days and the mycelial growth was examined on days 3, 5 and 7 after inoculation.

The mycelial growth inhibition was calculated using the formula:

- With a positive control being *C. gloeosporioides*

$$PI\ (\%) = \frac{\text{mycelial growth of control} - \text{mycelial growth with treatment}}{\text{x100 mycelial growth of control}}$$

Secondary screening of MLE2%, MLE2.5%, and MLE3% concentrations were conducted using the same procedure.

4.2.4. Effect of moringa leaf extract against *C. gloeosporioides* during the *in vivo*

Mature papaya fruits were collected from Mkhondeni Fresh Produce Market in Pietermaritzburg, South Africa. Only undamaged, healthy, unripe fruits were taken into the laboratory for experimental use. Fruits were disinfected with 70% (v/v) ethanol for 1 min, rinsed three times in sterile distilled water, and then air dried at room temperature before wounding. The fruits were wounded uniformly, approximately 3 mm deep and 3 mm wide, with a sterile pipette tip at the equatorial side. Four wounds were made in each fruit and dried for 2 hours.

The wounded fruit was then inoculated with 20 µℓ of MLE2.5% and MLE3% and allowed to air dry for 24 hours. After 24 hours, the fruits were inoculated with 20 µℓ of conidial suspension of *C. gloeosporioides* at a concentration of 1×10^5 conidia $mℓ^{-1}$. Water controls were inoculated with 20 µℓ of distilled water, whereas the pathogen controls were inoculated with the pathogen only. Inoculated papaya fruits were stored in cardboxes at 25°C with 95% relative humidity. There were 3 fruits per replicate and 3 replicates per treatment. At 3, 5, 7 and 10 days post-inoculation, wounds were examined for infection and the percentage of disease incidence (DI) was determined as follows:

DI (%) = (Number of Decayed wounds ÷ Number of Total wounds) × 100.

4.2.5. Interaction of *C. gloeosporioides* and moringa leaf extract under the scanning electron microscope

The effect of biological control agents on the hyphal development of *C. gloeosporioides* was studied using the method described by Capdeville *et al*. (2007), with few amendments.

Samples were cut into 3 mm x 3 mm blocks of the pathogen from the MLE2.5% treated and 1 non-treated PDA plated. The samples were primarily fixed in 3% (v/v) glutaraldehyde for 3 hours and then washed in 0.05M sodium cacodylate buffer for 5 minutes and dehydrated in different percentages (30%, 50%, 70%, 80%, 90%) of ethanol, 10 min each and 3 × 10 min in 100% ethanol in a fume hood. The samples were then transferred into a critical point dryer basket under 100% ethanol and dried using a Quorum K850 critical point dryer. All the samples were mounted onto the SEM stubs and coated with gold in a gold palladium sputter coater (Quorum Q150R ES). The dried coated samples were examined using a ZEISS, EVO LS 15 scanning electron microscope (Carl Zeiss SMT Ltd., Cambridge) operating at 5 kV.

4.2.6. Statistical analysis

All data were subjected to analysis of variance (ANOVA) using Genstat 18th edition. Differences between treatment means were separated using Duncan's multiple range test at $P<0.05$.

4.3. Results

4.3.1. Screening of moringa leaf extracts against *C. gloeosporioides in vitro*

The mycelial growth of the *C. gloeosporioides* on PDA plates treated with MLE2.5% and MLE3% was less than 20mm at day 7 after inoculation (Figure 4.1). The mycelial growth of the pathogen was greater than 40mm in MLE1%, MLE1.5% and MLE2%. All the concentrations tested managed to control *C. gloeosporioides* growth with mycelial growth less than 50mm during the preliminary screening (Figure 4.1). The control plates had no inhibition, whereas MLE2.5% and MLE3% concentrations had 93.4% and 87%, respectivelyTable4.1

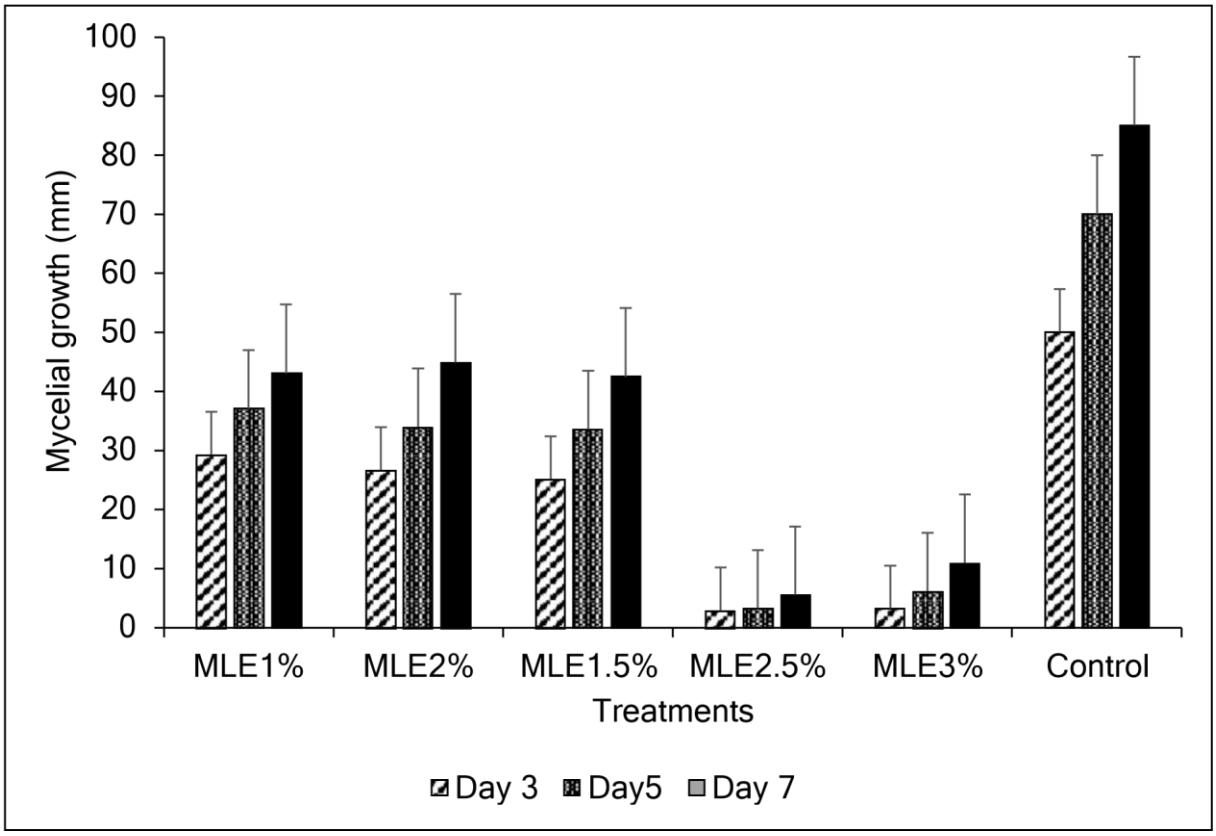

Figure 4.1 Mycelial growth of *Colletotrichum gloeosporioides* on potato dextrose agar amended with different concentrations of moringa leaf extracts at day 3, 5 and 7 at 25℃. Each concentration of moringa successfully inhibited the mycelial growth of the pathogen when compared to the control plates. Error bars indicate the significant differences between the treatments.

Table 4.1 Mycelial growth inhibition of *Colletotrichum gloeosporioides* by different concentrations of leaf extracts at day 7 at 25 C in primary screening.

