UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

Biocontrol agents in combination with *Moringa oleifera* leaf extract for integrated control of *Botrytis cinerea* of tomato

By

Nonkanyiso Malevu

(216002303)

Submitted in partial fulfilment of the

requirements for the degree of

Master of Science in Agriculture (Plant Pathology)

In the

Discipline of Plant Pathology

School of Agricultural, Earth and Environmental Sciences

College of Agriculture, Engineering, and Science

University of KwaZulu-Natal

Pietermaritzburg

December 2022

DISSERTATION SUMMARY

Tomatoes and tomato-based foods provide essential nutrients beneficial to human health. Despite these benefits from tomatoes, postharvest losses result in unprofitable tomato production in some parts of the world. During ripening and harvesting, tomato becomes susceptible to diseases resulting in shorter shelf life. Susceptibility of tomato plants to *Botrytis cinerea* which causes grey mould infection can occur at any growth stage and the most susceptible growth stage is during ripening and senescing. Factors such as mechanical injuries, inadequate storage conditions, inappropriate handling, and transport affect tomato quality. The use of chemicals not only negatively affects farmers' yield by further enhancing pesticide resistance to crop pathogens but also influences other sectors of communities through contamination of drinking water sources which is an environmental hazard.

There is a need to emphasise and encourage sustainable agricultural strategies such as biological control and plant extracts as alternative strategies which are eco-friendly and economically sustainable. Therefore, the main aim of this research was to examine the effect of biocontrol agents and *Moringa oleifera* leaf extract, individually and in combination, to control *B. cinerea* on tomatoes *in vitro* and *in vivo*. A total of 48 biocontrol agents were isolated from different parts of tomato leaves, citrus leaves, mushrooms and erect prickly pear. The isolates were screened against *B. cinerea* for the inhibitory effect and as potential control of the pathogen on potato dextrose agar (PDA) and tomato fruits. *Serratia marcescens*, *Bacillus pumilus* and *Bacillus safensis* inhibited *B. cinerea* by more than 50% *in vitro*. During *in vivo* screening, *Serratia* and *Bacillus* isolates inhibited grey mould incidence on ‘Jam’ tomatoes by more than 70%. The scanning electron microscopy images of the pathogen samples treated with biocontrol agents showed swollen and lysed mycelia.

Moringa leaf extracts (MLE) were prepared into four concentrations MLE 1%, MLE 2%, MLE 3% and MLE 4%. The MLE concentrations were tested for their antifungal activity on the pathogen growth during *in vitro* studies. High concentrations were found to have some inhibitory effect on the mycelial growth of *B. cinerea*. There was no significant difference observed in the control, MLE 1% and MLE 2% since no mycelial inhibition was observed after 7 days at 25°C. For *in vivo* studies, all the concentrations had some inhibitory effect against grey mould on ‘Jam’ tomatoes. This was evidenced

by lower disease incidence observed on the fruits treated with the moringa leaf extract compared to the control treatment. Scanning electron micrographs showed morphological changes in the hyphae on the samples treated with concentrations of MLE and there was also a breakage on the pathogen hyphae.

Furthermore, this study evaluated the integrated control of *B. cinerea* using *S. marcescens, B. safensis* and *B. pumilus* integrated with MLE 2% and MLE 3% *in vitro* and *in vivo*. *S. marcescens*, *B. safensis* and *B. pumilus* integrated with MLE 2% and MLE 3% successfully suppressed mycelial growth of *B. cinerea in vitro*. Treating tomato fruits with *S. marcescens, B. safensis* and *B. pumilus* integrated with MLE 2% and MLE 3% reduced the disease incidence of grey mould compared to the control. The SEM images of the mycelial growth of *B. cinerea* showed shrinkage, and breakage of pathogen mycelia and the spores were damaged showing breakage and immature spores both *in vitro* and *in vivo*. Integrating moringa leaf extract, *Serratia* spp. and *Bacillus* spp. have the potential to be an alternative to synthetic fungicides to control postharvest pathogens.

PREFACE

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

DECLARATION

I, NONKANYISO MALEVU, declare that

I. The research reported in this thesis, except where otherwise indicated, is my original work.
II. This thesis has not been submitted for any degree or examination at any other university.
III. This thesis does not contain other persons‟ data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.
IV. This thesis does not contain other persons‟ work unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
    a. Where their exact words have been used, their writing has been placed inside quotation marks and referenced.
    b. Their words have been re-written, but the general information attributed to them has been referenced.
V. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References sections.

…… ……

Nonkanyiso Malevu (MSc candidate)

……

Dr N.C. Mbili (Supervisor)

… ……

Prof L.S. Magwaza (Co-supervisor)

ACKNOWLEDGEMENTS

First and foremost, I would like to thank God Almighty for giving me such a great opportunity in life to complete the research successfully.

I would like to express my greatest appreciation towards my supervisors, Dr N.C. Mbili and Prof L.S. Magwaza for their inspiring guidance, support, encouragement, and patience they gave me towards the research.

I would like to thank Mr K. Mkhonza and Ms B.S. Mazibuko for their technical and laboratory assistance.

My sincere thanks to The National Research Foundation for financial support in the form of a scholarship for the duration of the study.

A big thanks to my family and friends for their love and support from the beginning to the end of my project.

I would like to thank Mr Kuhlekonke Mathenjwa, Mr Nigel Kombora and Mr Sydwell Sihlangu for their continuous support and motivation during the study.

I would like to thank Ms Londeka Mbatha, Ms Nokuthula Gamede, Ms Nomusa Sithole and UKZN Plant Pathology postgraduates for their encouragement and support.

Lastly, a big thank you to all my participants who were part of this study.

I can do all things through Christ
who strengthens me.

≈ ***Philippians 4:13***

DEDICATION

I dedicate my work to my father Mr K.B. Malevu, my mother Mrs T.N. Malevu, my sister Nothando and my brother Qhawe.

TABLE OF CONTENTS

CHAPTER 1

DISSERTATION INTRODUCTION

1.1 Background

Tomato (*Solanum lycopersicum L*.) is one of the most economically important crops grown worldwide for both fresh produce markets and processed food industries (Grandillo *et al*., 1999). Tomatoes and tomato products are rich in health-related food components as they contain carotenoids (lycopene), ascorbic acid (vitamin C), vitamin E, folate, flavonoids, and potassium (Beecher, 1998; Leonardi *et al*., 2000). Lycopene is an essential, red-coloured pigment primarily found in red fruits and vegetables, especially tomatoes and tomato products (Cadoni and Di Chiara 2000). According to the latest data available, in 2019, more than 5 million hectares was prepared for the production of tomato and over a million hectares were cultivated in China, followed by India, Turkey, the United States of America (USA) and Egypt, which represent more than 60% of the tomato production worldwide (FAO, 2019).

The tomato industry provides job opportunities and serves as a source of income in most developing countries worldwide (Arah *et al*., 2015). As of 2017, China, India, and Turkey are the world's leading tomato-producing countries. In 2016, Postharvest Innovation Programme (PHI) reported South Africa as one of the few countries that can produce tomatoes throughout the year. However, South Africa has reduced the number of tomatoes exported around Africa due to their short shelf life resulting in tomatoes mainly being grown for domestic use. In 2017, FAOSTAT (2019) reported that the tomato harvest was 608 306 tonnes with a value of R6221 per ton, which is the highest production and the average price reported since 2008 (DAFF, 2018). The most tomato-producing province is Limpopo (Mooketsi), followed by Mpumalanga province (Onderberg) and Eastern Cape province (Border area) (DAFF, (2016).

Limpopo province is growing most tomatoes produced in South Africa which is estimated to be 66% of the total annual tomato production (NDA, 2009). In South Africa, the contribution of tomato production to employment is estimated to be between 25 000 and 28 000 people a year which usually increases during seasons of higher production volumes. The main tomato varieties produced in South Africa are fresh market tomatoes, ‘Roma’ tomatoes and ‘Cherry’ tomatoes. Tomato colour is an

important marketing factor to consumers and a very important quality attribute for the processing industry (Arias *et al*., 2000). Postharvest diseases of tomatoes mainly affect the quantity and quality of fruits on the market.

Pathogenic fungi and bacteria cause infection on fresh fruit and vegetables during field growth till their consumption by a consumer (Barth *et al*., 2009). *Botrytis cinerea* Pers. Fr. is one of the major postharvest fungal pathogens affecting the quality and shelf-life of tomatoes and can destroy fruits during preharvest. In addition, *B. cinerea* is the second largest plant pathogen in the world and one of the primarily studied necrotrophic fungal pathogens (Williamson *et al*., 2007; Dean *et al*., 2012). This fungal pathogen can infect leaves, stems and fruit resulting in significant yield losses (Menzies and Jarvis, 1994). *B. cinerea* can result in significant losses in the field and during postharvest storage (Jarvis, 1977; Williamson *et al*., 2007; Sharma *et al*., 2009).

According to Walker (2016), the classification of different species of *Botrytis* into sympatry and their impact on disease development can contribute to developing control strategies. The pathogen is reported to have varying fungicide resistant characteristics between isolates. This pathogen is highly susceptible to competition during the control of the pathogen since external nutrients are required for conidial germination (Elad, 1996), germ tube growth and the success of infection (Redmond *et al*., 1987; Elad and Stewart, 2004). This study aimed to isolate yeast and bacteria from different plant parts and test their abilities to inhibit the mycelial growth of *B. cinerea* under *in vitro* and *in vivo* studies; to test antifungal activities of moringa leaf extract against *B. cinerea in vitro* and *in vivo*; to investigate the integrated control of the pathogen using biological control agents and moringa leaf extract.

1.2 Problem statement

Tomatoes have a short shelf life and are highly perishable resulting in infections by plant pathogens causing mechanical damage before and after harvest (Coates and Johnson, 1997). Several pathogens such as fungi, bacteria, viruses and nematodes infect tomatoes pre-harvest and postharvest. Great yield losses are caused by grey mould on fruit and vegetables (Myresiotis *et al*., 2007). Grey mould caused by *B. cinerea* is one of the most destructive diseases of tomatoes in the greenhouse and causes great losses during the pre-harvest and postharvest period (Elad *et al*., 2007;

Williamson *et al*., 2007; Jones *et al*., 2014;). *B. cinerea* produces conidia to spread the disease through the wind over a long distance to cause infection to the next host (Jarvis, 1997).

B. cinerea as a saprophyte can survive on plant debris in the soil for a longer time and when conditions are favourable, the pathogen sporulates and spread to another uninfected host (Sansone *et al*., 2005). An increase in the area cultivated with tomatoes causes an increase in the significance of the grey mould. From storage to marketing, the fungus is highly destructive to tomatoes resulting in postharvest rejection of fruits in the market. Various control strategies used against grey mould include chemical control, biological control, cultural control and other alternative methods such as plant extracts. Fungicides used against *B. cinerea* are an effective control strategy that affects the fungal respiration of *B. cinerea* (Leroux, 1996). The control of *B. cinerea* is highly dependent upon fungicides such as benzimidazole and dicarboximide.

Chemicals are used against pathogens to control different plant diseases resulting in the development of pathogen resistance to fungicides, the presence of chemical residues in fruits and environmental problems (Leroux, 2004; Adebayo *et al*., 2013). Some isolates of *B. cinerea* were reported to be resistant to dicarboximide because of the intensive use of fungicides (Sansone *et al*., 2005). *B. cinerea* is considered a high-risk pathogen because of the development of fungicide resistant strains (Leroux, 2004). Various synthetic fungicides become ineffective in controlling the pathogen due to the development of resistant *B. cinerea* isolates (Leroux, 2004). The ability of fungal pathogens to develop resistance to fungicides and the concern of the public about the potentially harmful impacts of fungicides on human health (Hahn, 2014; Romanazzi *et al*., 2016) have promoted the exploration of alternative environmentally safe biofungicides.

1.3 Justification

Over a decade, fruit and vegetable production around the world has increased because of population growth, improved living standards in most countries and the consumption of fruits and vegetables for health purposes (Wills, 2007). Tomato is a popular and extensively cultivated fruit crop of great economic importance in South

Africa. However, tomato production can be affected by abiotic factors such as high temperature, erratic rainfall, poor soil and biotic factors such as pests, fungal, bacterial and viral diseases. Furthermore, the tomato industry encounters obstacles of pre- and postharvest diseases caused by *B. cinerea, Colletotrichum coccodes* Wallr. Hughes and *Alternaria solani* Sorauer. *B. cinerea* infects plant tissues causing grey mould during pre- and postharvest in agronomically important crops such as *Vitis vinifera* L., *Solanum lycopersicum* L., *Cucumis sativus* L., *Pyrus communis* L. and *Prunus avium* L. (Jarvis, 1997).

Grey mould is one of the most studied tomato diseases among necrotrophic and polyphagous fungi (Van Kan, 2014; Fekete *et al*., 2011). This disease results in great economic losses for different tomato cultivars. Modern hybrid tomato cultivars are susceptible to *B. cinerea* although some tomato cultivars are showing a certain level of quantitative resistance (ten Have *et al*., 2007). After harvest, fruits for fresh market must be stored in cool storage to delay fruit decay and development of grey mould although *B. cinerea* can survive at low temperatures such as 0 °C (Elad *et al*. 2004). Chemical control using fungicides is the most effective way to control the occurrence of *Botrytis* spp (Leroux, 1996). However, the use of chemicals can result in environmental pollution and health hazards. Consumers require reduced use of pesticides in the food chain because of the formation of residues on fruits and vegetables (Sansone *et al*., 2005). A higher level of residue in food is hazardous to human health.

The continuous use of fungicides impedes the control of grey mould resulting to an increase in fungicidal residue on fruits and a rapid increase in resistance among pathogen populations (Tripathi and Dubey 2004). Most fungicide-resistant strains of *B. cinerea* commonly infect commercial strawberries and tomatoes worldwide (Amiri *et al*., 2013; Fan *et al*., 2016). Resistance is a problem every time a new fungicide is introduced to the field population, and this results in a need for the development of alternative non-chemical control strategies.

Biocontrol agents are safe, natural and can be used used to control postharvest pathogens. Bacteria such as *Bacillus spp*. are one of the biocontrol agents used against fungal pathogens. Various strains of *Bacillus spp*. are reported to have antifungal activity (Ge *et al*., 2016; Lee *et al*., 2006; Martinez Absalon *et al*., 2014).

Bacillus species are among the most extensively studied biocontrol agents which use antagonism and/or competition to suppress plant pathogens (Mnif and Ghribi, 2015). The beneficial effects of *Bacillus* spp. on plant growth and yield have been reported in several crops such as *Triticum aestivum* Linn., *Zea mays* L., *Glycine max* L., *Helianthus annus* L., *Solanum tubersum* L., *Phaseolus vulgars* L., *Cucumis sativus* and many others (Aloo *et al*., 2019).