Treatment	Mycelial growth inhibition (%)
Control	0.0
MLE1%	49.0
MLE1.5%	50.0
MLE2%	47.0
MLE2.5%	93.0
MLE3%	87.0

MLE2.5% and MLE3% inhibited the mycelial growth of the fungal pathogen compared to the untreated control and MLE2% at day 7 (Figures 4.2 and, Table 4.2)

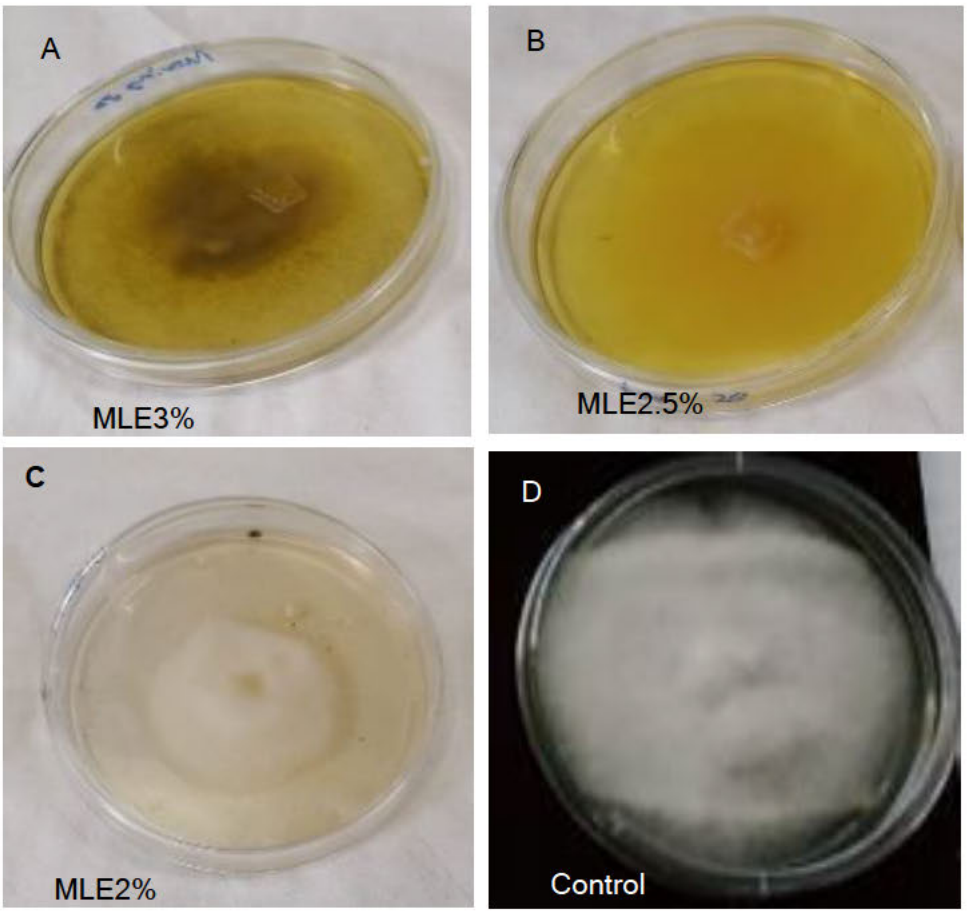

Figure 4.3 Control of *C. gloeosporioides* using moringa leaf extracts after 7 days at 25°C. Mycelial growth on PDA amended with MLE3% (A), MLE2.5% (B), MEL2% (C) and *C. gloeosporioides* control (D).

Table 4.2 Mycelial growth inhibition of Colletotrichum gloeosporioides by different concentrations of leaf extracts at day 7 at 25 C in secondary screening.

Treatments	Mycelil growth inhibition (%)
Control	0.0
MLE2%	48.0
MLE2.5%	96.9
MLE3%	97.5

4.3.2. Effect of *Moringa* leaf extracts on *C. gloeosporioides in vivo*

There was no significant difference in anthracnose incidence between papaya fruit wounds treated with MLE3% and the untreated fruits (Figure 4.4). However, there was a significant difference in MLE2.5% treated fruits compared to MLE3% at day 3 and day 5 only (Figure 4.4). The water control treatment had the lowest anthracnose incidence of 16.67% and it was significantly different (P≤0.05) from the other treatments (Figure 4.5).

The infection in Figure 4.6A is not as severe as in Figure 4.5C.

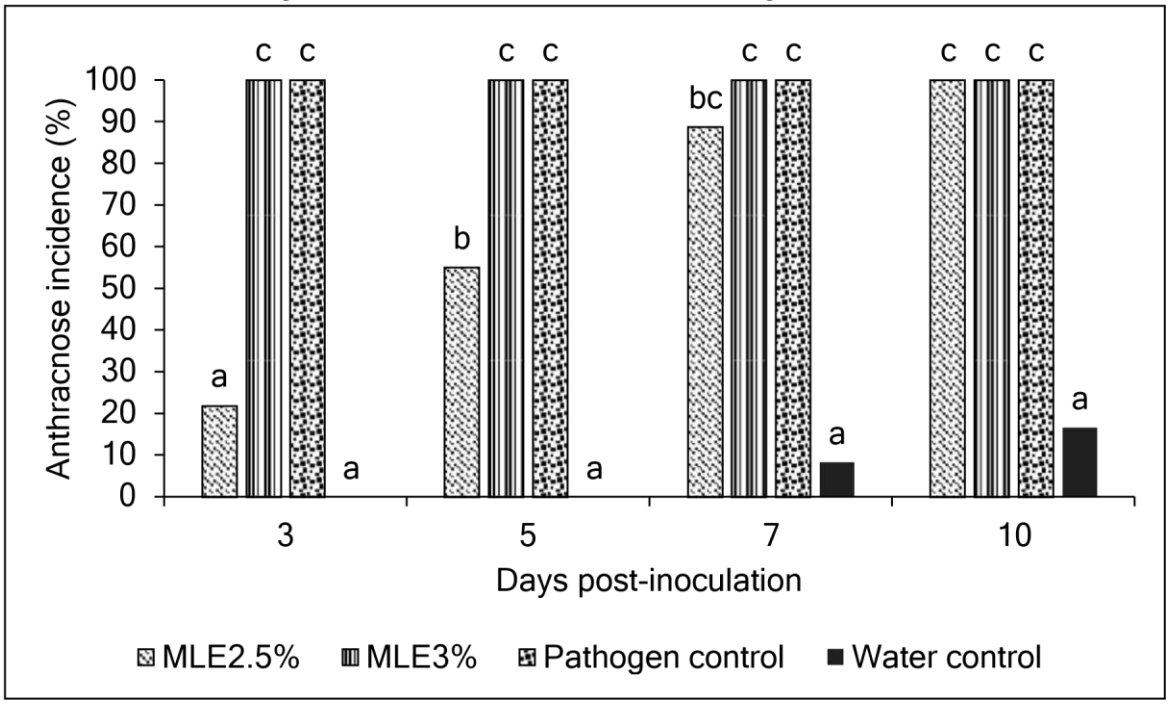

Figure 4.4 Anthracnose incidence on papaya fruit treated with different concentrations of moringa leaf extract after 10 days at 25°C. Means followed by the same letter are not significantly different according to Duncan's multiple range test (P = 0.05).

MLE2.5% provided a better control (88.67%) at day 7 and a delayed disease incidence when compared to MLE3% (100%) and pathogen control (100%). There was a significant difference between MLE2.5 and MLE3% at day 3 and day 5, but at day 7 and day 10 there was no significant difference (Figure 4.4).

Figure 4.5 Effect of moringa leaf extracts on anthracnose incidence in artificially inoculated papaya fruits after 10 days at 25°C. Fruits treated with MLE2.5% and pathogen (A); internal part of the fruit treated with MLE2.5% and pathogen (B); MLE3% and pathogen (C); internal part of the fruit treated with the MLE3% and pathogen (D).

4.3.3. Interaction of *C. gloeosporioides* and moringa leaf extract under the scanning electron microscope

MLE2.5% caused morphological changes in the mycelial growth of *C. gloeosporioides* compared to the control (Figure 4.6 A) after 10 days at 25°C. Shrinkage in hyphal size, and severe detrimental effects on conidia formation (Figure 4.6 B) were observed under SEM. The observed effects include lysis, abnormal growth, disruption, shrinkage, aggregation, reduced hyphal diameters and length and spore formation as compared to the control with a normal growth of *C. gloeosporioides*.