Different commercial products have been produced from *Bacillus spp*. and tested to confirm their properties and abilities to control grey mould. Commercial *Bacillus*-based products are developed and distributed worldwide and contain beneficial strains of *Bacillus subtilis Cohn*, *Bacillus amyloliquefaciens*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus velezensis*, *Bacillus cereus*, *Bacillus thuringiensis*, etc (Mazzola and Freilich, 2017). *B. amyloliquefaciens* induced salicylic acid- dependent resistance in tomato plants, reduced the incidence of *tomato spotted wilt virus*, and delayed systemic accumulation of *potato virus Y*(Beris, 2018). Treating tomato with *B. cereus* significantly reduced disease incidence caused by *B. cinerea* through activation of induced systemic resistance (ISR) (Nie *et al*., 2017).

The use of plant materials and other natural substances with antimicrobial activity is considered a promising new strategy to reduce economic losses caused by postharvest diseases such as grey mould. Moringa plant is regarded as a miracle plant since all plant parts are used for medical purposes and other purposes such as antifungal activities against plant pathogens (Price, 2000; Anwar *et al*., 2007; Garima *et al*., 2011). *Moringa oleifera* Lam. leaf extract has significantly shown activity against *Saccharomyces cerevisiae* Hansen (Patel, 2014). Antifungal activity of drumstick leaf extract was reported on the growth of *Aspergillus niger.* van Tieghen (Arowora and Adetunji, 2014). Antifungal activity of crude leaves extracts of *M. oleifera* on *Trichophyton rubrum* var. Nigricans and *Microsporium canis* are fungi that cause superficial infection in the human body (Nneka, 2006). This research will contribute to developing methods of controlling postharvest pathogens affecting the tomato industry worldwide.

1.4 Research aim and objectives

The main objective of this research was to investigate the effect of integrating biocontrol agents and *M. oleifera* extract to control *B. cinerea* of tomatoes *in vitro* and postharvest storage.

The specific objectives of this research were to:

i. Evaluate the *in vitro* and *in vivo* effect of antagonistic microorganisms against *B. cinerea* of tomato.
ii. Evaluate the *in vitro* and *in vivo* effect of moringa leaf extracts against *B. cinerea* of tomato.
iii. Determine the mode of action of biocontrol agents and moringa extract against *B. cinerea* of tomato
iv. Evaluate the integration of biocontrol agents and moringa extract on postharvest grey mould of tomato fruit.

1.5 Dissertation structure

The dissertation has 6 chapters, and the details of each chapter are described as follows: Chapter 1 is the general introduction of the dissertation; Chapter 2 is the literature review focusing on tomatoes and their production and information on grey mould; Chapter 3 focused on isolating antagonistic microorganisms from different plant parts and testing their inhibitory effect against *B. cinerea in vitro* and *in vivo*; Chapter 4 evaluated the antifungal properties of moringa leaf extract against *B. cinerea in vitro* and *in vivo*; Chapter 5 evaluated the effect of integrating biocontrol agents and moringa leaf extract against *B. cinerea*; Chapter 6 summarises the major findings of the study and their implications.

1.6 References

Adebayo, O., Dang, T., Belanger, A. and Khanizadeh, S. (2013). Antifungal studies of selected essential oils and a commercial formulation against *Botrytis cinerea*. *Journal of Food Research*, 2, 217-226.

Aloo, B.N., Makumba, B.A. and Mbega, E.R. (2019). The potential of *Bacilli rhizobacteria* for sustainable crop production and environmental sustainability. *Microbiology Research*, 219, 26-39.

Amiri, A., Heath, S. M., and Peres, N. A. (2013). Phenotypic characterization of multifungicide resistance in *Botrytis cinerea* isolates from strawberry fields in Florida. *Plant Disease*, 97,393-401.

Anwar, F., Latif, S., Ashraf, M. and Gilani, A.H. (2007). *Moringa oleifera*: A food plant with multiple medicinal uses. *Phytotherapy Research*, 25, 17-25.

Arah, K.L., Kumah, E.K., Anku, E.K. and Amaglo, H. (2015). An overview of post-harvest losses in tomato production in Africa causes and possible prevention strategies. *Journal of Biology, Agriculture and Healthcare*, 5, 78-88.

Arias, R., Lee, T., Logendra, L. and Harry, J. (2000). Correlation of lycopene measured by HPLC with the L*, a*, b* colour readings of a hydroponic tomato and relationship of maturity with colour and lycopene content. *Journal of Agricultural and Food Chemistry*, 48, 1697-1702.

Arowora, K.A. and Adetunji, C.O.A. (2014). Antifungal effects of crude extracts of *Moringa oleifera* on *Aspergillus niger* v. tieghem associated with postharvest rot of onion bulb. *SMU Medical Journal*, 1, 214-223.

Barth, M., Hankinson, T. R., Zhuang, H., and Breidt, F. (2009). Microbiological spoilage of fruits and vegetables. In *Compendium of the microbiological spoilage of foods and beverages*. Springer, New York, United States.

Beecher, G.R. (1998). Nutrient content of tomatoes and tomato products, Nutrient content of tomatoes. *Proceedings of the Society of Experimental Biology and Medicine*, 218, 98–100.

Beris, D., Theologidis, I., Skandalis, N. and Vassilakos, N. (2018). *Bacillus amyloliquefaciens* strain MBI600 induces salicylic acid dependent resistance in tomato plants against Tomato spotted wilt virus and *Potato virus Y*. *Scientific Reports*, 8, 1-11.

Cadoni, C. and Di Chiara, G. (2000). Differential changes in accumbens shell and core dopamine in behavioral sensitization to nicotine. *European Journal of Pharmacology*, 387, R23-R25.

Coates, L. and Johnson, G. (1997). Postharvest diseases of fruit and vegetables. *Plant Pathogens and Plant Diseases*, 533-548.

Dean, R., van Kan, J. and Pretorius, Z.A. (2012). The top 10 fungal pathogens in molecular plant pathology*. Molecular Plant Pathology,* 13, 1-17.

Department of Agricultural and Forestry. (2018). Abstract of agricultural statistics. National Department of Agriculture, Forestry and Fisheries, Pretoria, South Africa

Department of Agricultural and Forestry., (2016). A profile of the South African tomato market value chain. South Africa. https://www.dalrrd.gov.za/doaDev/sideMenu/Marketing/Annual%20Publications/Tomato%20Market%20Value%20Chain%20Profile%202019.

Elad, Y. (1996). Mechanisms involved in the biological control of *Botrytis cinerea* incited diseases. *European Journal of Plant Pathology,* 102, 719-32.

Elad, Y. and Stewart, A. (2004). Microbial control of *Botrytis spp.* In: Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. (Eds.), *Botrytis*: Biology, Pathology and Control. Kluwer Academic Publishers, Dordrecht, Netherlands.

Elad, Y., Williamson, B., Tudzinski, P. and Delen, N. (2004). *Botrytis* spp. and diseases they cause in agricultural systems an introduction. In: Elad, Y., Williamson, B., Tudzinski, P. and Delen, N. (Eds). *Botrytis*: Biology, Pathology and Control. Kluwer Academic Publishers, Dordrecht, Netherlands.

Elad, Y., Williamson, B., Tudzynski, P., and Delen, N. (2007). Botrytis: Biology, Pathology and Control. Springer, Dordrecht, Netherlands.

Fan, F., Li, N., Li, G.Q. and Luo, C.X. (2016). Occurrence of fungicide resistance in *Botrytis cinerea* from greenhouse tomato in Hubei Province, China. *Plant Disease*, 100, 2414-2421.

Food and Agriculture Organization (FAO). (2019). Food and Agriculture Organization of the United Nations. Retrieved from http://www.fao.org/faostat/en/#home. Date accessed: 25 October 2022.

Food and Agriculture Organization Corporate Statistical Database (FAOSTAT). (2019). Online statistical database of the Food and Agricultural Organisation of the United Nation: http://www.fao.org/faostat/en/. Date accessed: 30 October 2022.

Fekete, E., Fekete, E., Irinyi, L., Karaffa, L., Árnyasi, M. and Asadollahi, M. (2011). Genetic diversity of a *Botrytis cinerea* cryptic species complex in Hungary. *Microbiology Research*, 167, 283-91.

Garima, M., Pradeep, S., Ramesh, V., Sunil, K., Saurabh, S., Jha, K.K. and Khosa, R.L. (2011). Traditional uses, phytochemistry and pharmacological properties of *Moringa oleifera* plant: An overview*. Der Pharmacia Letter*, 3, 141-164.

Ge, B., Liu, B., Nwet, T.T., Zhao, W., Shi, L. and Zhang, K. (2016). *Bacillus methylotrophicus* strain NKG-1, isolated from Changbai Mountain, China, has potential applications as a biofertilizer or biocontrol agent. *PloS One*, 11, e0166079.

Grandillo, S., Zamir, D. and Tanksley, S.D. (1999). Genetic improvement of processing tomatoes: A 20-year perspective," *Euphytica*, 110, 85-97.

Hahn, M. (2014). The rising threat of fungicide resistance in plant pathogenic fungi: *Botrytis* as a case study. *Journal of Chemical Biology,* 7, 133-141.

Jarvis W.R. (1977). Botryotinia and *Botrytis* species taxonomy, physiology and pathogenicity. Monograph No. 15, Canadian Department of Agriculture, Ottawa, Canada.

Jarvis, W.R. (1997). Botrytinia and *Botrytis* species: Taxonomy, physiology and pathogenicity Monograph No. 15. Ottawa: *Canada Department of Agriculture*.

Jones, J.B., Zitter, T.A., Momol, T.M. and Miller, S.A. (2014). Compendium of tomato diseases and pests, 2nd edition; APS Press,St. Paul, MN, USA

Lee, J.P., Lee, S.W., Kim, C.S., Son, J.H., Song, J.H., Lee, K.Y. and Moon, B.J. (2006). Evaluation of formulations of *Bacillus licheniformis* for the biological control of tomato grey mould caused by *Botrytis cinerea*. *Biological Control*, 37, 329-337.

Leonardi, C., Ambrosino, P., Esposito, F. and Fogliano, V. (2000). Antioxidant activity and carotenoid and tomatine contents in different typologies of fresh consumption tomatoes. *Journal of Agricultural and Food Chemistry,* 48, 4723-4727.

Leroux, P. (2004). Chemical control of Botrytis and its resistance to chemical fungicides. *Botrytis*: Biology, Pathology and Control. In: Elad, Y. Williamson, B. Tudzynski P. and Delen N (eds). Kluwer Academic Publishers, London, UK.

Leroux, P. (1996). Recent developments in the mode of action of fungicides. *Pesticide Science*, 47, 191-197.

Martinez-Absalon, S., Rojas-Solis, D., Hernandez-Leon, R., Prieto-Barajas, C., Orozco-Mosqueda, M. D. C., Pena-Cabriales, J. J. and Santoyo, G. (2014). Potential use and mode of action of the new strain *Bacillus thuringiensis* UM96 for the biological control of the grey mould phytopathogen *Botrytis cinerea*. *Biocontrol Science and Technology*, 24, 1349-1362.

Mazzola, M. and Freilich, S. (2017). Prospects for biological soilborne disease control: Application of indigenous versus synthetic microbiomes. *Journal of Phytopathology*, 107, 256-263.

Menzies, J. and Jarvis, W. (1994). Grey Mould. Diseases and Pests of Vegetable Crops In: Howard, R., Garland, J. and Seaman, W. (Eds). The Canadian Phytopathological Society and Entomological Society, Ottawa, Canada.

Mnif, I. and Ghribi, D. (2015). Potential of bacterial derived biopesticides in pest management. *Crop Protection*, 77, 52-64.

Myresiotis, C.K., Karaoglanidis, G.S. and Tzavella-Klonari, K. (2007). Resistance of *Botrytis cinerea* isolates from vegetable crops to anilinopyrimidine, phenylpyrrole, hydroxyanilide, benzimidazole, and dicarboximide fungicides. *Plant Disease*, 91, 407-413.

National Department of Agriculture. (2009). Directorate: Agricultural Statistics Section. Pretoria, South Africa.

Nie, P., Li, X.; Wang, S., Guo, J., Zhao, H. and Niu, D. (2017). Induced systemic resistance against *Botrytis cinerea* by *Bacillus cereus* AR156 through a JA/ET- and NPR1-dependent signalling pathway and activates pamp-triggered immunity in Arabidopsis. *Frontiers in Plant Science*, 8, 238.

Nneka, V.C. (2006). Potentials of the leaf extracts of *Azadirachta indica* and *Ocamum gratissimum* L. for the control of some potato (*Solanum tuberosum* L.) fungal diseases. *Nigerian Journal of Botany*, 19, 68-73.

Patel, P., Patel, N., Patel, D., Desai, S. and Mushram, D. (2014). Phytochemical analysis and antifungal activity of *Moringa oleifera*. *International Journal of Pharmacy and Pharmaceutical Sciences*, 6, 144-147.

Price, M. L., (2000). The moringa tree purification and activity assay of the coagulant protein from *Moringa oleifera* seed. *Water Research*, 39, 2338-2344.

Redmond, J.C., Marois, J.J. and Macdonald, J.D. (1987). Biological control of *Botrytis cinerea* on roses with epiphytic microorganisms. *Plant Disease,* 71, 799-802.

Romanazzi, G., Smilanick, J.L., Feliziani, E. and Droby, S. (2016). Integrated management of postharvest grey mould on fruit crops. *Postharvest Biology and Technology*, 113, 69-76

Sansone, G., Rezza, I., Calvente, V., Benuzzi, D., Maria, I. and Sanz de, T. (2005). Control of *Botrytis cinerea* strains resistant to iprodione in apple with rhodotorulic acid and yeasts. *Postharvest Biology and Technology*, 35, 245-251.

Sharma, R.R., Singh, D. and Singh, R. (2009). Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: a review. *Biological Control*, 50, 205-221.

ten Have, A., van Berloo, R., Lindhout, P., van Kan, J.A.L. (2007). Partial stem and leaf resistance against the fungal pathogen *Botrytis cinerea* in wild relatives of tomato. *Europeean Journal of Plant Pathology*, 117, 153-166.

Tripathi, P. and Dubey, N.K. (2004). Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. *Postharvest Biology and Technology*, 32, 235-245.

Van Kan, J.A., Shaw, M.W. and Grant Downton, R.T. (2014). Botrytis species: Relentless necrotrophic thugs or endophytes gone rogue. *Molecular Plant Pathology*, 15, 957-961.

Walker, A.S. (2016). Diversity within and between species of *Botrytis*. In: Fillinger, S, Elad, Y, eds. *Botrytis* – the fungus, the pathogen and its Management in Agricultural Systems. Springer International Publishing, Cham, Switzerland.

Williamson, B., Tudzynski, B., Tudzynnski, P. and van Kan, J.A. (2007). *Botrytis cinerea*: the cause of grey mould disease. *Molecular Plant Pathology,* 8, 561-580.

Wills, R. B. H. (2007). Postharvest: an introduction to the physiology and handling of fruit, vegetables and ornamentals, 5th ed. New Zealand, Australia.

Chapter 2
Literature review

2.1 Introduction

Tomato (*Solanum lycopersicum* L.) is one of the most important fruits worldwide and belongs to the family Solanaceae (Knapp, 2002). Tomato has become one of the most widely cultivated and extensively consumed horticultural crops globally (Grandillo *et al*., 1999). The nutritional and economic importance of this crop has resulted in high global tomato production. Cool and dry climates are required for higher yield and good quality tomatoes (Nicola *et al*., 2009), although tomatoes are adapted to climatic conditions from temperate to hot humid tropical (Naika *et al*., 2005). High moisture content in the fruit causes the tomato to be perishable with shorter shelf life mostly under tropical conditions (Muhammad *et al*., 2011).

Texture is one of the key components of the perception of tomato fruit quality by consumers (Causse *et al*., 2003; Chaib *et al*., 2007; Sinesio et al., 2010). Tomato is an important cash crop comprising essential vitamins for both smallholders and medium-scale commercial farmers (ACIAR, 2005). Tomato fruit can be consumed in several ways such as an extract, cooked or uncooked (Alam and Goyal, 2007). This fruit is nutritious and beneficial to the human body as it contains lycopene and carotenoids with antioxidant properties (Arab and Steck, 2000). Beneficial vitamins and minerals in tomato include vitamin A and C, potassium, phosphorus, magnesium and calcium. The main antioxidants in tomato are carotenoids, ascorbic acid and phenolic compounds (Giovanelli *et al*., 1999). The primary antioxidant activity of tomatoes differs considerably according to the genetic variety, ripening stage and growing conditions (Leonardi *et al*., 2000).

Lycopene is an important and abundant compound in the ripened tomato and is highly concentrated on the skin of the fruit. Various epidemiological studies show a link between increased consumption of tomato-based products and lycopene with reduced prostate cancer risk (Suwanaruang, 2016; Urbonavičienė *et al*., 2018). Fruit colour can be affected by abiotic factors such as temperature, altitude, ripening season length, and UV-B radiation (Lu *et al*., 2019). The content of mineral nutrients present within the fruit is known to affect the ripening, storage behaviour and quality of tomatoes (Mignani *et al*., 1995; Zdravkovic *et al*., 2007; Paul *et al*., 2012).

Various pigments present on fruits can serve an active defence, for example anthocyanins can significantly provide photoprotection against severe solar radiation and also increase the absorbance of solar radiation when the temperature is low which improve metabolic and developmental processes (Willson and Whelan, 1990). Hence, environmental factors may also be responsible for the variation in fruit colour.

Tomato is mostly harvested at the orange and red stage and stored in the refrigerator (10 °C to 15 °C) for less than 14 days before consumption. Although tomatoes are not recommended to be stored in the refrigerator lower than 10 °C because of their sensitivity to chilling temperatures (Parnell *et al*., 2004), however, it is prevalent in practice. The quality of tomato is affected by postharvest handling, poor storage practices, transportation, and improper marketing. There have been reports of numerous pathogens that infect tomatoes across the globe. The most significant in terms of the economy are bacteria, viruses, nematodes, and fungi (Jones *et al*., 1991; Chupp and Sherf, 1960).

Among all factors which reduce food supply, postharvest diseases of fruits and vegetables are major causes of postharvest losses and reduce shelf life (Karabulut and Baykal, 2004; Liu *et al*., 2005;). Postharvest losses occur in the food supply chain due to pathogen infections, handling, storage, transportation, and processing, resulting in the reduction in quality, quantity and market value of agricultural commodities (Kader, 2005; Parfitt *et al*, 2010). Another concern about postharvest diseases is their effect on consumer prices and modes of transportation (Gatto *et al*., 2011). Great losses of fruits and vegetables have motivated researchers to execute studies on effective methods to control postharvest diseases and other losses in tomato production (Wilson and Wisniewski, 1989).

2.2 Origin and economic importance of tomatoes

Tomato crop originated in the Andes regions of South America, extending from central Ecuador across Peru to northern Chile (Denham, 2014), and it was then spread to other parts of the world. In 2016, United States of America (USA), China, India and Turkey were the countries with the largest tomato production area (FAO, 2016). The crop is ranked as the second most important crop following potatoes in global production (Tan *et al*., 2010). Tomato contains tomatine, an alkaloid with fungicidal

properties, which its concentration decreases as the fruit matures. In 2014, about 5 million hectares of the global area were cultivated with tomatoes with a production of 171 million tonnes and the major producing countries were China and India (FAOSTAT, 2017). Prices of fresh tomatoes have more variation than processed tomatoes and prices also depend on the shipping-point price. Production losses differ from a Developed Country (DC) to a Less Developed Country (LDC). In DCs, production losses can reach 23% and are concentrated on the treatment of the product by the consumers (Kader, 2005). The most significant losses in LDCs are up to 50% and are concentrated in the treatment of the product by the producers (Hodges, 2010).

2.3 Production of tomato in South Africa and production constraints

Tomato is one of the highest ranked vegetables grown worldwide and it contributes more than 15% of the global vegetable production (Rothan *et al*., 2019). In the Southern African Development Community region, South Africa is the dominant tomato producer growing 54% of tomatoes on 11% of the total cropped area. In Limpopo province, cultivating tomatoes is an important economic activity. Economic sustainability of the tomato industry locally and nationally is essential to maintain the economic and social stability of employees and their dependents. However, tomatoes are susceptible to various fruit decays occurring in the field and during postharvest handling. Consequently, the production of tomato is affected by diseases which are limiting factors for an economic increase and mostly fungal diseases which are found to be severe on crops.

Tomato crop production can be affected by more than 200 diseases caused by numerous pathogens both in the field and postharvest processing (Singh *et al*., 2017). These diseases can be observed with different symptoms, and different management strategies have been developed. *Botrytis cinerea* Pers. is one of the common postharvest fungal pathogens affecting the quality and quantity of tomatoes. *B. cinerea* is known as second most significant fungal pathogen causing both preharvest and postharvest infections after *Penicillium expansum.* (Dean *et al*., 2012; Fillinger and Elad, 2016). It is an opportunistic pathogen infecting many fruits and vegetables by causing infection through the wounded part of the crop (Pollastro *et al*., 1996). The susceptibility of tomato to necrotrophic fungal infection increases during transition from unripe to ripe fruit resulting in great losses during postharvest. Most losses of tomato occur after the harvest of healthy fruits and during transportation to markets.

2.4 Grey mould

Grey mould is caused by *B. cinerea* which is the fungal pathogen belonging to the group hyphomycetes and consists of about 28 species of important pathogens (Dewey and Grant-Downton, 2016). *B. cinerea* is identified as the second most important plant pathogen of fruits and vegetables because of its economic importance (Dean *et al*., 2012). This fungus has a wide host range resulting in severe pre- and postharvest losses globally. *B. cinerea* as a saprophyte may survive on plant debris in the soil for a longer period.

2.4.1 Taxonomy and morphology

B. cinerea consists of macroconidia and microconidia where microconidia can function as a primary inoculum (Shirane *et al*., 1988). It is a phytopathogenic fungus that has haploid and heteroallelic ascomycete (Munoz *et al*., 2010) with high genetic variability. *B. cinerea* grows as white to grey mycelia on potato dextrose agar (Figure 2.1A). Mycelium consists of conidiophores and can penetrate intercellular spaces of the host tissues. Conidiophores are spore-producing structures (Figure 2.1B) and conidia are regarded as the main produced and dispersed inoculum (Holz *et al*., 2004). Conidia is unicellular, sharp at one end, brown coloured with a size between 9.0 - 12.0 x 6.5 - 10.0 µm. The pathogen can reside in the soil as mycelium and on the diseased material as the sclerotia.

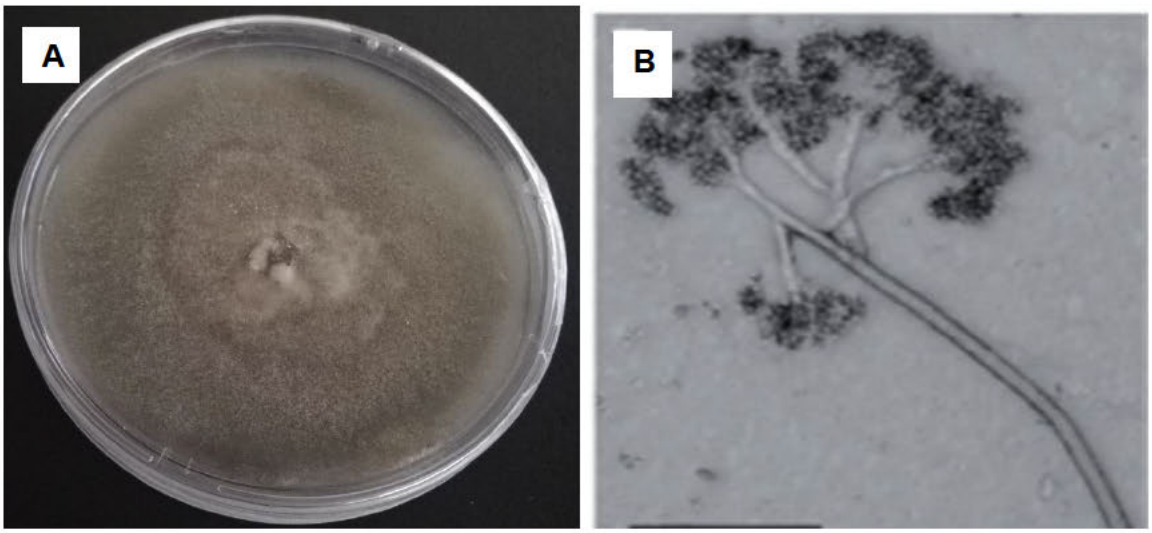


Figure 2.1: Mycelial growth of *Botrytis cinerea* on potato dextrose agar (A) and conidiophores with conidia (B) (Holz *et al*., 2004).

2.4.2 Distribution and host range

B. cinerea is widely distributed and can infect more than 200 crop species including tomato plants (Elad *et al*., 2004). Characteristics of *Botrytis* species such as sclerotia and conidium size are essential to determine certain species since some species are morphologically similar and growing conditions significantly influence their variation (Beever and Weeds, 2004). *Botrytis* diseases are probably the most common and widely distributed diseases of vegetables, ornamentals, fruit and even field crops throughout the world (Agrios, 1997). These species are described to cause infection to about 596 genera of vascular plants consisting of more than 1400 plant species (Elad *et al*., 2016). Among *Botrytis* species, *B. cinere*a is reported to infect more than 230 host species (Choquer *et al*., 2007; Elad and Stewart, 2007; Walker *et al*., 2015) which belong to over 170 plants families of agriculture and agri-food (Elad *et al*., 2016).

2.4.3 Economic importance

Fungal pathogens result in large economic losses of fruits and vegetables which occur during pre- and postharvest (Dean *et al*., 2012; Nabi *et al*., 2017). Great losses are evident after the harvest of fruits and vegetables during storage and transportation to the market place. The economic importance of *B. cinerea* is more intense in areas with cool and humid weather (Pande *et al*., 2006).

2.4.4 Disease cycle and epidemiology

B. cinerea reproduces asexually by releasing conidia which can be dispersed by wind or water and reproduce sexually by releasing sclerotia which is essential for survival during unfavourable conditions (Brandhoff *et al*., 2017). The fungus can survive as conidia for a shorter period and as sclerotia in crop debris and senescent leaves of the plant for a longer period (Leroch *et al*., 2011). The pathogen invades the host in the autumn and during wet summer periods. Development of grey mould is mostly conducive under optimal temperatures between 15-25°C (Janisiewicz and Korsten, 2002). However, the optimal temperature of the infection process in the field is a minimum of 12°C (Domsch *et al*., 1980). The spread of disease can increase through frequent handling of fruits.

The infection of *B. cinerea* on the crop begins when the pathogen enters the host through stomata and other host openings. Conidia germinate and produce germ tubes which penetrate the host during the infection process to kill host tissues resulting in

lesion formation and sporulation (Van Kan, 2006). The spore produces an extracellular matrix used for attachment through hydrophobic interactions and several extracellular enzymes are released to facilitate penetration (Doss, 1999). *B. cinerea* produces degrading enzymes during entry and interaction with the host causing oxidative burst (Fourie and Holz, 1995; Tenberge, 2004). These enzymes include cutinases, pectinases and proteases utilised by the pathogen to penetrate host tissues through host natural openings and wounds. Infection can occur on the belowgroundare parts such as bulbs, corms, tubers, and roots and they may rot while they still in the soil or at harvest (Agrios, 2006) (Figure 2.2). After infection, the fungus can repeat its life cycle through the sporulation of the infected tissues by producing conidiophores and conidia (Holz *et al*. 2004).

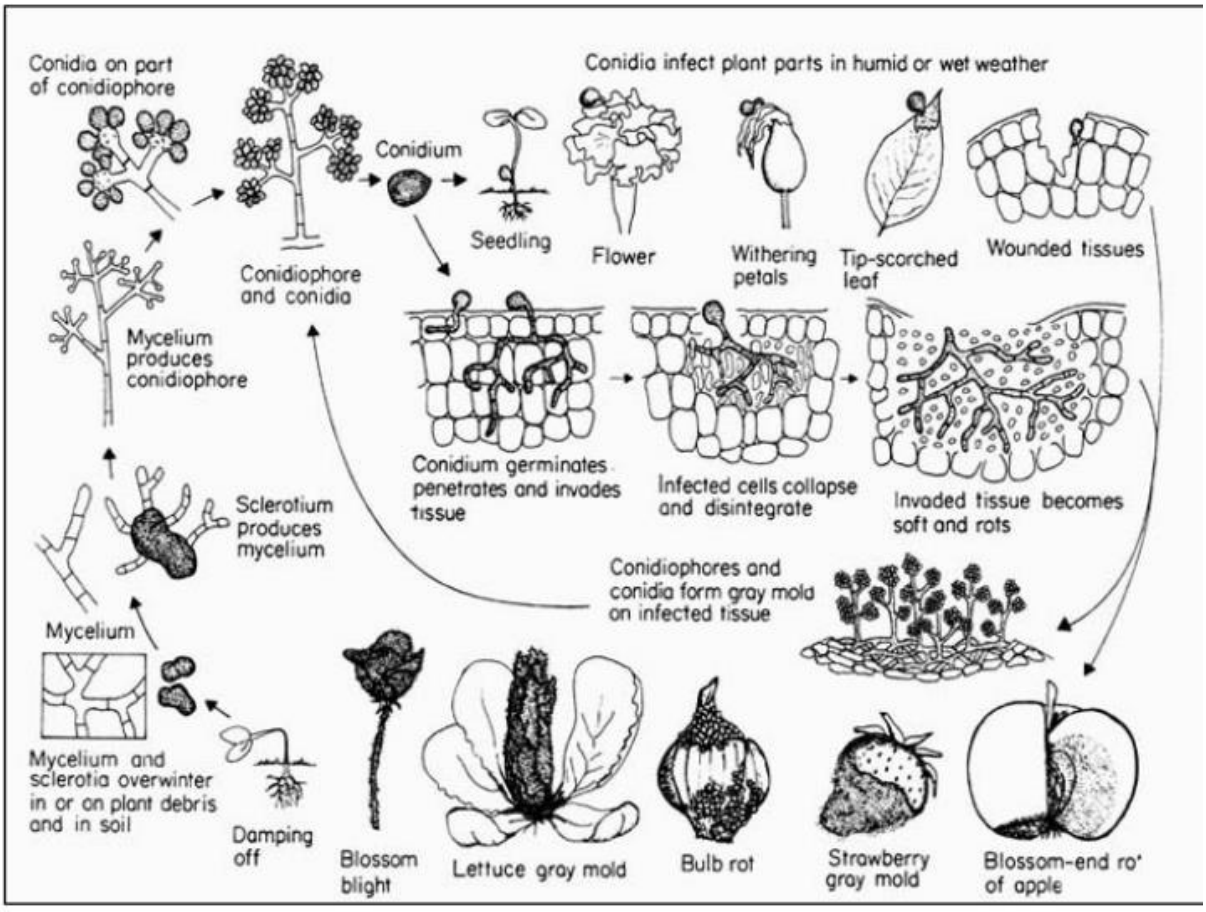


Figure 2.2: Life cycle of *Botrytis cinerea* on fruits and vegetables (Agrios, 2006).