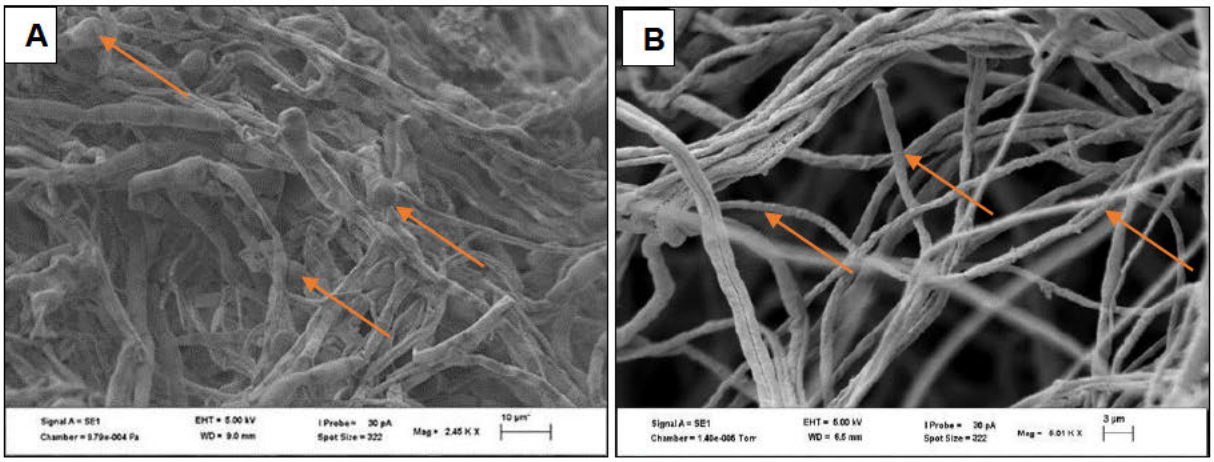

Figure 4.7: Effect of MLE2.5% on mycelia of *C. gloeosporioides* (B) and the control (A) under the scanning electron micrographs. The arrows indicated the formation of spores on control (A) and shrinkage in mycelia and the absence of spores when treated with MLE2.5% (B) indicated with orange arrows.

4.4. Discussion

The objective of this research was to investigate the efficacy of different concentrations of *Moringa* leaf extract in controlling *C. gloeosporioides in vitro* and *in vivo*. In this study the extracts from moringa leaf powder showed adequate antifungal activity on *C. gloeosporioides*, *in vitro* and *in vivo*. *Moringa* leaf extracts have successfully controlled *C. gloeosporioides* and proved their antifungal properties against this fungal pathogen. There was a significant reduction (>50%) in disease development due to the amending PDA with moringa leaf extracts used and the fungicidal effect of moringa among concentrations of extracts (Figure 4.1). MLE2.5% and MLE3% inhibited *C. gloeosporioides* after 7 days (Table 4.1). Increasing the concentration of moringa extracts was more effective and had a higher inhibitory effect in controlling the growth of the pathogen and this agrees with the report by Suleiman (2010) who reported significant differences between the results recorded on *C. gloeosporioides* mycelial growth due to different concentrations of extracts used. This is in agreement with the results found on MLE2.5% and MLE3% inhibiting *C. gloeosporioides*.

Bautista-Baños *et al*. (2000) reported that spore formation and germination, mycelial growth and infection can sometimes be inhibited by leaf extracts. The ethanolic moringa leaf extracts that were found to inhibit *C. gloeosporioides* mycelial growth in this study were selected for further evaluation at the *in vivo* trial. There were significant differences among treatments ($P< 0.05$) for fruits inoculated with moringa leaf extract compared to control fruits. The MLE2.5% concentration showed the best control of *C. gloeosporioides* compared to the pathogen control and the MLE3% at day 3 and 5 at 25^{o}C, which might be due to the enzymatic activities induced and the antifungal mechanism of moringa extract (Figure 4.4 and figure 4.5). The study by Mosch *et al.,* 1996 agrees that plant extracts rather than being effective in controlling diseases caused by pathogens, also induce several host defence mechanisms such enzymatic activities. Montes-Belmont *et al* (2000) reported that antimicrobial properties of plant extracts from various species have been proven to affect fungal development *in vitro* and *in vivo*.

The scanning electron microscopy trial showed the effect of MLE2.5% in successfully inhibiting the mycelial growth of *C. gloeosporioides,* causing severe changes in the shapes and sizes of the mycelia and conidia. Jabeen *et al*. (2008) observed and reported a destroyed *Fusarium solani* hyphae when treated with moringa extracts. Dwivedi and Enespa (2012) also indicated that *Moringa oleifera* extracts showed significant inhibition in the mycelial growth and development of many fungal pathogens. This may be associated with plant biochemical compounds such as phenols as they are involved in plant defence. In addition, the results indicated that moringa leaf extract can be used as one of the antifungal strategies and contribute to reducing the use of synthetic fungicides. These findings were similar to the results of El-Mohamedy and Abdalla (2014) which showed that plant extracts can be used as natural fungicides to control pathogenic fungi and thus reduce the dependence on the use of synthetic fungicides.

4.5. Conclusion

The results of the study have established that MLE2.5% have antifungal efficacy on mycelial growth and spore germination of *C. gloeosporioides* of papaya fruits and may be recommended as natural-based and alternative method to control postharvest fungal pathogens such as *C. gloeoporioides*.

4.6. References

Baños, S.B., López, M.H. and Necha, L.L.B., 2000. Antifungal screening of plants of the State of Morelos, Mexico against four fungal postharvest pathogens of fruits and vegetables. *Revista Mexicana de Fitopatología*, 18, 36-41.

Bautista-Baños, S., Hernández-López, M., Díaz-Pérez, J.C., and Cano-Ochoa, C.F., 2000. Evaluation of the fungicidal properties of plant extracts to reduce Rhizopus stolonifer of 'ciruela' (*Spondias purpurea L*.) during storage. *Postharvest Biological Technology. 20*, 99-106.

Bautista-Baños, S., Sivakumar, D., Bello-Perez, A., Villanueva-Arce, R. and HernandezLopez, M., 2013. A review of the management alternatives for controlling fungi on papaya fruit during the postharvest supply chain. *Crop Protection*. 49, 8-20.

Bommarito, S.G., Greenough, D.R., and Tudela, A.F., 1998. Inhibition of mango anthracnose (*Colletotrichum gloeosporioides*) and stem-end rot (*Phomosis mangniferae*) by ethanolic leaf extracts of neem (*Azadirachta indica* Juss) in the Northern Mariana Islands. *Phytopathology.*3, *88* 9.

Bong, P.S., and Min, C.I., 1997. Effect of medicinal plant extracts on apple storage diseases. *Korean Journal of Plant Pathology*. 13, 57-62.

Capdaville, G.D., Souza Junior, M.T., Santos, J.R.P., Miranda, S.P., Caetano, A.R., Falcao, R. and Gomes, A.C.M.M., 2007. Scanning electron microscopy of the interaction between Cryptococcus magnus and *Colletotrichum gloeosporioides* on papaya fruit. *Pesquisa Agropecuaria Brasileira*. 42, 1537-1544.

Djioua, T., Charles, F., Freire Jr, M., Filgueiras, H., Ducamp-Collin, M.N. and Sallanon, H., 2010. Combined effects of postharvest heat treatment and chitosan coating on quality of fresh-cut mangoes (*Mangifera indica L*.). *International Journal Food Science and Technology.* 45, 849- 855.

Dwivedi, S.K. and Neetu, D., 2012. Antifungal activity of some plant extracts against guava wilt pathogen. *International Journal of Environmental Science*. 3, 312-320.

El Khetabi, A., Lahlali, R., Ezrari, S., Radouane, N., Lyousfi, N., Banani, H., Askarne, L., Tahiri, A., El Ghadraoui, L., Belmalha, S. and Barka, E.A., 2022. Role of plant extracts and essential oils in fighting against postharvest fruit pathogens and extending fruit shelf life: A *Rev*iew *Trends in Food Science andTechnology*. *120*, 402-417.

El-Mohamedy, R.S. and Abdalla, A.M., 2014. Evaluation of antifungal activity of *Moringa oleifera* extracts as natural fungicide against some plant pathogenic fungi in- vitro. *Journal of Agricultural Technology*. 10, 963-982

Jabeen, R., Shahid, M., Jamil, A. and Ashraf, M., 2008. Microscopic evaluation of the antimicrobial activity of seed extracts of *Moringa oleifera*. *Pakistan Journal of Botany*. *40*,1349-1358.

Mohamed, S., Saka, S., El-Sharkawy, S.H., Ali, A.M.A.M., and Muis, S. 1996. Antimycotic screening of 58 Malasyan plants against plant pathogens. *Journal of Pest Science*. 47,259- 264.

Moyo, B., Masika, P.J., Hugo, A. and Muchenje, V., 2011. Nutritional characterization of Moringa (*Moringa oleifera Lam*.) leaves. *African Journal of Biotechnology.* 10, 12925-12933.