2.4.5 Symptoms

Fungal symptoms of *B. cinerea* may vary depending on the host and plant part attacked although symptoms can be observed in all plant parts (ten Have *et al*., 2002). The fungus causes spots, seedling damping off, blossom blight or fruit rot (ten Have *et al*., 2002). Grey mould causes water-soaked spots and can result in the collapsing of infected plant tissues (ten Have *et al*., 2002). During postharvest, the infection begins on the wounds caused by the bruising or puncturing of the fruits created during harvest and handling (Xiao, 2006). Grey mould is identified as a light grey fuzzy growth that appears on stems and leaves. The fungus shows grey-brown furry mould which produces masses of grey mould spores that covers the infected area. These spores spread from the infected part of the plants (Butler, 2015) (Figure 2.3A). Leaves of the crop exhibit irregular V-shaped areas of dead tissues which spread towards the main veins and yellowing on leaf margins (Cattlin, 2007) (Figure 2.3B). Fruits show white faint, pale halos (Butler, 2015) (Figure 2.3C) which become yellow during the ripening of the fruit and small necrotic fleck may appear along with the halo on the fruit (Cattlin, 2008) (Figure 2.3D).

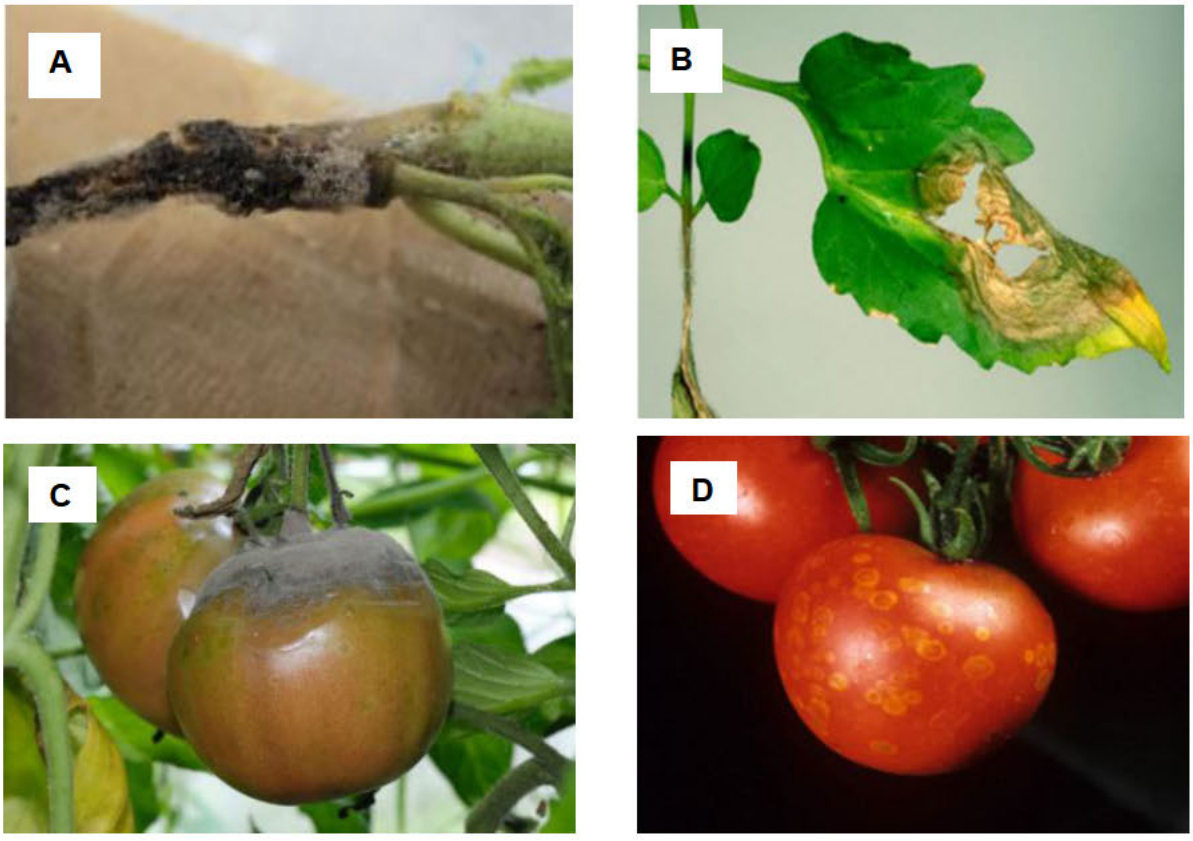


Figure 2.3: Symptoms of the grey mould of tomato showing brownish grey mould with masses of spores and girdling on the stem (Butler, 2015) (A), tomato leaf with dead

tissues and yellowing (Cattlin, 2007) (B), water-soaked rotten tomato producing spores (Butler, 2015) (C) and matured tomato fruit with ghost spots (Cattlin, 2008) (D).

2.5 Management strategies

The control of *B. cinerea* can be affected by modes of attack of the pathogen, diverse hosts and inoculum sources resulting in difficulty in managing the pathogen (Leroch *et al*., 2011). *B. cinerea* remains catastrophic to tomatoes in the greenhouse and challenging to control since greenhouse environmental conditions may favour the development of fungal diseases.

2.5.1 Fungicides

Chemical control is a currently used and more effective way compared to other methods of managing grey mould (Mertely *et al*., 2000). The control of *B. cinerea* is highly dependent upon fungicides such as benzimidazole and dicarboximide, e.g., iprodione. However, the intensive use of fungicides results in the resistance of some *B. cinerea* strains against dicarboximide (Sansone *et al*., 2005). There are numerous fungicides which use different modes of action to control *B. cinerea* on crops. Mixing fungicides of different modes of action is recommended to reduce the development of fungicide resistance and combining both protectant and systemic chemicals to reduce resistant fungal populations (Roy *et al*., 2010). The most effective fungicide that is currently used against *B. cinerea* is Fenhexamid which is a sterol biosynthesis-inhibiting fungicide (Rosslenbroich and Stuebler, 2000).

Various synthetic fungicides become ineffective in managing the pathogen due to the development of resistant *B. cinerea* isolates (Leroux, 2004). However, inappropriate application of fungicides and genetic variability of *B. cinerea* strains resistant to fungicides have become a threat to producers. Since 1998, China has been using pyrimethanil to control grey mould on fruits and vegetables (Ji *et al*., 2002). Pyrimethanil is believed to have the properties of inhibiting biosynthesis and secretion of chemicals during *B. cinerea* infection process (Latorre *et al*., 2002). Many countries, including South Africa, are reported to have *B. cinerea* strains resistant to pyrimethanil (Ji *et al*., 2002; Latorre *et al*., 2002; Ji *et al*., 2003).

2.5.2 Cultural practices

Cultural control is used to reduce favourable environmental conditions for the development of the disease. This method can alter environmental conditions to reduce grey mould on fruits and vegetables (Legard *et al*., 2000). Increasing planting space allows more airflow which reduces the incidence of grey mould although this may reduce the total yield per acre (Legard *et al*., 2000). Sanitation reduces the amount of initial inoculum of *B. cinerea* in the field by removing decayed plant material (Mertely *et al*., 2000). Pruning the canopy allows adequate air movement and a good light interception which reduces leaf wetness. Leaf wetness is required for germination of sporangia, formation of primary infectious structures, and the process of penetration (Cohen 1977; Arauz et al., 2010).

2.5.3 Biological control

Currently, a potential alternative for fruit protection against phytopathogens at the postharvest stage is provided by biocontrol control technologies, which protect plants against fungal diseases (Ghazanfar *et al*., 2016). Biological control of plant diseases involves the suppression of populations of plant pathogens by living organisms (Heimpel and Mills, 2017). Biocontrol agents are safe, natural and a currently effective alternative to chemical fungicides against grey mould. Biofungicide is the general name for microorganisms (microbial pesticides) and natural compounds which can control plant diseases (biochemical pesticides) (Roger and Keinath, 2010). Understanding the mechanisms and mode of action used by biocontrol agents under field conditions is important for the successful application and improvement of biocontrol organisms (Droby and Chalutz 1994; Spadaro and Droby 2016; Wisniewski *et al*. 2007).

Mechanisms of biocontrol include killer toxins (Walker *et al*., 1995), predation (Lachance and Pang, 1997), competition for nutrients (Filonow, 1998), secretion of cell wall degrading enzymes (Masih *et al*., 2001) and production of syringotoxins and syringomycins (Woo *et al*., 2002). Microbial pesticides consist of the majority of commercial *B. cinerea* control products with active ingredients ranging from fungi, yeasts, bacteria and actinomycetes. Biofungicides are known to be good alternatives to chemical fungicides for the control of *B. cinerea*. Biofungicides significantly reduce

fungicide residues if any are present and microbial pesticide residues are less harmful to living organisms and the environment even when they are applied before harvest (Koul, 2011).

2.5.3.1 Yeasts as a biocontrol agent

Several yeast species have already been shown to be effective biological control agents in protecting plants against fungal diseases (Droby *et al*., 1989; McGuire, 1994; Wisniekwski *et al*., 1991). Antagonistic yeasts such as *Pichia anomala* NCYC 435, *S. cerevisiae* 28, and *P. membranifaciens* 333 are inhibitory to some wood-decay and plant pathogenic fungi including *B. cinerea*, *Rhizoctonia solani* Kühn., *Ophiostoma ulmi* (Buisman) Nannf. (Walker *et al*., 1995). Several studies have indicated the efficient antagonistic activity of yeast against *B. cinerea* (Saligkarias *et al*., 2002; Santos *et al*., 2004; Elmer and Reglinski, 2006; Dal Bello *et al*., 2008). Yeast can colonise fungal hyphae and affect the germination of the pathogen conidia (Janisiewicz and Korsten, 2002). And natural epiphytic yeasts have shown auspicious biocontrol activity against numerous postharvest pathogens such as *B. cinerea* (McLaughlin *et al*., 1992; McGuire, 1994; Lurie *et al*., 1995; Arras, 1996; Cheah *et al*., 1996; Lima *et al*., 1997; Schena *et al*., 1999), although there is less focus on the biological control of the preharvest disease.

Antagonistic yeast competes for nutrients and space with other organisms such as plant pathogens (Sparado and Dropy, 2016); (Muccilli, and Restuccia, 2015). Competition for nutrients causes the inhibition of fungal spore germination of the fungal pathogens. For example, *Aureobasidium pullulans* inhibited the growth of *P. expansum* inhibited (Benchegroun et al., 2007) and *Monilinia laxa* (Di Francesco *et al*., 2017). A study conducted have shown that PM4 strain of the yeast *Rhodotorula glutinis* was effective in inhibiting different isolates of *B. cinerea* on tomato (Buck and Jeffers, 2004). Antagonistic yeasts can also be potential alternative to synthetic chemical fungicides because of the present antimicrobial properties (Dukare *et al*., 2019; Zhang *et al*., 2020).

2.5.3.2 Bacteria as a biocontrol agent

Biological control of *B. cinerea* is thoroughly investigated in economically important crops such as tomatoes, strawberries and grapes over 30 years (Sylla *et al*., 2015; Haidar *et al*., 2016; Marín *et al*., 2016; Nicot *et al*., 2016; Passera *et al*., 2017). *Bacillus*

spp. is known to be effective in controlling several plant diseases because they produce numerous broad-spectrum antibiotics and their ability to extend shelf life because of their endospore forming ability (Emmert and Handelsman, 1999). *Bacillus* have shown the greatest potential for *Botrytis* disease control (Elad and Stewart, 2007), using several mechanisms such as secretion of lytic enzymes, siderophores and antibiosis, competition for space and nutrient, defence plant stimulating and a combination of mechanisms (Akhtar and Siddiqui, 2010).

Various commercial products produced from *Bacillus* spp. were tested to confirm their properties and abilities to control grey mould. These products include Kodiak HB from *B. subtilis* GB03 (Mahaffee and Backman, 1993) and Serenade from *B. subtilis* QST-713 (Marrone, 2002; Percival *et al*., 2016). *B. subtilis* S1-0210 was tested on strawberries and was found to reduce grey mould infection by 85 % in both *in vitro* and field studies (Hang *et al*., 2005). France currently has six biocontrol products registered for viticulture where three products are based on the microbial antagonists *Bacillus subtilis Cohn, Aureobasidium pullulans*, and *Bacillus - amyloliquefaciens* (Serenade Max®, Botector®) and the other three based on Potassium bicarbonate, essential oils (eugenol, thymol, and geraniol) and gibberellic acid (Armicarb®, Mevalone®, and Berelex 40 SG®) (Index-Acta-phytosanitaire, 2017).

Bacillus species such as *B. cereus* can induce resistance of the host against *B. cinerea* (Nie *et al*., 2017). *Bacillus* species are known as plant growth-promoting bacteria in a wide range of plants (Kloepper *et al*; 2002; Kokalis-Burelle *et al*., 2004; Bai *et al*., 2003; Deepa *et al*., 2010) and it is one of the principal plant-growth promoting rhizobacteria (PGPR) group that control several pathogenic fungi when applied as a biocontrol. Despite the large number of scientific papers published on the control of *B. cinerea* using biocontrol agents, the number of efficient bacteria commercialized to be used as microbial fungicides against *B. cinerea* during pre- and postharvest remains limited (Nicot *et al*., 2011; Romanazzi *et al*., 2016) (Table 2.1).

Table 2.1 : Commercially available biopesticides based on the bacteria for control of grey mould on different crops.

®Trade name	Bacterial strain	Company (and/or country)
Pantovital®	*Pantoea agglomerans*	IRTA (Spain)
Serenade Max®	*B. subtilis*	Bayer, formerly BASF (Germany)
Bio-save®	*Pseudomonas syringae*	Jet harvest solutions (USA)
Amylo-X®	*B. amyloliquefaciens*	Biogard CBC (Italy)
DoubleNickel 55WDG/LC™	*B. amyloliquefaciens*	Certis (USA)
Companion®	*B. subtilis* GB03	Growth products (USA)
Botokira Wettable Powder®	*B. subtilis* IK-1080	(Idemitsu Kosan Inc., Japan)
Bio Arc®	*B. megaterium6*	Sphere Bio-Arc PVTLtd (India)
Mycostop®	*Streptomyces griseoviridis* strain K61	Verdera Oy (Finland)
Actinovate®	*S. lydicus* WYEC 108	Novozymes (Denmark)

2.5.4 Plant extracts

The problem of pathogen resistance against antibiotics has resulted in more research on natural substances extracted from plants, as they possess potential antibacterial and antifungal activities (Maiyo et al., 2010; Erfan and Marouf, 2019). Plant extracts of medicinal plants contain important biodegradable compounds which are effective against fungal and bacterial pathogens and can be used for integrated pest management programs as they contain bioactive chemicals such as flavonoids, phenols, tannins, alkaloids, quinones, saponins and sterols (Burt, 2004). Currently, researchers are interested in the development of safe antifungal agents produced from natural plant products to control fungal plant diseases

Moringa oleifera Lam. is a genus of the fast-growing tropical deciduous plant in the family Moringaceae is known to have tuberous roots, light green leaves and abundant flowering with elongated, pendulous fruits and seeds (Asensi *et al*., 2017). Moringa

plant is considered a miracle plant since all plant parts are used for medical purposes (Price, 2000; Anwar *et al*., 2007; Garima *et al.*, 2011). *M. oleifera* produces compounds such as zeatin, quercetin, b-sitosterol, caffeoylquinic acid and kaempferol which have antifungal and antibacterial activities (Anjorin *et al*., 2010). *M. oleifera* plant consists of phytochemicals including alkaloids and flavonoids which have antioxidant activity that inhibits the growth of pathogenic fungi and bacteria (Sreelatha and Padma, 2009). Antimicrobial activity of moringa leaf extracts involves bounding of lipophilic compounds to cytoplasmic membrane which reduces growth of the pathogen (Huang *et al*., 2000). Furthermore, bark juice of the tree is reported to show high antibacterial activity against pathogenic organisms especially *Staphylococcus aureus* and leaf juice is reported to inhibit the growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Mehta *et al.*, 2003). Fungicidal effect of moringa extract is reported on some soil-borne fungi including *Rhizoctonia*, *Pythium* and *Fusarium* (Moyo *et al*., 2012). A study by Dwivedi and Enespa (2012) has shown that *M. oleifera* extracts (leaves, bark and seeds) 75 % (v/v) inhibited mycelial growth of *Fusarium solani f. sp.* radicicola and *Fusarium oxysporum* f. sp. lycopersici.

2.5.5 Integrated control

There is currently limited research on the integrated control of *B. cinerea* using *M. oleifera* plant extracts and biocontrol agents. Moringa leaf extract has been combined with *Trichoderma* as an integrated biocontrol agent against *Sclerotium* damping-off and stem rot disease of Vigna unguiculata L. in the field condition (Adandonon *et al*., 2006). In addition to reducing disease severity and disease incidence, plant extracts can increase shoot and root mass (Obongoya *et al*., 2010). Screening plant extracts and plant products for antimicrobial activity in plants is reported by Afolayan (2003) to contain potential sources of novel antibiotic prototypes. A study reported by Hassanein *et al*. (2010) had shown that neem (*Azadirachta indica* A Juss.) extracts had high efficacy of antifungal activities as compared to other plant extracts because of the variation in chemical compounds present in plants. This phenomenon also applies to biocontrol agents and reports show that their effectiveness differs based on the nature, quality and quantity of antibiotics or inhibitory substances secreted (Vidyasagar and Tabassum, 2013). Latha *et al*. (2009) reported that biocontrol agents, *Trichoderma viride* Pers. and *Pseudomonas fluorescens*, showed high compatibility with zimmu leaf

plant extract. The combination of biocontrol agents and zimmu leaf extract effectively reduced the disease incidence of *Alternaria* leaf spot of tomato (Latha *et al*., 2009).

2.6 Conclusion

Grey mould is one of the most important postharvest affecting yield and quality of tomato fruits. The problem of fungicidal resistance and residues on tomato fruits results in an urgent need for economically and environmentally friendly alternative control measures. These measures can be achieved using biological control agents against *B. cinerea* and integration of biocontrol agents with plant extracts to reduce *B. cinerea* on tomato fruits.

2.5 References

ACIAR, (2005). Partners in research for development magazine. Summer 2005/6. Online publication: http://aciar.gov.Au/publication/pmg023. Date accessed:15 September 2021.

Adandonon, A, Aveling, T.A.S., Labuschagne, N. and Tamo, M. (2006). Biocontrol agents in combination with *Moringa oleifera* extract for integrated control of *Sclerotium* caused cowpea damping-off and stem rot. *European Journal of Plant Pathology*, *115*, 409-418.

Afolayan, A.J. (2003). Extracts from the shoots of *Arctotis arctoides* inhibit the growth of bacteria and fungi. *Pharmaceutical Biology*, 41, 22-25.

Agrios, G.N. (1997). Plant Pathology, 4th edition. Academic Press, New York, USA.

Agrios, G.N.(2006). Plant Pathology, sixth Edition. Elsevier Academic Press, Burlington, Ma, USA.

Akhtar, M.S. and Siddiqui, Z.A. (2010). Role of lant Growth Promoting *Rhizobacteria* in Biocontrol of Plant Diseases and Sustainable Agriculture. In: D.K. Maheshwari (Ed). In Plant Growth and Health Promoting Bacteria. Springer, Berlin, Heidelberg.

Alam, T. and Goyal, G.K. (2007). Packaging and storage of tomato puree and paste. *Stewart Postharvest Review*, 3, 1-8.

Anjorin, T.S., Ikokoh, P. and Okolo, S. (2010). Mineral composition of *Moringa oleifera* leaves, pods and seeds from two regions in Abuja, Nigeria. International *Journal of Agricultural Biology*, 12, 431-434.

Anwar, F., Latif, S., Ashraf, M. and Gilani, A.H. (2007). *Moringa oleifera*: A food plant with multiple medicinal uses. *Phytotherapy Research*, 25, 17-25.

Arab, L. and Steck, S. (2000). Lycopene and cardiovascular disease. *The American Journal of Clinical Nutrition*, 71, 1691S-1695S.

Arauz, L. F., Neufeld, K. N., Lloyd, A. L., and Ojiambo, P. S. (2010). Quantitative models for germination and infection of *Pseudoperonospora cubensisin* response to temperature and duration of leaf wetness. *Phytopathology*.100, 959-967

Arras, G. (1996). Mode of action of an isolate of *Candida famata* in biological control of *Penicillium digitatum* in orange fruits. *Postharvest Biology and Technology*, 8, 191-198.

Asensi, G.D., Villadiego, A.M.D. and Berruezo, G.R. (2017). *Moringa oleifera*: Review on applications and uses in food. *Archives Latin De Nutrition*, 67, 86-97.

Bai, Y., Zhou, X. and Smith. D.L. (2003). Enhanced soybean plant growth resulting from co-inoculation of *Bacillus* strains with *Bradyrhizobium japonicum*. *Crop Science*, 43, 1774-1781.

Beever, R.E. and Weeds, P.L. (2004). Taxonomy and genetic variation of *Botrytis* and Botryotinia. *Botrytis*, Biology, Pathology and Controls. Kluwer Academic Publisher. Netherland, 29-52.

Brandhoff, B., Simon, A., Dornieden, A., Schumacher, J. (2017). Regulation of conidiation in *Botrytis cinerea* involves the light-responsive transcriptional regulators bcltf3 and bcreg1. *Current Genetics*, 63, 931-949.

Buck, J. W. and Jeffers, S. N. (2004). Effect of pathogen aggressiveness and vinclozolin on efficacy of *Rhodotorula glutinis* PM4 against *Botrytis cinerea* on geranium leaf disks and seedlings. *Plant Disease*. *88*, 1262-1268.

Burt, S. (2004). Essential oils: their antibacterial properties and potential applications in foods are view. *International Journal of Food Microbiology*, 94, 223-253.

Butler, S. (2015). *Botrytis* infection on tomato fruit. North Carolina State University PDIC.https://content.ces.ncsu.edu/botrytis-gray-mold-of-tomato#section_heading_5275. Date accessed: 06 March 2018

Cattlin, N. (2007). Grey mould *Botrytis cinerea* necrosis and mycelium on late season tomato leaf. https://www.alamy.com/stock-photo-grey-mould-botrytis-cinerea-necrosis-and-mycelium-on-late-season-tomato-13997044.html. Date accessed: 07 September 2021.

Cattlin, N. (2008). Ghost spot *Botrytis cinerea* spotting on ripe red tomatoes, Alamy Stock Photo. https://www.alamy.com/stock-photo-ghost-spot-botrytis-cinerea-spotting-on-ripe-red-tomatoes-16250461.html. Date accessed: 25 August 2021.

Causse, M., Buret, M., Robini, K. and Verschave, P. (2003). Inheritance of nutritional and sensory quality traits in fresh market tomato and relation to consumer preferences. *Journal of Food Science*, 68, 2342-2350.

Chaib, J., Devaux, M.F., Grotte, M.G., Robini, K., Causse, M., Lahaye, M. and Marty, I. (2007). Physiological relationships among physical, sensory, and morphological attributes of texture in tomato fruits. *Journal of Experimental Botany*, 58, 1915-1925.

Cheah, L.H., Wilson, C.L. and Marshall, A.P. (1996). Rapid screening for antagonists against *Botrytis* storage rots using leaf and fruit tissue. *Postharvest Biology and Technology*, 8, 223-228.

Choquer, M., Fournier, E., Kunz, C., Levis, C., Pradier, J., Simon, A., and Viaud, M. (2007). *Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphageous pathogen. *FEMS Microbiology Letters*, 277, 1-10.

Chupp, C. and Sherf, A. F. (1960) Vegetable Diseases and Their Control, Ronald Press Company, New York, USA.

Cohen, Y. (1977). The combined effects of temperature, leaf wetness, and inoculum concentration on infection of cucumbers with *Pseudoperonospora cubensis*. *Canadian Journal of Botany*. 55,1478-1487.

Dal Bello, G., Mónaco, C., Rollan, C., Lampugnani, G., Arteta, N. and Abramoff, C. (2008). Biocontrol of postharvest grey mould on tomato by yeasts. *Journal of Phytopathology*, 156, 5257-5263.

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

Deepa, C.K., Dastager, S.G. and Pandey. A. (2010). Isolation and characterization of plant growth promoting bacteria from non-rhizospheric soil and their effect on cowpea (*Vigna unguiculata* (L.) Walp.) seedling growth. *World Journal of Microbiological Biotechnology*, 26, 1233-1240.

Denham, T. (2014). Tomatoes: Origins and development. In: Smith, C. (Ed.), Encyclopedia of global archaeology. Springer, New York, USA.

Dewey, F.M. and Grant-Downton, R. (2016). *Botrytis*-biology, detection and quantification. In: Fillinger, S., Elad, Y., (Eds.) *The Fungus, the Pathogen and its Management in Agricultural Systems*. Springer International Publishing, Cham, Switzerland.

Di Francesco, A.; Ugolini, L.; D'Aquino, S.; Pagnotta, E.; Mari, M. (2017). Biocontrol of *Monilinia laxa* by *Aureobasidium pullulans* strains: Insights on competition for nutrients and space. *International Journal of Food Microbiology*. 248, 32-38.

Domsch, K.H., Gams, W. and Anderson, T. (1980). Compendium of Soil Fungi. Academic Press, London, UK.

Doss, R.P. (1999). Composition and enzymatic activity of the extracellular matrix secreted by germlings of *Botrytis cinerea*. *Applied and Environmental Microbiology*, 65, 404-408.

Droby, S. and Chalutz, E. (1994). Mode of action of biocontrol agents of postharvest diseases. In: Wilson C.L., Wisniewski M.E. (Eds). Biological control of postharvest diseases: Theory and Practice. CRC Press, Boca Raton, USA.

Droby, S., Chalutz, E., Wilson, C.L., Wisniewoski, M.E. (1989). Characterization of the biological control activity of *Debaryomyces hansenii* in the control of *Penicillium digitatum* on grapefruit. *Canadian Journal of Microbiology*, 35, 794-800.

Dukare, A. S., Paul, S., Nambi, V. E., Gupta, R. K., Singh, R., Sharma, K. and Vishwakarma, R. K. (2019). Exploitation of microbial antagonists for the control of

postharvest diseases of fruits: a review. *Critical Reviews in Food Science and Nutrition*. *59*, 1498-1513.

Dwivedi, S.K. and Enespa, A. (2012). Effectiveness of extract of some medical plants against soil borne fusaria causing diseases on *Lycopersicon esculantum* and *Solanum melongena* plants. *International Journal of Pharmacy and Biological Sciences*, 3, 171-1180.

Elad, Y. and Stewart, A. (2007). Microbial control of Botrytis spp. In Elad, Y., Williamsom, B. and Tudzynski, P. (Eds.). *Botrytis*: *Biology, Pathology and Control*. Springer, Dordrecht, Netherlands

Elad, Y., Pertot, I., Prado, A. M. C., and Stewart, A. (2016). Plant hosts of *Botrytis* spp. In: Fillinger, S.; Elad, Y (Eds). The Fungus, The Pathogen and its Management in Agricultural Systems. Springer, Cham, Switzerland.

Elad, Y., Williamson, B., Tudzinski, P. and Delen, N. (2004). *Botrytis* spp. and diseases they cause in agricultural systems an introduction. In: Elad, Y., Williamson, B., Tudzinski, P. and Delen, N. (Eds). *Botrytis*: Biology, Pathology and Control. Kluwer Academic Publishers, Dordrecht, Netherlands.

Elmer, P.A.G. and T. Reglinski. (2006). Biosuppression of *Botrytis cinerea* in grapes. *Plant Pathology,* 55, 155-177.

Emmert, E.A. and Handelsman, J. (1999). Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiology Letters*, 171, 1-9.

Erfan, A.M., Marouf, S. (2019). Cinnamon oil downregulates virulence genes of poultry respiratory bacterial agents and revealed significant bacterial inhibition: An *in vitro* perspective. *Veterinary World.*12, 1707–1715.

Fillinger S. and Elad Y. (2016). *Botrytis*-The Fungus, the Pathogen and its Management in Agricultural Systems. Springer International Publishing, Cham, Switzerland.

Filonow, A.B. (1998). Role of competition for sugars by yeasts in biocontrol of grey mould of apple. *Biocontrol Science Technology*. 8, 243-256.