Palou, L., Valencia-Chamorro, S.A. and Pérez-Gago, M.B., 2015. Antifungal edible coatings for fresh citrus fruit: A Review in *Coatings*. *5*, 962-986.

Šernaitė, L., 2017. Plant extracts: antimicrobial and antifungal activity and appliance in plant protection. *Sodinink. Daržinink*, *36*, 58-68.

Sitara, U., Hassan, N. and Naseem, J., 2011. Antifungal activity of *Aloe vera* gel against plant pathogenic fungi. *Pakistan Journal of Botany*, 43, 2231-2233.

Suleiman, M.N., 2010. Fungitoxic activity of neem and pawpaw leaves extracts on *Alternaria solani*, causal organism of yam rots. *Advances in Environmental Biology*.159-162.

Talibi, I., Askarne, L., Boubaker, H., Boudyach, E.H., Msanda, F., Saadi, B. and Aoumar, A.A.B., 2012. Antifungal activity of some Moroccan plants against *Geotrichum candidum*, the causal agent of postharvest citrus sour rot. *Crop Protection*. *35*,41-46.

Vongsak, B., Sithisarn, P., Mangmool, S., Thongpraditchote, S., Wongkrajang, Y. and Gritsanapan, W., 2013. Maximizing total phenolics, total flavonoids contents and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method. *Indian Crops Production*. 44, 566-571.

Chapter 5

Integrated control of *Colletotrichum gloeosporioides* on papaya fruit using *Bacillus megatarium* and moringa leaf extracts

Abstract

Colletotrichum is one of the major plant pathogenic genera that is responsible for causing anthracnose and plant disease on a variety of hosts from trees to grasses. The moringa leaf extract MLE2.5%, MLE3% and *Bacillus megatarium* treatments, alone or in combination, were investigated for the control of *C. gloeosporioides* both *in vitro* and *in vivo*. *In vitro* studies indicated that *B. megatarium* was compatible with MLE2.5% and MLE3% on postharvest treatments. *B. megatarium* in combination with MLE2.5% reduced the mycelial growth of *C. gloeosporioides* by 80.6% after day 10 days at 25°C. The mycelial growth of the pathogen was also inhibited by *B. megatarium* integrated with MLE3% (71%). Papaya fruits treated with MLE2.5%+ *B. megatarium* showed the lowest anthracnose incidence (55%) compared to MLE2.5% alone (100%) and *B. megatarium* alone (100%) and MLE3% (100%) after 10 days at 25°. The physicochemical properties of papaya fruits (weight loss and total soluble solids) were not negatively affected by MLE2.5% + *B. megatarium* treatment.

Keywords: *Colletotrichum gloeosporioides; Moringa oleifera*; Inhibition; Integrated control; *Bacillus megatarium;* Physicochemical parameters

5.1. Introduction

Fruits and vegetables are a rich source of fibre, health compounds, vitamins, and minerals and their consumption has increased in the past years (Septembre- Malaterre *et al*., 2018). Consumers have a right to good quality produce that is safe for consumption and therefore they are increasingly interested in the nutritional value, good taste and flavour of the fruit and vegetables they consume (Rahman *et al*., 2021). According to consumers, fruit quality is referred to fruits with a perfect size, shape, aroma, colour and an absence of defects such as bruises, cuts or decays (Prasad *et al*., 2018). Post-harvest losses on climacteric fruits are normally caused by physiological disorders, parasitic diseases, mechanical damage, and overripe fruit (Akyem-peprah, 2012).

Papaya is one of the climacteric fruits and there are many changes occurring during its ripening, and ethylene emission activate the infection process, which negatively affects their quality, shelf life, and market value and they are perishable (Ghebreslassie, 2003; Sivakumar and Wall, 2013). Papaya is susceptible, like almost every crop grown throughout the world is susceptible to one or more species of *Colletotrichum* and it causes anthracnose symptoms of small, sunken, dark-brown lesions on fruits (Kugui, 2021). Several management practices have been developed and used to control this pathogen, synthetic fungicides were used as one of the controls in reducing losses from post-harvest diseases (Darshan *et al*., 2019).

The extensive use of synthetic fungicides has been restricted to reduce risks to human health, environmental pollution and to reduce the aggravating problem of fungicide resistance (Spadaro and Gullino, 2004, Siddiqui and Ali, 2014). Therefore, alternative management methods are required to avoid the danger of excessive toxic residues, while considering the fact that papaya fruits has a short life span after harvested as a raw fruit. The present investigation is focused on eco-friendly management strategies that facilitate to reduce the chemical usage and additionally increased consumer demands for agricultural commodities without pesticide residues (Siddiqui and Ali, 2014) and reducing concerns about the potential risk fungicides pose for human health, environmental contamination, and the development of fungicide resistance by pathogens (da Cunha *et al*., 2018). A control strategy based on antagonistic microorganisms, or biological control and plant extracts has become an attractive alternative approach.

Biological control has emerged as a promising alternative in the control of post-harvest diseases of fruit applied or in pre-harvest or post-harvest (Magallon-Andalon *et al*., 2012). This control contributes to the extended period of firmness and inhibition of ethylene production, which are often correlated with the shelf-life extension of fruits, suggesting an active role for these physiological changes in defence against pathogenic fungi (Bautista-Baños *et al*., 2003). The antimicrobial properties of plant extracts from various species have been proven to affect fungal development *in vitro* and *in vivo* (Bautista-Baños *et al*., 2003) and the spore formation and germination, mycelial growth and infection can be inhibited by plant extracts (Bautista-Baños *et al*., 2000a).

The management strategy of integrating different strategies has been proposed as one of the possible ways to deliver a satisfactory level of fruit disease control, while allowing the growers to avoid applying synthetic chemical fungicides as the plant disease control strategy (Palou, 2016; Wisniewski *et al*., 2016). Thus, the aim of the current study was to investigate the efficacy of combinations of *Bacillus megatarium* and moringa leaf extract to control *C. gloeosporioides in vitro and in vivo.*

5.2. Materials and methods

5.2.1. Fruit

Mature papaya fruits were collected from Mkhondeni Fresh Produce Market, Pietermaritzburg, South Africa. Only the undamaged, healthy and mature fruits were used in the experiments.

5.2.2. Isolation of *Bacillus* isolates

An Erlenmeyer bottle with 100ml of distilled water was placed in a water bath set at 80°C for 1 h at 130 rotations min^{-1} (rpm). Serial dilutions were performed to four concentrations (including the stock solution) from 10^0 to 10^{-3}. A 20 µL aliquot of each concentration was pipetted and spread onto PDA Petri dishes (three replicates for each concentration), incubated at 25°C for 3 days. Single colonies were identified and picked using a flamed inoculation loop and sub-cultured at 25°C.

5.2.3. Fungal pathogen

C. gloeosporioides was isolated from infected papaya fruits and cultures were directly plated and maintained on potato dextrose agar (PDA), then incubated at 28°C for 7 days. The pure cultures of the pathogen were obtained by subculturing on fresh PDA plates. These pure cultures were maintained on McCartney bottles three-quarter filled and incubated at 4^{o}C temperature for long-term storage. The inoculum was prepared from 14 days old culture dishes incubated at 28^{o}C, and spores were viewed under the light microscope (Carl Zeiss, Geschäftsbereich, Germany)

5.2.4. The effect of *B. megatarium* and moringa leaf extract *in vitro*

MLE2.5% was dispensed on a Petri dish and 9ml of the media (PDA) was added while carefully spread evenly over the plate to form a PDA-extract mixture. The plates were then gently rotated to ensure the even dispersion of the extracts. The agar extract mixture was allowed to solidify for 24 hours and then inoculated at the centre with a 4mm diameter mycelial block obtained from the 7-day old pathogen cultures and the 3-day old *B. megatarium* culture was streaked on either side of the plate. There were three replicates per treatment. All the plates were incubated at 25 C̊ for 7 days and examined at day 3, 5 and 7 for growth and the presence of inhibition.

5.2.5. The effect of *B. megatarium* and moringa leaf extract *in vivo*

Papaya fruits were disinfected with 70% ethanol for 1 min, rinsed three times in sterile distilled water, and air dried at room temperature before wounding. The fruits were wounded uniformly, approximately 3 mm deep and 3 mm wide, with a sterile pipette tip at the equatorial side. Four wounds were made in each fruit. The wounded fruit was then inoculated with 20 µℓ of the *B. megatarium* at a concentration of 1×10^{8} cells mℓ$^{-1}$ and 20 µℓ of MLE2.5% and allowed to air dry for 24 hours. After 24 hrs, they were inoculated with a suspension of 20 µℓ of conidial suspension of *C. gloeosporioides* at concentrations of 1×10^{5} conidia mℓ$^{-1}$. Water controls were inoculated with 20 µℓ of distilled water, whereas the pathogen controls were inoculated with the pathogen only. Inoculated fruits were stored in enclosed plastic trays to maintain a high relative humidity (RH) of about 90% at room temperature.

There were fruits per replicate and 3 replicates per treatment. At 3, 5, 7- and 10 days post-inoculation, wounds were examined, and the percentage of disease incidence (DI) was determined as follows:

DI (%) = (Number of Decayed Wounds ÷ Number of Total Wounds) × 100.

5.2.6 Determination of physical and biochemical quality

The weight loss (%) and total soluble solids (TSS) (°Brix) of papaya fruits treated with *B. megatarium*, MLE2.5% and MLE3% (alone and in combinations) were determined after 10 days at 25°C. The peel and seeds of papaya fruits treated with *B. megatarium*, MLE2.5% and MLE3% (alone and in combinations) were removed, cut into halves, and homogenized using a blender. A digital refractometer (Atago, Tokyo) was used to measure the TSS (°Brix)) of papaya juice. Weight loss was calculated using the following equation (Mohammed et al. (1999):

Weight loss (%) = (Initial weight – Final weight) ÷ Initial weight)

5.2.7 Statistical analysis

All data were subjected to analysis of variance (ANOVA) using Genstat 18th edition. Differences between treatment means were separated using Duncan's multiple range test at $P<0.05$.

5.3 Results

5.3.1 The effect of *B. megatarium* and moringa leaf extract on mycelial growth of *C. gloeosporioides in vitro*

The integration of MLE2.5%+B16 showed the lowest mycelial growth (16.33 mm) and the highest inhibition of anthracnose incidence (80.6%) at day 10 after inoculation (Figure 5.1 and Table 5.1). Integration of MLE3%+B16 showed the least inhibition (71.0%) of pathogen mycelial growth at day 10 after inoculation when compared to the pathogen control 0% (Table 5.1).

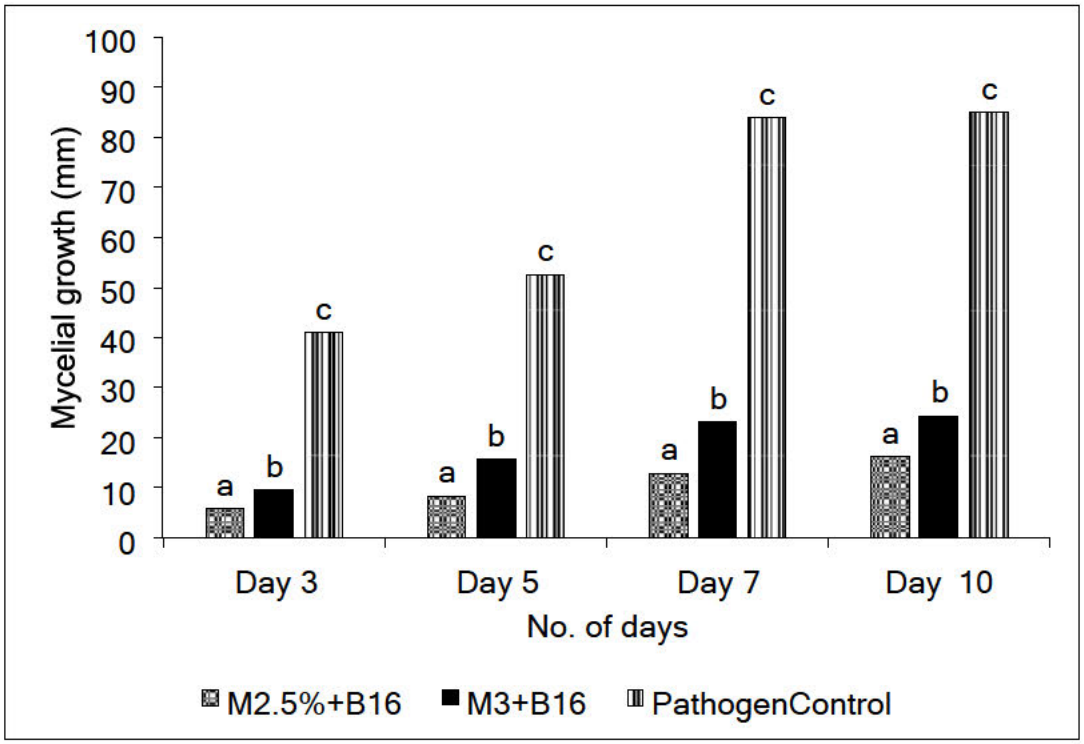

Figure 5.1 Effect of integrated control of M2.5%+ B16 and M3% + B16 on the mycelial growth of *C. gloeosporioides* during the *in vitro trial*. Means followed by the same letter are not significantly different according to Duncan's multiple range test (P = 0.001).

Table 5.1 Mycelial growth inhibition of *C. gloeosporioides* by Moringa leaf extracts and *Bacillus megatarium* (B16) at 25°C at day 3, 5, 7, and 10 days post-inoculation.

Treatments	Mycelial growth inhibition (%)			
	Day 3	Day 5	Day 7	Day 10
Control	0.0	0.0	0.0	0.0
MLE2.5% + B16	85.8	83.7	84.5	80.6
MLE3% + B16	76.4	69.9	72.2	71.0

5.3.2 Effect of *Bacillus megatarium* and *Moringa* leaf extract *in vivo*

The combination of MLE2.5% +B16 was the most effective treatment for the control of anthracnose at day 3, 5, 7 and 10 days post-inoculation (Figure 5.2). Anthracnose incidence on papaya fruits treated with MLE2.5%+B16 was 55%, compared to 100% anthracnose incidence in control fruit at day 10 after inoculation (Figure 5.2). MLE2.5% treated fruit developed anthracnose incidence of 66%, compared to *B. megatarium*

treated fruit with 55% at day 7 after inoculation (Figure 5.2). However, the anthracnose incidence increased with each successive day of the trial leading to an incidence of 33% for water control. MLE2.5% integrated with *B. megatarium* (Figure 5.3 A) provided a better control and delayed anthracnose incidence when compared to MLE3% integrated with *B. megatarium* (Figure 5.3 B).