Food and Agriculture Organization Corporate Statistical Database. (2016). Food and Agriculture Organisation of the United Nations. Rome -Italy. http://faostat.fao.org/default.aspx. Date accessed: 15 February 2022.

Food and Agriculture Organization Corporate Statistical Database. (2017). Production – Crops – Area harvested / Production quantity – Tomatoes – 2014. Food and Agriculture Organization. Available online at: www.fao.org/faostat/en (accessed 19 September 2020).

Fourie, J.F. and Holz, G. (1995). Initial infection processes by *Botrytis cinerea* on nectarine and plum fruit and the development of decay. *Journal of Phytopathology*, 85, 82-87.

Garima, M., Pradeep, S., Ramesh, V., Sunil, K., Saurabh, S., Jha, K.K. and Khosa, R.L. (2011). Traditional uses, phytochemistry and pharmacological properties of *Moringa oleifera* plant. *Der Pharmacia Lettre,* 3, 141-164.

Gatto, M.A., Ippolito, A., Linsalata, V., Cascarano, N.A., Nigro, F., Vanadia, S. and Di Venere, D. (2011). Activity of extracts from wild edible herbs against postharvest fungal diseases of fruit and vegetables. *Postharvest Biology and Technology*, 61, 72-82.

Ghazanfar, M.U., Hussan, M., Hamid, M.I.and Ansari, S.U. (2016). Utilization of biological control agents for the management of postharvest pathogens of tomato. *Pakistan Journal of Botany*. 48, 2093-2100.

Giovanelli, G., V. Lavelli, C. Peri and S. Nobili. (1999). Variation in antioxidant components of tomato during vine and post-harvest ripening. *Journal of the Science of Food and Agriculture*,79, 1583-1588.

Grandillo, S., Zamir, D. and Tanksley, S.D. (1999). Genetic improvement of processing tomatoes: a 20years perspective. *Euphytica*, 110, 85-97.

Haidar R, Fermaud M, Calvo-Garrido C. (2016). Modes of action for biological control of *Botrytis cinerea* by antagonistic bacteria. *Phytopathologia Mediterranea* 55, 13-34.

Hang, N. T.T., Oh, S.O., Kim, G. H., Hur, J.S. and Koh, Y.J. (2005). *Bacillus subtilis* S1-0210 as a biocontrol agent against *Botrytis cinerea* in strawberries. *The Plant Pathology Journal*, 21, 59-63.

Hassanein N.M., Ali, M.M., Youssef, K.A. and0 Mahmoud, D.A. (2010). Control of tomato early blight and wilt using aqueous extract of neem leaves. *Phytopathologia Mediterranea*.49, 143-151

Heimpel, G.E. and Mills, N. (2017). *Biological Control – Ecology and Application .* Cambridge. Cambridge : University Press. https://doi.org/10.1017/9781139029117. Date accessed : 19 October 2021

Hodges, R.J. (2010). Postharvest losses and waste in developed and less developed countries: opportunities to improve resource use. *Journal of Agricultural Science*, 149, 37-45.

Holz, G., Courtze, S. and Williamson, B. (2004). The ecology of *Botrytis* on plant surface. *Botrytis* biology pathology and control. Kluwer, Dordrecht, 9-27

Huang, X., Xie, W. and Gong, Z. (2000). Characteristics and antifungal activity of a chitin binding protein from Ginkgo biloba. *FEBS Letter*, 478, 123-126

Index-Acta-phytosanitaire (2017). Index Acta phytosanitaire 2018. Acta Editions, Flers, France.

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

Ji, M. S., Qi, Z.Q., Zhao, P., Cheng, G.W., Gu, Z.M. and Wang, Y. Z. (2002). Primary study on resistance of *Botrytis cinerea* to pyrimethanil in tomato. *Journal of Shenyang Agricultural University*, 33, 345-347.

Ji, M.S., Qi, Z.Q., Wang, Y.Z., Cheng, G.W., and Zu, M. (2003). Resistance of *Botrytis cinerea* to pyrimethanil in tomato. *Acta Phytophylacica Sinica*, 30, 396-400.

Jones, J. B., Jones, J. P., Stall, R. E., & Zitter, T. A. (1991). Compendium of Tomato Diseases. American Phytopathological Society Press, St. Paul, Minnesota, USA.

Kader, A.A. (2005). Increasing food availability by reducing postharvest losses of fresh produce. *Acta Horticulturae*, 682, 2169-2178.

Karabulut, O.A. and Baykal, N. (2004). Integrated control of postharvest diseases of peaches with a yeast antagonist, hot water and modified atmosphere packaging. *Crop Protection*, 23, 431-435.

Kloepper, J.W, Ryu, C.M. and Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. *Journal of Phytopathology,* 94, 1259-1266.

Knapp, S. (2002). *Solanum* section *Geminata*. Flora Neotropica, Monograph, 84, 1-405

Kokalis-Burelle, N., Vavrina, E.N., Rosskopf, E.N. and Shelby. R.A. (2002). Field evaluation of plant growth-promoting rhizobacteria amended transplant mixes and soil solarization for tomato and pepper production in Florida. *Plant and Soil*, 238, 257-266.

Koul, O. (2011). Microbial biopesticides: Opportunities and challenges. *CABR Views Perspectives in Agriculture Veterinary Science Nutrition and Natural Resources*, 6, 1-26

Lachance, M.A. and Pang, W.M. (1997). Predacious yeasts. *Yeast*, 13,225-232.

Latha, P., Anand, T., Ragupathi, V., Prakasam, R. and Samiyappan, R. (2009). Antimicrobial activity of plant extracts and induction of systemic resistance in tomato plants by mixtures of PGPR strains and zimmu leaf extract against *Alternaria solani*. *Biological Control*. 50, 85-93.

Latorre, B.A., Spadaro, I. and Rioja, M. E. (2002). Occurrence of resistance strains of *Botrytis cinerea* to anilinopyrimidine fungicides in table grapes in Chile. *Crop Protection*, 21, 957-961.

Legard, D.E., Xiao, C.L., Mertely, J.C., Chandler, C.K., (2000). Effects of plant spacing and cultivar on incidence of *Botrytis* fruit rot in annual strawberry. *Plant Diseases*, 84, 531-538.

Leonardi, C., Ambrosino, P., Esposito, F. and Fogliano. V. (2000). Antioxidative activity and carotenoid and tomatine contents in different typologies of fresh consumption tomatoes. *Journal of Agricultural and Food Chemistry*, 10, 4723-4727.

Leroch, M., Kretschmer, M. and Hahn, M. (2011). Fungicide resistance phenotypes of *Botrytis cinerea* isolates from commercial vineyards in Southwest Germany. *Journal of Phytopathology*, 159, 63-65.

Leroux, P. (2004). Chemical control of Botrytis and its resistance to chemical fungicides. In: Botrytis: Biology, Pathology and Control. Kluwer Academic Publishers, London, UK.

Lima, G., Ippolito, A., Nigro, F. and Salerno, M. (1997). Effectiveness of *Aureobasidium pullulans* and *Candida oleophila* against postharvest strawberry rots. *Postharvest Biology and Technology*, 10, 169-178.

Liu, H.X., Jiang, W.B., Bi, Y. and Luo, Y.B. (2005). Postharvest BTH treatment induces resistance of peach (*Prunus persica L.* cv. Jiubao) fruit to infection by *Penicillium expansum* and enhances activity of fruit defence mechanisms. *Postharvest Biology and Technology*, 35, 263-269.

Lu, L., Fritsch, P.W., Matzke, N.J., Wang, H., Kron, K.A., Li, D.Z. and Wiens, J.J. (2019). Why is fruit colour so variable? Phylogenetic analyses reveal relationships between fruit-colour evolution, biogeography and diversification. Global Ecology and Biogeography, 28, 891- 903.

Lurie, S., Droby, S., Chalupowicz, L., Chalutz, E. (1995). Efficacy of *Candida oleophila* strain-182 in preventing *Penicillium expansum* infection of nectarine fruits. *Phytoparasitica*, 23, 231-234.

Mahaffee, W. F. and Backman, P. A. (1993). Effects of seed factors on spermosphere and rhizosphere colonization of cotton by *Bacillus subtilis* GB03. *Journal of Phytopathology*, 83, 1120-1125.

Maiyo, Z., Ngure, R., Matasyoh, J. and Chepkorir, R. (2010). Phytochemical Constituents and antimicrobial activity of leaf extracts of three Amaranthus plant species. *African Journal of Biotechnology.*9,3178–3182.

Marín, A., Chafer, M., Atares, L., Chiralt, A., Torres, R., Usall, J. (2016). Effect of different coating-forming agents on the efficacy of the biocontrol agent *Candida* sake CPA-1 for control of *Botrytis cinerea* on grapes. *Biological Control*, 96, 108-119.

Marrone, P. G. (2002). An effective biofungicide with novel modes of action. *Pesticide Outlook*, 13, 193-194.

Masih, E.I., Slezack-Deschaumes, S., Marmaras, I., Ait Barka, E., Vernet, G., Charpentier, C., Adholeya, A., Paul, B. (2001). Characterisation of the yeast *Pichia*

membranifaciens and its possible use in the biological control of *Botrytis cinerea*, causing the grey mould disease of grapevines. *FEMS Microbiology Letters*, 202, 227-232.

McGuire, R.G. (1994). Application of *Candida guilliermondii* in commercial citrus coatings for biological control of *Penicillium digitatum* on grapefruits. *Biological Control*, 4, 1-7.

McLaughlin, R.J., Wilson, C.L., Droby, S., Ben-Arie, R. and Chalutz, E. (1992). Biological control of postharvest diseases of grape, peach, and apple with the yeasts *Kloeckera apiculata* and *Candida guilliermondii*. *Plant Disease*, 76, 470-473.

Mehta L.K., Balaraman, R., Amin, A.H., Baffa, P.A. and Gulati, O.D. (2003). Effects of fruits *Moringa oleifera* on the lipid profile of normal and hypercholesterolaemic rabbits. *Journal of Ethnopharmacology,* 86, 191-195.

Mertely, J. C., Chandler, C. K., Xiao, C. L., and Legard, D. E. (2000). Comparison of sanitation and fungicides for management of *Botrytis* fruit rot of strawberry. *Plant Disease*, 84, 1197-1202.

Mignani, I., Greve, L. C., Ben-Arie, R., Stotz, H. U., Li, C., Shackel, K., and Labavitch, J. (1995). The effects of GA3 and divalent cations on aspects of pectin metabolism and tissue softening in ripening tomato pericarp. *Physiologia Plantarum*, 93, 108–115.

Moyo, B., Masika, P. J. and Muchenje, V. (2012). Antimicrobial activities of *Moringa oleifera* Lam leaf extracts. *African Journal of Biotechnology*, 11, 2797-2802.

Muccilli, S., and Restuccia, C. (2015). Bioprotective role of yeasts. *Microorganisms*. *3*, 588-611.

Muhammad, R.H., Bamisheyi, E. and Olayemi, F.F. (2011). The effect of stage of ripening on the shelf life of tomatoes (*Lycopersicon esculentum*) stored in the evaporative cooling system (E.C.S). *Journal of Dairying, Foods and Home Sciences*, 30, 299-301.

Munoz, C., Gamez Talquenca, S., Oriolaniand, E. and Combina, M. (2010). Genetic characterization of grapevine-infecting *Botrytis cinerea* isolates from Argentina. *African Journal of Biotechnology*, 27, 66-70.

Nabi, S.U., Raja, W.H., Kumawat, K.L., Mir, J.I., Sharma, O.C. and Singh, D.B. (2017). Postharvest diseases of temperate fruits and their management strategies-a review. *International Journal of Pure and Applied Bioscience*, 5, 885-898.

Naika, S., Van Lidt de Jeude, J., de Goffau, M., Hilmi, M. and Van Dam B. (2005). Cultivation of tomato. Production processing and marketing. In: Van Dam B (Eds.), Wageningen, Digigrafi, Netherlands.

Nicola, S., Tibaldi, G. and Fontana, E. (2009). Tomato production systems and their application to the tropics. *Acta Horticulturae*, 821, 27-34.

Nicot, P. C., Stewart, A., Bardin, M., and Elad, Y. (2016). "Biological control and biopesticide suppression of *Botrytis*-incited diseases." In: Fillinger, S. and Elad, Y. (Eds). In *Botrytis* – the Fungus, the Pathogen and its Management in Agricultural Systems. Springer International Publishing, Cham, Switzerland.

Nicot, P., Bardin, M., Alabouvette, C., Köhl, J. and Ruocco, M. (2011). Potential of biological control based on published research. 1. Protection against plant pathogens of selected crops. In: Nicot, P. (Ed.). Classical and augmentative biological control against diseases and pests: critical status analysis and review of factors influencing their success.IOBC/WPRS, Zurich, Switzerland.

Nie, P., Li, X., Wang, S., Guo, J., Zhao, H. and Niu, D. (2017). Induced systemic resistance against *Botrytis cinerea* by *Bacillus cereus* AR156 through a JA/ET-andNPR1-dependent signalling pathway and activates PAMP-triggered immunity in Arabidopsis. *Frontiers in Plant Science*, 8, 238.

Obongoya, B., Wagai, S. and Odhiambo, G. (2010). Phytotoxic effect of selected crude plant extracts on soil-borne fungi of common bean. *African Crop Science Journal,* 18, 15–22.

Palti, J., and Cohen, Y. (1980). Downy mildew of cucurbits: the fungus and its hosts, distribution, epidemiology and control. *Phytoparasitica*. 8, 109-147.

Pande, S., Galloway, J., Gaur, P.M. (2006). *Botrytis* grey mould of chickpea: a review of biology, epidemiology and disease management. *Australian Journal of Agricultural Research,* 57, 1137-1150.

Parfitt, J., Barthel, M. and Macnaughton, S. (2010). Food waste within food supply chains: Quantification and potential for change to 2050. *Philosophical Transactions of the Royal Society*, 365, 3065-3081.

Parnell, T.L., Suslow, T.V. and Harris, L.J. (2004). Tomatoes: safe methods to store, preserve, and enjoy, ANR publication 8116.

Passera, A., Venturini, G., Battelli, G., Casati, P., Penaca, F., Quaglino, F. (2017). Competition assays revealed *Paenibacillus pasadenensis* strain R16 as a novel antifungal agent. *Microbiological Research*, 198, 16-26.

Paul, V., Pandey, R., Ramesh, K. V. and Singh, A. (2012). Role of mineral nutrients in physiology, ripening and storability of fruits. An International Treatise Series – Nutriophysiological and Molecular Interventions for Crop Improvement Under Changing Climate. *Advances in Plant Physiology*.13, 56-96.

Percival, D. C., Abbey, J., Lu, H. and Harris, L. (2016). Use of biofungicides to address conventional *Botrytis* blight control challenges in wild blueberry production. *In XI international Vaccinium symposium*, 1180, 241-248.

Pollastro, S., Faretra, F., Di Canio, V. and De Guido, A. (1996). Characterization and genetic analysis of field isolates of *Botryotinia fuckeliana* (*Botrytis cinerea*) resistant to dichlofluanid. *European Journal of Plant Pathology,* 102, 607-613.

Price, M. L., (2000). The Moringa Tree purification and activity assay of the coagulant protein from *Moringa oleifera* seed. *Water Research*, 39, 2338-2344.

Roger, F. and Keinath, A. (2010). Biofungicides and chemicals for managing diseases in organic vegetable production. Clemson University Cooperative Ext. Information Leaflet 88.

Romanazzi, G., Smilanick, J.L., Feliziani, E. and Droby, S. (2016). Integrated management of postharvest grey mould on fruit crops. *Postharvest Biology and Technology* 113, 69-76.

Rosslenbroich, H.J. and Stuebler, D. (2000). *Botrytis cinerea*—history of chemical control and novel fungicides for its management. *Crop Protection*, 19, 557– 561.

Rothan, C., Diouf, I. and Causse, M. (2019). Trait discovery and editing in tomato. *The Plant Journal*, 97, 73-90.

Roy, S., Banerjee, A., Tarafdar, J. and Samanta, S.K. (2010). Superior bio-efficacy of a combined formulation of carbendazim and mancozeb in inducing defense responses in chilli seedlings against *Sclerotium rolfsii* in comparison with methyl jasmonate. *Crop Protection*, 29, 163-167.

Saligkarias, I.D., Gravanis, F.T., Epton, H.A.S. (2002.). Biological control of *Botrytis cinerea* on tomato plants by the use of epiphytic yeasts *Candida guilliermondii* strains 101 and US 7 and *Candida oleophila* strain I-182: I. *in vivo* studies. *Biological control*, 25, 143-150.

Sansone, G., Rezza, I., Calvente, V., Benuzzi, D., Maria, I. and Sanz de, T. (2005). Control of *Botrytis cinerea* strains resistant to iprodione in apple with rhodotorulic acid and yeasts. *Postharvest Biology and Technology*, 35, 245-251.

Santos, A., Sánchez, A. and Marquina. D. (2004). Yeasts as biological agents to control *Botrytis cinerea*. *Microbiological Research,* 159, 331-338.

Schena, L., Ippolito, A., Zahavi, T., Cohen, L., Nigro, F. and Droby, S. (1999). Genetic diversity and biocontrol activity of *Aureobasidium pullulans* isolates against postharvest rots. *Postharvest Biology and Technology*, 17, 189-199.

Shirane, N., Masuko, M. and Hayashi, Y. (1988). Nuclear behaviour and division in germinating conidia of *Botrytis cinerea*. *Journal of Phytopathology*, 78, 1627-1630.

Sinesio, F., Cammareri, M., Moneta, E., Navez, B., Peparaio, M., Causse, M. and Grandillo, S. (2010). Sensory quality of fresh French and Dutch market tomatoes: a preference mapping study with Italian consumers. *Journal of Food Science*, 75, S55-S67.

Singh, V. K., Singh, A. K. and Kumar, A. (2017). Disease management of tomato through PGPB: current trends and future perspective. *Biotechnology*, *7*, 1-10.

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 Technology*, 47, 39-49.

Sreelatha, S. and Padma, P.R. (2009). Antioxidant Activity and Total Phenolic Content of *Moringa oleifera* Leaves in Two Stages of Maturity. *Plant Foods for Human Nutrition*, 64, 303-311

Suwanaruang T. (2016). Analyzing lycopene content in fruits. *Agricultural Science Procedia*, 11, 46-8.

Sylla, J., Alsanius, B. W., Kruger, E., and Wohanka, W. (2015). Control of *Botrytis cinerea* in strawberries by biological control agents applied as single or combined treatments. *European Journal of Plant Pathology*. 143, 461–471

Tan, H.L., Thomas Ahner, J.M. and Graingeretal, E.M. (2010). “Tomato based food products for prostate cancer prevention: what have we learned?” *Cancer and Metastasis Reviews*, 29, 553-568.

ten Have, A., Tenberg, K.B., Benen, J.A.E., Tudzynski, P., Visse, J. and van Kan, J.A.L. (2002). The Contribution of Cell Wall Degrading enzymes to pathogenesis of fungal plant pathogens. In: Kempken, F. (ed). Agricultural applications (The Mycota volume 11) Sprinter Science and business media, New York, USA.

Tenberge, K.B. (2004). Morphology and cellular organisation in Botrytis interactions with plants. Botrytis: Biology, Pathology and Control (Elad Y, Williamson B, Tudzynski P and Delen N, eds), Kluwer Academic Publishers, Dordrecht, The Netherlands,67–84.

Urbonavičienė, D., Bobinaitė, R., Trumbeckaitė, S., Raudonė, L., Janulis, V. and Bobinas, C. (2018). Agro-industrial tomato by-products and extraction of functional food ingredients. *Zemdirbyste Agriculture*, 105, 63-70.

Van Kan, J.A.L. (2006). Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends in Plant Science*, 11, 247-253.

Vidyasagar, G.M. and Tabassum, N. (2013). Antifungal investigations on plant essential oils. A review: *International Journal of Pharmacy Research*. 5, 19–28.

Walker A.S., Gladieux P., Decognet V., Fermaud M., Confais J., Roudet J. (2015). Population structure and temporal maintenance of the multi-host fungal pathogen *Botrytis cinerea*: causes and implications for disease management. *Environmental Microbiology.* 17 1261-1274.

Walker, G.M., McLeod, A.H., Hodgson, V.J. (1995). Interactions between killer yeasts and pathogenic fungi. *FEMS Microbiology Letters*, 127, 213-222.

Willson, M.F. and Whelan, C.J. (1990). The Evolution of Fruit Colour in Fleshy-Fruited Plants. *The American Naturalist 136*, 790-809.

Wilson, C.L. and Wisniewski, M.E. (1989). Biological control of postharvest diseases of fruits and vegetables: an emerging technology. *Annual Review of Phytopathology*, 27, 425-441.

Wisniekwski, M.E., Biles, C.L., Droby, S., McLaughlin, R.J., Wilson, C.L., Chalutz, E. (1991). Mode of action of the postharvest biocontrol yeast *Pichia guilliermondii*. Characterization of attachment to *Botrytis cinerea*. *Physiological and Molecular Plant Pathology,* 39, 245-248.

Wisniewski, M., Wilson, C., Droby, S., Chalutz E., El Ghaouth, A. and Stevens, C. (2007). Postharvest biocontrol: new concepts and applications. In: Vincent, C., Goettel, M.S. and Lazarovits, G. (Eds). Biological control: A global perspective. Centre for Agriculture and Bioscience International, Wallingford, UK.

Woo, S., Fogliano, V., Scala, F., Lorito, M. (2002). Synergism between fungal enzymes and bacterial antibiotics may enhance biocontrol. *Antonie Van Leeuwenhoek*, 81, 353-356.

Xiao, C. L. (2006). Postharvest fruit rots in d'Anjou pears caused by *Botrytis cinerea*, *Potebniamyces pyri*, and *Sphaeropsis pyriputrescens*. *Plant Health Progress*, *7*, 40.

Zdravkovic, J., Markovic, Z., Zdravkovic, M., and Pavlovis, N. (2007). Relation of mineral nutrition and content of lycopene and b-carotene in tomato *(Lycopersicon esculentum* Mill.) fruits. *Acta Horticulturae,* 729, 177-181.

Zhang, X., Li, B., Zhang, Z., Chen, Y., and Tian, S. (2020). Antagonistic yeasts: A promising alternative to chemical fungicides for controlling postharvest decay of fruit. *Journal of Fungi*. *6*, 158.

CHAPTER 3

In vitro and *in vivo* screening of antagonistic microorganisms against *Botrytis cinerea* of tomato

Abstract

Tomatoes have a short shelf life and highly perishable resulting in infections caused by plant pathogens causing mechanical damage before and/or after harvest. This research evaluated the *in vitro* and *in vivo* effect of antagonistic microorganisms against *B. cinerea* of tomatoes. A total of 48 antagonistic microorganisms were isolated from different plant parts and tested for inhibitory effect against *B. cinerea* of tomato *in vitro*. *Serratia marcescens*, *Bacillus pumilus* and *Bacillus safensis* inhibited *B. cinerea* by more than 50% in secondary screening. These isolates were further characterized and identified as best performing isolates. *In vivo* screening of *Serratia* and *Bacillus* isolates inhibited grey mould incidence on 'Jam' tomatoes by more than 70%. The results obtained from this study showed that biological control agents of *Serratia spp* and *Bacillus spp* were inhibitory against grey mould. The scanning electron micrographs showed the breakage of the mycelia *in vitro*, and the spores of *B. cinerea* were damaged on tomato fruits. Biocontrol agents are potential alternatives against postharvest grey mould of tomatoes.

Keywords: Grey mould**,** *Botrytis cinerea*, antagonistic microorganisms, tomato, *Serratia* spp and *Bacillus* spp.

3.1 Introduction

Tomato, scientifically known as *Solanum lycopersicum* L., is one of the most widely cultivated and extensively consumed horticultural crops globally (Grandillo *et al*., 1999). This fruit is nutritious and beneficial to the human body as it contains lycopene and carotenoids with antioxidant properties. The main antioxidants in tomatoes are carotenoids, ascorbic acid and phenolic compounds (Collins *et al*., 2022). Several pathogens such as fungi, bacteria, viruses, and nematodes infect tomatoes both pre-harvest and postharvest (Parnell *et al*., 2016). Postharvest decay of fruits mostly occurs between flowering and fruit maturity or during harvesting, handling, and storage (Eckert and Ogawa, 1988). *Botrytis cinerea*, the causal agent of grey mould, is the second most important fungal pathogen worldwide and one of the most studied fungal

phytopathogens (Dean *et al*., 2012; Williamson *et al*., 2007). This pathogen results in substantial yield and quality losses during field production and postharvest storage worldwide (Jarvis, 1977; Williamson *et al*., 2007; Sharma *et al*., 2009).

B. cinerea is controlled using pre and postharvest fungicide treatments resulting in the development of resistant strains of the pathogen worldwide (Walker *et al*., 2013). The top ten countries consuming pesticides around the world are China, the USA, Argentina, Thailand, Brazil, Italy, France, Canada, Japan and India. Some chemical agents in 2011 were rejected mainly in the USA because of environmental protection and consumer health regulations (Carbu *et al*., 2016). In South Africa, fungicides such as Iprodione (Rovral™), Fungazil (Imazalil™), Mertect (Thiabendazole™) and Scholar (Fludioxinol™) significantly control postharvest diseases of most fruits (Eckert and Ogawa, 1985; Kupferman, 1998). However, the continuous use of chemical fungicides can result in reduced efficacy because of the development of pathogen- resistance (Manso and Nunes, 2011; Carmona-Hernandez *et al*., 2019)

Previous studies have given evidence that yeasts have biocontrol activity when applied to tomato leaves (Elad *et al*., 1994). Yeast has shown promising biocontrol activity on numerous postharvest pathogens such as *B. cinerea* (Lima *et al*., 1997; Schena *et al*., 1999), but there is less research on biological control of preharvest diseases. There is still a small number of biocontrol products marketed as biofungicides to manage *B. cinerea* (Haidar *et al*. 2016). Postharvest biocontrol is convenient to treat harvested fruits since many postharvest pathogens cause infection after harvest through wounds (Janisiewicz and Jeffers, 1997; Nunes *et al*., 2001). The biocontrol market consists mostly of bacteria-based and fungi-based products of approximately 85% products and the rest are other products based on viruses, predators and other organisms (Glare *et al*., 2012).

When biocontrol agents encounter the surface of the fruit, they colonize the wounds and affect the germination of the pathogenic fungal spores through niche exclusion and competition for nutrients (Liu *et al*., 2012). Bacteria and yeasts have been shown to use competition for nutrients to reduce conidial germination (Blakeman and Fokkema, 1982). *Bacillus* and *Paenibacillus* species produce different antifungal substances including lipopeptide antibiotics, antifungal proteins, volatile compounds, lytic enzymes, other antibiotics and plant defence-related enzymes (Selim *et al*., 2005;

Huang and Chen, 2008; Ongena and Jacques, 2008; Raza *et al*., 2008; Govindasamy *et al*., 2010; Malfanova *et al*., 2012; Zhang *et al*., 2013). Biological control of postharvest diseases of fruits is an effective and environmentally friendly alternative to fungicides (Köhl, *et al*., 1995; Janisiewicz and Korsten, 2002). Yeasts are specifically suitable for the control of postharvest diseases among the antagonistic agents since it consists of high inhibitory capacity, rapid colonization of fruit wounds and modes of action mainly based on competition for nutrients, direct physical interaction with fungal hyphae and production of cell wall lytic enzymes (Droby and Chalutz, 1994; Castoria *et al*., 1997).

Yeast species have been used to manage *B. cinerea* on several crops such as grapes (An Long *et al*., 2005), *Phaseolus vulgaris* L. (Elad *et al*., 1994), *Pyrus communis* L. (Chand-Goyal and Spotts, 1996), *Malus malus* L., *Actinidia chinensis* A. Chev. (Lima *et al*., 1999) and *Fragaria x ananassa* Duch. (Helbig, 2002). Furthermore, Elad *et al*. (1994); Dik and Wubben (2001) reported epiphytic yeasts controlled the grey mould of tomatoes caused by *Botrytis* spp under *in vivo* studies. Extensive studies on the use of antagonistic microorganisms to control fungal diseases of several crops may provide an environmentally friendly supplement to chemicals. This research aimed to investigate the antifungal effect of antagonistic microorganisms *in vitro* and *in vivo* against *B. cinerea* in tomatoes.

3.2 Materials and methods

3.2.1 Collection of diseased tomato samples and isolation of *B. cinerea*

Tomato fruits were collected from local fresh produce markets in Pietermaritzburg, KwaZulu-Natal, South Africa. Fruits showing symptoms of grey mould were selected for isolation of the fungal pathogen. The fruits were stored at 25°C for three days at the Postharvest laboratory, Plant Pathology Department, University of KwaZulu-Natal. After three days, fruits were sterilised with 70% ethanol and rinsed with sterile distilled water and allowed to dry. The fruit parts showing grey mould symptoms were cut into (1 cm × 1 cm) pieces using a flame-sterilized scalpel. Potato dextrose agar (PDA) from Merck was prepared and poured into sterile Petri plates (20 mL/plate) and allowed to solidify. Pieces of decayed tomatoes were conveyed into freshly prepared PDA plates and were incubated for 7 days at 25°C.

3.2.2 Identification of isolated *B. cinerea*

After obtaining pure cultures of *B. cinerea*, light microscopy (Carl Zeiss, Germany) light microscope was used to identify *B. cinerea* isolate. For microscopic identification, a drop of lactophenol cotton blue dye was placed on a clean glass slide and a thin smear of *B. cinerea* mycelia from 7-day old cultures were placed aseptically on the dye. The suspension was covered with a coverslip and viewed under the light microscope at 10x and 40x magnification to observe the spores of *B. cinerea*. *B. cinerea*, pure culture was prepared by single sporing and stored in glycerol (70%) for long-term storage.