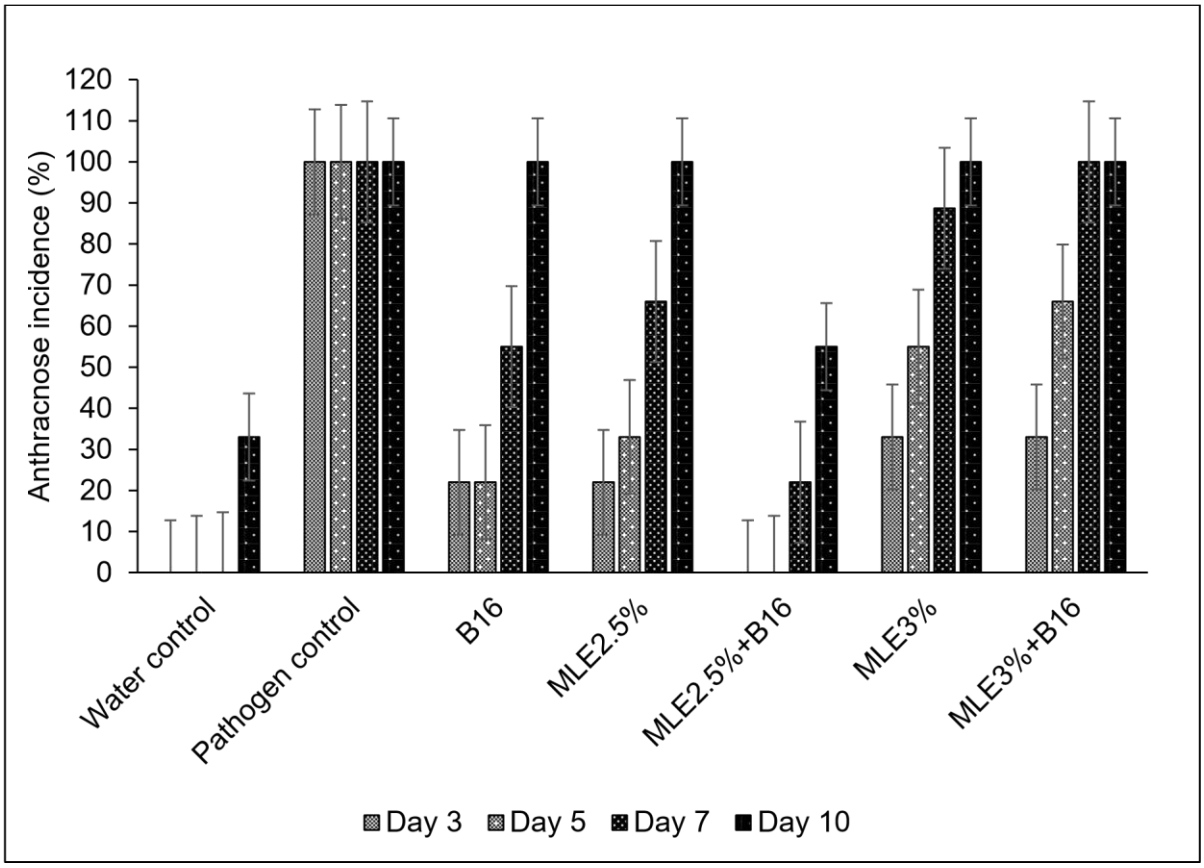

Figure 5.2 Anthracnose incidence on papaya fruits treated with *Moringa* leaf extracts and *Bacillus megatarium* (B16) at 25°C at day 3, 5, 7 and 10 post-inoculation.

Figure 5.3 Effect of anthracnose on papaya fruits treated with MLE2.5% integrated with *B. megatarium* (A) and fruits treated with MLE3% + *B. megatarium* (B) at day 10 post-inoculation at 25°C.

The percentage weight loss of the control fruit was low (4.75%) compared to fruits treated with MLE3% with the percentage weight loss of 9%. This was not significantly different from papaya fruits treated with MLE2.5% (8.06%), MLE2.5%+B16 (6.79%) and MLE3%+B16 (7.47%). It was observed that the total soluble solids on papaya fruit treated with B16 (21.32 °Brix), MLE2.5% (19.85 °Brix), MLE2.5%+B16 (20.53°Brix) was not significantly different from the control (22.02 °Brix) after 7 days at 25°C. The fruits treated with MLE3% had the lowest TSS (14.25 °Brix) and significantly different from other treatments.

Table 5.2: The effect of *Moringa* leaf extracts and *Bacillus megatarium* on physicochemical parameters of papaya fruit after 10 days at 25°C.

Treatments	Weight loss (%)	TSS (°Brix)
Control	4.75 b	22.02 a
B16	8.40 a	21.32 a
MLE2.5%	8.06 ab	19.85 a
MLE2.5%+B16	6.79 ab	20.53 a
MLE3%	9.00 a	14.25 c
MLE3%+B16	7.47 ab	15.98 b
P value	<0.001	<0.001
LSD	5.32	7.51
CV%	41.4	27.0

5.4 Discussion

The objective of this study was to evaluate the efficacy of the integrated biological control with *Moringa* leaf extract in the control of *C. gloeosporioides in vitro* and *in vivo*. *C. gloeosporioides* infection is initiated on developing fruit in the field, but the symptoms do not appear until the fruit ripens (Sharma and Kulshrestha, 2015). The results of this trial show that *Bacillus megatarium* (B16) has potential biological control properties in combination with moringa leaf extract against the growth of *C. gloeosporioides*. Yousef et al. (2015) state that moringa extracts applied in fruits and vegetables improve textural quality, retain volatile compounds, suppress respiration, and reduce microbial growth. Therefore *B. megatarium* (B16) integrated with MLE2% and MLE3%, screened in this study showed significant antagonistic activity against *C. gloeosporioides* both *in vitro* and *in vivo.*

The effect of the B16 integrated with MLE2.5% and with MLE3% had high mycelial growth inhibition percentage of *C.gloeorioides* as shown in Table 5.2. The reduction in the mycelial growth of *C. gloeosporioides* can be attributed to the antagonistic mechanism that *Bacillus* employ against pathogenic microorganisms (Kai *et al*., 2007.; Islam *et al*., 2012; Batista *et al*., 2014; Kadhim and Al-Shammaa, 2014). Tesfay *et al*. (2017) demonstrated the inhibition of *C. gloeosporioides* mycelia by moringa leaf extract *in vitro*. Similarly, Chiejina and Onaebi (2016) reported ethanolic moringa leaf extract as the best controlling agent with 100% inhibition of *Geotrichum candidum* and significantly reduced the mycelial growth of both *Mucor micheli* and *Rhizopus stolonifera*. The inhibitory effect of plant extract on mycelial growth of various pathogens has been largely attributed to the high phytochemical constituents, which include alkaloids, tannins and phenols among a few others (Anokwuru *et al*., 2011; Tesfay *et al.*, 2017).

The incidence of the anthracnose disease, caused by *C. gloeosporioides* on papaya fruits *in vivo* trial was successfully inhibited by MLE2.5%+B16 with 45% anthracnose inhibition after 10 days at 25°C, while fruits treated with MLE3%+B16 had a percentage inhibition of 34% at day 7 post-inoculation (Figure 5.2). This could be corroborated by the findings of Maqbool *et al.* (2010) and Manzoor *et al*. (2023), who reported that the incorporation of plant-based active ingredients, such as plant extracts, significantly improves the antimicrobial activity of edible coatings against postharvest pathogens. *Bacillus* is used for different purposes such as increasing

productivity in agriculture or plant protection from fungal diseases (Kai *et al*., 2007). The integration of B16 (*B. megatarium*) and MLE2.5% was the best treatment to inhibit*C. gloeosporioides* causing anthracnose of papaya fruits. *B. megaterium* strains are characterized by high diversity of antimicrobial compounds and lysates which enables *B. megaterium* to grow exponentially.

5.5 Conclusion

The use of moringa leaf extract at a concentration of 2.5% in combination with *Bacillus* isolate B16 (*B. megatarium*) resulted in the control of *C. gloeosporioides* of papaya fruits compared to MLE3% in combination with B16 treated fruits. The two in combination could be usefull in controlling and extending the postharvest shelf life of papaya fruits. Thus, can be considered as effective and potential controls for papaya postharvest *C. gloeosporioides*.

5.6. References

Akyem-Peprah, M.K., 2012. Assessment of postharvest losses in citrus (*Citrus sinensis* (L) Osbeck) in the Birim North District: A Case Study of Adventist Development and Relief Agency Supported Citrus Farmers. *Doctoral Dissertation.*

Anokwuru, C.P., Anyasor, G.N., Ajibaye, O., Fakoya, O. and Okebugwu, P., 2011. Effect of extraction solvents on phenolic, flavonoid and antioxidant activities of three Nigerian medicinal plants. *Natural Science. 9*, 53-61.

Batista, A.B., Oliveira, J.T., Gifoni, J.M., Pereira, M.L., Almeida, M.G., Gomes, V.M., Da Cunha, M., Ribeiro, S.F., Dias, G.B., Beltramini, L.M. and Lopes, J.L.S., 2014. New insights into the structure and mode of action of Mo-CBP3, an antifungal chitinbinding protein of *Moringa oleifera* seeds. *Plant Journal*. *9*, 111427.

Bautista-Baños, S., Hernández-López, M., Bosquez-Molina, E. and Wilson, C.L., 2003. Effects of chitosan and plant extracts on growth of *Colletotrichum gloeosporioides*, anthracnose levels and quality of papaya fruit. *Crop Protection. 22*, 1087- 1092.

Bautista-Baños, S., Hernández-López, M., Dıaz-Pérez, J.C. and Cano-Ochoa, C.F., 2000a. Evaluation of the fungicidal properties of plant extracts to reduce *Rhizopus stolonifer* of 'ciruela'fruit (*Spondias purpurea* L.) during storage. *Postharvest Biology and Technology. 20*, 99-106.

Chiejina, N.V. and Onaebi, C.N., 2016. Phytochemical constituents and antifungal properties of *Chromolaena odorata L*. and *Moringa oleifera Lam* on fungal rot of cucumber (*Cucumis sativus* L.) fruit. *Asian Journal Plant Science. 15*, 35.

da Cunha, T., Ferraz, L.P., Wehr, P.P. and Kupper, K.C., 2018. Antifungal activity and action mechanisms of yeasts isolates from citrus against *Penicillium italicum*. *International Journal Food Microbiology.* 276, 20-27.

Darshan, K., Vanitha, S., Venugopala, K.M. and Parthasarathy, S., 2019. Strategic ecofriendly management of post-harvest fruit rot in papaya caused by *Colletotrichum gloeosporioides. Journal of Biology Control*. 3, 225-235.

Ghebreslassie, B.M., 2003. *Postharvest maintenance of the shelf life and quality attributes of banana and papaya*. *Doctoral Dissertation*.

Islam, M.R., Jeong, Y.T., Lee, Y.S. and Song, C.H., 2012. Isolation and identification of antifungal compounds from *Bacillus subtilis* C9 inhibiting the growth of plant pathogenic fungi. *Mycobiology. 40*, 59-65.

Kadhim, E.J. and AL-Shammaa, D.A., 2014. Phytochemical characterization using GCMS analysis of methanolic extract of *Moringa oleifera* (Family *Moringaceae*) plant cultivated in Iraq. *Chemistry Mater Research. 6*, 9-26.

Kai, M., Effmert, U., Berg, G. and Piechulla, B., 2007. Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. *Archives of Microbiology. 187*, 351-360.

Kugui, S.K., 2021. *Prevalence and incidence of anthracnose of pawpaw (carica papaya), characterization of colletotrichum gloesoporoides and control using plant extracts in baringo and elgeyo marakwet counties (kenya)* (doctoral dissertation, university of eldoret).

Magallon-Andalon, C.G., Luna-Solano, G., Ragazzo-Sanchez, J.A. and Calderon-Santoyo, M., 2012. Parasitism and substrate competitions effect of antagonistic yeasts for biocontrol of *Colletotrichum gloeosporioides* in papaya (*Carica papaya L*.) var Maradol. *Mexican Journal of Science Research. 1*, 2-9.

Manzoor, A., Yousuf, B., Pandith, J.A. and Ahmad, S., 2023. Plant-derived active substances incorporated as antioxidant, antibacterial or antifungal components in coatings/films for food packaging applications. *Food Bioscience*.102717.

Maqbool, M., Ali, A., Ramachandran, S., Smith, D.R. and Alderson, P.G., 2010. Control of post-harvest anthracnose of banana using new edible composite coating. *Crop protection*. 29, 1136-1141.

Palou, L., Ali, A., Fallik, E. and Romanazzi, G., 2016. GRAS, plant-and animal-derived compounds as alternatives to conventional fungicides for the control of postharvest diseases of fresh horticultural produce. *Postharvest Biological Technology.* 122, 41-52.

Prasad, K., Jacob, S. and Siddiqui, M.W., 2018. Fruit maturity, harvesting, and quality standards. *In Preharvest Module and Postharvest Fruit Vegetable Quality*. 41-69.

Rahman, S.M.E., Mele, M.A., Lee, Y.T. and Islam, M.Z., 2021. Consumer preference, quality, and safety of organic and conventional fresh fruits, vegetables, and cereals. *Foods*, *10*, 105.

Septembre-Malaterre, A., Remize, F. and Poucheret, P., 2018. Fruits and vegetables, as a source of nutritional compounds and phytochemicals: Changes in bioactive compounds during lactic fermentation. *Food Research International. 104*, 86-99.

Sharma, M. and Kulshrestha, S., 2015. *Colletotrichum gloeosporioides* causing pathogen of fruits vegetables. *Bioscience Biotechnology Research Asia*. 12, 12331246.

Siddiqui, Y. and Ali, A., 2014. *Colletotrichum gloeosporioides* (Anthracnose). In *Postharvest Decay*. 337-371.

Sivakumar, D. and Wall, M.M., 2013. Papaya fruit quality management during the postharvest supply chain. *Food Review International. 29*, 24-48.

Spadaro, D. and Gullino, M.L., 2004. State of the art and future prospects of the biological control of postharvest fruit diseases. *International Journal in Food Microbiology. 91*, 185-194.

Tesfay, S.Z., Magwaza, L.S., Mbili, N. and Mditshwa, A., 2017. Carboxyl methylcellulose (CMC) containing *Moringa* plant extracts as new postharvest organic edible coating for Avocado (*Persea americana Mill.)* fruit. *Scientia Horticulturae. 226*, 201-207.

Wisniewski, M., Droby, S., Norelli, J., Liu, J. and Schena, L., 2016. Alternative management technologies for postharvest disease control: The journey from simplicity to complexity. *Postharvest Biological Technology. 122*, 3-10.

Yousef, A. R. M., Abd El-Moniem, E. A. A., and Saleh, M. M. S. 2015. The effect of some natural products on storability and fruit properties of ‘Fuerte’ avocado. *International Journal of Chemical Technology Research.* 8, 1454-1462.

Chapter 6

General overview

6.1. Introduction

Papaya is one of the major fruit crops cultivated in both tropical and sub-tropical zones and one of the common fruits due to its excellent nutritional value (Silva *et al*., 2007). This fruit crop consists of phytonutrients, which are present in papaya leaves, and unripe and ripe fruits (Karunamoorthi *et al*., 2014). Papaya is a source of vitamins (A, B1, B2, and C), fibre, riboflavin thiamine, folate, and niacin. It is in the top five fruits list, together with watermelon, kiwi, guava and grapefruit based on nutritional scores (Saeed *et al*., 2014; Alara *et al*., 2020). Increased negative effects are limiting the production of papaya and affecting postharvest quality due to various factors such as chilling injury, mechanical damage, fruit overripeness and diseases. The main factor of these losses is contributed by the postharvest diseases caused by fungal pathogens (Singh and Singh, 2016).

Postharvest losses due to postharvest disease may occur at any time during handling, from harvest to consumption. These disease losses lead to a reduction in fruit quantity and quality, as some diseases may not render produce unsaleable yet still reduce product value (Vindanapathirana *et al*., 2018). Plant pathogens, such as *C. gloeosporioides*, causes the fungal disease known as anthracnose, infecting vegetables and fruit crops both in the field and during the postharvest handling and commercialization phases. This is a complex disease of the major clades of the genus *Colletotrichum*, which comprises more than 22 species to date (Peralta-Ruiz *etal*., 2023).

6.2. Research objectives and major findings

The current study investigated the effect of non-chemical and eco-friendly controls against *C. gloeosporioides,* with the following objectives:

- Isolation of antagonistic yeasts and *Bacillus* isolates from different medicinal plants
- Investigation of the *in vitro* and *in vivo* effect of yeast and *Bacillus* isolates on *C. gloeosporioides*.
- Investigation of the effect of different concentrations of moringa leaf extract on *C. gloeosporioides in vitro* and *in vivo.*

- Investigating the independent and combined effects of *Bacillus* or yeast isolates with moringa leaf extracts for the control of *C. gloeosporioides* of papaya.

Chapter 3: Investigating the *in vitro* and *in vivo* effects of antagonistic microorganisms on *Colletotrichum gloeosporioides* of papaya

- Yeast YCR3 and YSY1 and *Bacillus* isolates BMR2 and B16 provided the best *in vitro* control and inhibition of the *C. gloeosporioides*.
- B16, identified as *Bacillus megaterium*, was highly antagonistic against *C. gloeosporioides* both *in vitro* and *in vivo*.
- SEM images showed inhibition of mycelial growth and conidia formation of *C. gloeosporioides* by BMR2, B16 and YCR3.
- Broken and shrunken hyphae were successfully caused by the application of the biocontrol agents against *C. gloeosporioides*.

Chapter 4: *In vitro* and *in vivo* antifungal activity of *Moringa oleifera* leaf extract against *C. gloeosporioides*.

- Two concentrations of *Moringa* leaf extract (MLE2.5% and MLE3%) showed a great antifungal effect against the mycelial growth of *C. gloeosporioides*.
- *Moringa* leaf extract at a concentration of 2.5% showed higher efficacy in inhibiting the fungal growth of *C. gloeosporioides* both *in vitro* and *in vivo.*
- SEM showed an effect of MLE2.5% on the mycelia of *C. gloeosporioides* with shrinkage in mycelia and absence of spores compared to the control.
- B16 was compatible with *Moringa* leaf extract on the amended PDA.
- B16 (*B. megatarium*) was highly antagonistic in inhibiting *C. gloeosporioide*s and its efficacy was improved when combined with MLE2.5% and MLE3%.

Chapter 5: Integrated control of *Colletotrichum gloeosporioides* on papaya fruit using *Bacillus megatarium* and *Moringa* leaf extracts

- Scanning electron microscope showed an effect of MLE2.5% on the mycelia of *C. gloeosporioides* with shrinkage in mycelia and absence of spores.
- Isolate B16 *(Bacillus megatarium*) was compatible with *Moringa* leaf extracts (MLE2.5% and MLE3%) on the amended PDA plate.

- *B. megatarium* was highly antagonistic in inhibiting *C. gloeosporioides* and its efficacy was highly improved when combined with MLE2.5% and MLE3%.
- The weight loss and total soluble solids of fruits treated with MLE2.5% + *B. megatarium* treatment were not negatively affected.
- *Bacillus megatarium* can be integrated with *Moringa* leaf extracts to manage anthracnose of papaya.

6.3. Discussion

The isolated *Bacillus* and yeasts from the medicinal plants provided a better control of *C. gloeosporioides* both *in vitro* and *in vivo* and based on the results by Ravindran *et al* 2023 *Bacillus* species isolated successfully inhibited the *C. gloeosporioides* with more than 80% inhibition percentage. B16 identified and confirmed from Inqaba as *Bacillus megaterium* and was highly antagonistic against *C. gloeosporioides* both *in vitro* and *in vivo*. The SEM images showed inhibition of mycelial growth and conidia formation of C. gloeosporioides by BMR2, B16 and YCR3. Results from Mannaa *et al*., 2017 shows that *B. megaterium* strain exhibited significant biocontrol activity against grain fungi.

Two concentrations of ethanol *Moringa* leaf extract (MLE2.5% and MLE3%) had a great antifungal effect against the mycelial growth of *C. gloeosporioides*. The ethanol extracts has the great impact in controlling and inhibiting *C. gloeosporioides* (Okigbo and Ogbonnaya, 2006), and specifically the leaf extracts completely inhibited postharvest rots of papaya (Banos *et al*., 2002). Concentration of MLE 2.5% showed higher efficacy in inhibiting the fungal growth of *C. gloeosporioides* both in vitro and in vivo and the SEM results showed the same effect on the mycelia of *C. gloeosporioides* with shrinkage in mycelia and absence of spores compared to the control. The mycelial growth, spore formation, germination and infection can be inhibited by plant extracts (Banos *et al*., 2002).

B. megatarium highly inhibited the *C. gloeosporioides* and its efficacy was then highly improved when integrated with MLE2.5% and MLE3%. During postharvest, dipping fruits in leaf or plant extracts inhibited the rot development during storage (Bautista-Banos *et al*., 2003). The weight loss and total soluble solids of fruits treated with MLE2.5% + *B. megatarium* treatment were not negatively affected. Therefore *B. megatarium* can be integrated with *Moringa* leaf extracts to manage anthracnose of papaya both *in vitro* and *in vivo* studies.

6.4. Conclusion

The study evaluated the antifungal activity of the screened Bacillus, yeasts isolates and moringa leaf extracts against C. gloeosporioides of papaya during the in vitro and in vivo trials. These controlling techniques are easily accessible to be used as potential controlling measures, even when integrated.

6.5. Recommendations

For the reduction of postharvest anthracnose incidence on papaya fruit, the implementation of economically friendly control strategies must be considered. The use of antifungal and antimicrobials such as biological control agents and Moringa plant extracts for disease control is required. This study showed that biocontrol agents and Moringa leaf extract have an inhibitory and antifungal effect on C. gloeosporioides. However, their combination suggested a possible postharvest method for controlling this pathogen on papaya fruits.

A residual effect of synthetic chemicals during storage and the pathogen strains of C. gloeosporioides have developed resistance to fungicides and there is a need to develop alternative methods. Therefore, the papaya fruit industry needs non-chemical control options in order to minimize C. gloeosporioides. These control options include the integrated use of Bacillus megatarium and Moringa during postharvest. However, future research should investigate the effect of integrated control of these treatments on the physiological parameters of papaya including phenolics, ascorbic acid, sugar content, defence-related enzymes, titrable acidity and sensory attributes of papaya.

6.6. References

Alara, O.R., Abdurahman, N.H. and Alara, J.A., 2020. *Carica papaya*: Comprehensive overview of the nutritional values, phytochemicals and pharmacological activities. *Advance Traditional Medicine*. 2020, 22, 1–31.

Banos, S.B., Necha, L.L.B., Luma, L.B. and Torres, K.B., 2002. Antifungal activity of leaf and stem extracts from various plant species on the incidence of *Colletotrichum gloeosporioides* of papaya and mango fruits after storage. *Revista Mexicana de Fitopatologia*. 20, 8-12.

Bautista-Banos, S., Hernandez-Lopez, E., Bosquez-Molina, E. and Wilson, C.L., 2003. Effect of chitosan and plant extracts on growth of *Colletotrichum gloeosporioides,* anthracnose levels and quality of papaya fruit. *Crop Protection*. 22, 1087-1092.

Fortunati, E., Mazzaglia, A., Balestra, G.M., 2019. Sustainable Control Strategies for

Plant Protection and Food Packaging Sectors by Natural Substances and Novel Nanotechnological Approaches. *Journal of Science in Food Agriculture.* 99, 986–1000.

Karunamoorthi, K., Kim, H.M., Jegajeevanram, K., Xavier, J. and Vijayalakshmi, J., 2014. Papaya: A gifted nutraceutical plant-a critical review of recent human health research. *Cellellular Medicine*. 4, 2-1.

Manna, D.K., Maity, P., Nandi, A.K., Pattanayak, M., Panda, B.C., Mandal, A.K., Tripathy, S., Archarya, K., Sahoo, A.K., Gupta, N. and Roy, S., 2017. Structural elucidation and immunostimulating property of a novel polysaccharide extracted from edible mushroom lentinus fusipes. *Carbohydrate polymers*. 157, 1657- 1665.

Okigbo, R.N. and Ogbonnaya, U.O., 2006. Antifungal effects of two tropical plant leaf extracts (Ocimum gratissium and Aframomum melegueta) on postharvest yam (Dioscorea spp.) rot. *African Journal of Biotechnology*. 5, 9.

Peralta-Ruiz, Y., Rossi, C., Grande-Tovar, C.D. and Chaves-López, C., 2023. Green management of postharvest anthracnose caused by *Colletotrichum gloeosporioides*. *Journal of Fungi*, 9, 623.

Ravindran, A.P., Lajapathy, J.M., Lalithakumari, S.G., Mohan, A.K., Cryjac, T., and Usha,

S.S., 2023. Efficacy of Bacillus licheniformis: a biocontrol agent against Colletotrichum gloeosporioides Penz. (Penz. and Sacc.) causing anthracnose in greater yam (Dioscorea alata L.). *Egyptian Journal of Biological Pest Control*. 33, 112.

Saeed, F., Arshad, M.U., Pasha, I., Naz, R., Batool, R., Khan, A.A., Nasir, M.A. and Shafique, B., 2014. Nutritional and phyto-therapeutic potential of papaya (*Carica papaya* Linn.): An overview. *International Journal of Food Property*. 17, 16371653.

Silva, J.D., Rashid, Z., Nhut, D.T., Sivakumar, D., Gera, A., Souza, M.T. and Tennant, P., 2007. Papaya (*Carica papaya* L.) biology and biotechnology. *Tree and Forestry Science in Biotechnology.* 1, 47-73.

Singh, D. and Singh, R.P., 2021. Management of Postharvest Diseases of Fruits and

Vegetables through Chemicals. *In Postharvest Handling Dissertation in Horticulture. Prod*. 57-78.

Vidanapathirana, R., Champika, P.J., Rambukwella, R. and Wijesooriya, N., 2018. Quality and safety issues in fruit and vegetable supply chains in Sri Lanka: A Review. *Hector Kobbekaduwa Agrarian Research and Training Institute*.