3.2.3 Pathogenicity test of *B. cinerea* on tomato cultivar

Tomato “Jam” cultivar was disinfected with 70% ethanol and rinsed with distilled water and allowed to dry out at room temperature. Fruits were dipped for 5 min in the inoculum of *B. cinerea* isolate with a conidial suspension of the concentration 1×10^4 and 1×10^5 spores/mL. The control fruits were dipped into autoclaved distilled water for 5 min. The fungal pathogen was inoculated into five tomatoes with 3 replicates. The inoculated fruits were stored for 7 days at 25 °C with a relative humidity of 95%. Results were recorded after 7 days to measure the lesion diameter of the disease incidence for the pathogen.

3.2.4 *In vitro* screening of biocontrol agents against *B. cinerea*

Biological control agents were isolated from different plant materials of *Solanum lycopersicum*, *Ganoderma resinaceum* and *Opuntia stricta* and nutrient agar was used as selective media. These isolates were tested for their abilities to inhibit the mycelial growth of *B. cinerea* on PDA plates. The *in vitro* primary screening of 53 biocontrol agents was performed to determine the degree of inhibition of bacteria and yeast isolates against *B. cinerea* mycelial growth. From the primary screening, 32 isolates with % inhibition of ≥ 40% were selected for secondary screening. This was done by using a 3-day-old mycelial plug of *B. cinerea* excised into 2 mm x 2 mm using a sterile scalpel and placed at the centre of freshly prepared PDA plates. Pure cultures of biocontrol isolates were grown on PDA and incubated at 25°C, and streaked after 48 hours using the inoculating loop on the two opposite sides about 3 cm from the pathogen mycelial plug. For control plates, only a mycelial plug of *B. cinerea* was

placed at the centre of the fresh PDA plate. The plates were sealed using parafilm and incubated at 25°C for 7 days. The experiments were replicated 3 times and were repeated twice for both primary and secondary screening. The mycelial growth was measured after 7 days and mycelial growth inhibition was calculated.

3.2.5 Molecular identification of bacterial isolates

The molecular identification of the isolated biocontrol agents was done according to Stephen et al., (1997). Genetic DNA was extracted from the pathogen cultures using the Quick-DNA™ Fungal/Bacterial Miniprep Kit (Zymo Research, Catalogue No. D6005). The 16S target region was amplified using the OneTaq@ Quick-load@ 2X Master mix (NEB, Catalogue No M0486) with the primers presented in Table 3.1. The PCR products were run on a gel extracted with the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Catalogue No. D4001). The extracted fragments were sequenced in the forward and reverse direction (Nimagen, BrillianDye™ Terminator Cycle Sequencing Kit V3.1, BRD3-100/1000) and purified (Zymo Research, ZR-96 DNA Sequencing Clean-up Kit™, Catalogue No. D4050). The purified fragments were analysed on the ABI 3500x1 Genetic Analyser (Applied Biosystems, ThermoFisher Scientific) for each reaction for every sample. CLC Bio Main Workbench v7.6 was used to analyse the files with the samples generated by the ABI 3500XL Genetic Analyser and results were obtained by a BLAST search (NCBI).

Table 3.1: 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 *In vivo* screening of biocontrol agents against *B. cinerea*

A conidial suspension was prepared using a 10-day old *B. cinerea* culture. The surface of the sporulating culture was flooded with sterile distilled water and rubbed with a glass rod. The hemocytometer was used to enumerate conidial suspension concentration and adjusted to 1×10^5 spores/mL. Healthy and undamaged 'Jam' tomato fruits were harvested at Port Shepstone, KwaZulu-Natal, South Africa. Fruits were surface sterilised with 70% ethanol for 1 min, rinsed in distilled water for 1 min

and allowed to dry at room temperature. There were 11 biocontrol isolates suspensions that were prepared. Fruits were dipped into suspensions of biocontrol agents 1 × 10^8 cells/mL for 5 min and allowed to dry for 24 hours at 25°C. After 24 hours, fruits were inoculated with *B. cinerea* by dipping fruits in the inoculum for 5 min. For the control set, fruits were only inoculated with *B. cinerea* inoculum. Inoculated fruits were placed in boxes covered with plastics and sealed. Fruits were stored at 25°C storage with 95% relative humidity for 10 days. Disease incidence was measured after day 3, 5, 7 and 10 of post-inoculation. The experiments were performed in three replicates with three fruits per replicate and were repeated twice. The percentage of disease incidence on tomato fruits was obtained by dividing the number of infected fruits in a sample by the total number of tomato fruits in a sample according to the reference for the formula. Therefore, the formula was as follows:

$$\text{Overall average \% incidence} = \frac{\text{The number of infected fruits in a sample}}{\text{Total number of fruits in a sample}} \times 100$$

3.2.7 Scanning electron microscopy analysis of the interaction between *B. cinerea* and biocontrol agents *in vitro* and *in vivo*

Biocontrol agents that successfully inhibited *B. cinerea* in dual culture and on tomato fruits were grown on a freshly prepared PDA petri dish. The mycelial disc (2 mm x 2 mm) was placed at the centre of the PDA plate and each biocontrol agent was streaked 3 cm away from the mycelial plug on both sides. For *in vivo* trial, tomato fruits were disinfected with 70% ethanol, wounded and inoculated with 1 x 10^5 spores/mL suspension. The inoculated PDA plates and fruits were incubated at 25°C. After 7 days, the inhibition of mycelial growth and sporulation of *B. cinerea* was observed under scanning electron microscopy (SEM) Zeiss EVO LS15, Carl Zeiss NTS Ltd., Germany conducted at the Microscopy and Microanalysis Unit, University of KwaZulu-Natal, Pietermaritzburg, South Africa. Samples were cut from inoculated PDA plates and fruit samples, held for 2 hours in fixation of 3% buffered glutaraldehyde and washed twice in 0.05M sodium cacodylate buffer for 5 minutes. The samples were then dehydrated with approximately 2 mL aliquots of 10%; 30%; 50% and 70% ethanol for 10 minutes per concentration. The samples were rinsed three times with 100% ethanol for 10 minutes to complete the dehydration process. Following that, the samples were placed in the Quorum K850 critical drying point dryer (CPD) basket with

100% ethanol. The ethanol was replaced with liquid carbon dioxide (CO_2) during CPD. The liquid CO_2 was heated and pressurized to the critical point at which the liquid turned into a gas without damaging the samples due to surface tension, leaving the samples dry and undamaged. Using black double-sided tape, the dried samples were carefully mounted onto SEM stubs. The sample stubs were transferred to the Quorum Q150R ES sputter coater. In this step, the samples were coated twice with gold and palladium to make them conductive to the electron beam. After drying, the samples were examined under the Zeiss EVO LS15 SEM.

3.2.8 Statistical analysis

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

3.3 Results

3.3.1 Pathogenicity and morphology of *B. cinerea*

The pathogenicity test showed that *B. cinerea* was pathogenic to 'Jam' tomatoes (Figure 3.2) after 10 days post inoculation (Figure 3.2). There was no significant difference in grey mould incidence of tomatoes dipped in a conidial suspension of *B. cinerea* at concentrations of 1×10^4 and 1×10^5 conidia/mL with P-value ≤0.001 (Figure 3.1). There was low disease incidence (less than 5%) in tomato fruits for the control treatment from contamination and significantly different from the pathogen inoculated fruits (Figure 3.2).

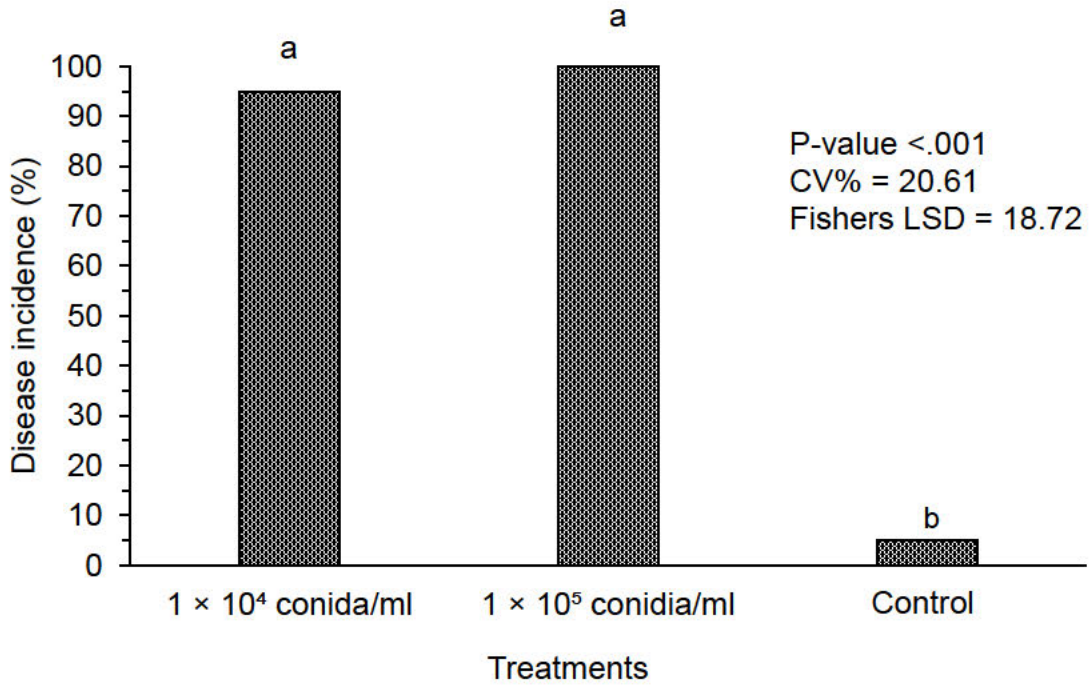


Figure 3.1: Pathogenicity test of *B. cinerea* on tomato fruits after 10 days of inoculation at 25°C.

Figure 3.2: Disease incidence of grey mould on tomatoes inoculated with 1 × 10^5 conidia/mL (A) and uninoculated tomatoes (B) after 10 days

3.3.2 *In vitro* screening of biocontrol agents against *B. cinerea*

There were 53 biocontrol agents isolated from different plants where 27 isolates were the bacteria and 26 isolates were the yeast used for primary screening (Table 3.2). For tomatoes there were 25 bacteria isolates, 18 isolates were from pickly pear where 16 were yeast and 2 were bacteria and 10 isolates were from mushroom. The

biocontrol agents from primary screening are grouped according to the average percentage inhibition of *B. cinerea* mycelial growth (Table 3.2). 27% of biocontrol agents inhibited *B. cinerea* by 51-75% *in vitro*. Isolates from tomato gave the most antagonistic bacteria strains and isolates from pickly pear gave the most antagonistic yeast. Most biocontrol isolates inhibited the pathogen by less than 50%.

Table 3.2: The grouping of yeast and bacterial isolates according to their average percentage inhibition.

Groups	**Ranges of average percentage inhibition**	**Percentage of bacterial isolates (%)**	**Percentage of yeast isolates (%)**
Group 1	0 - 25%	29.63	50.00
Group 2	26 - 50%	48.15	26.92
Group 3	51 - 75%	22.22	23.08
Group 4	76 - 100%	0.00	0.00

In vitro screening of *B. cinerea* against some of the antagonistic microorganisms grown on PDA after 7 days at 25 °C is shown in Figure 3.3 A, B, and C, and the control plate of *B. cinerea* (Figure 3.3 D).

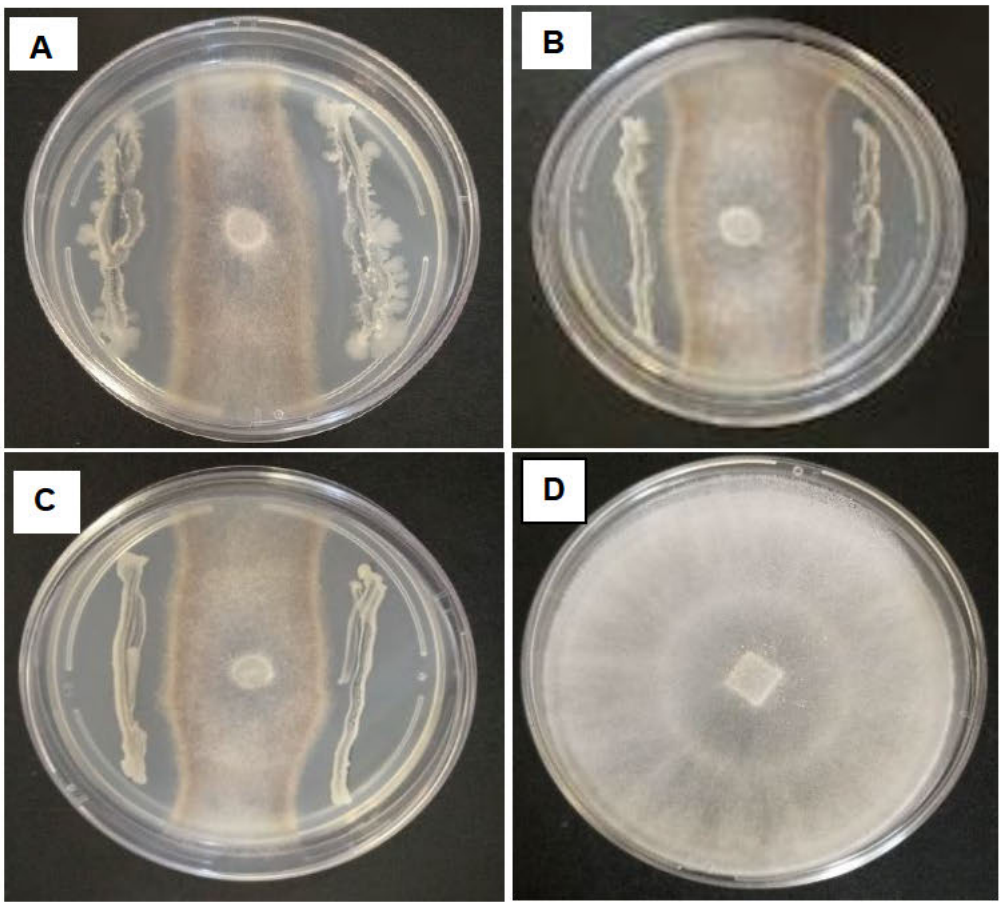


Figure 3.3: Best performing isolates of biocontrol agents B23 (A), B1 (B) and B11 (C) against *B. cinerea* on potato dextrose agar media (D) after 7 days at 25°C *in vitro* secondary screening.

Mycelial growth of *B. cinerea* was inhibited by both bacteria and yeast isolates (Tables 3.3 and 3.4). There was no significant difference between bacteria and yeast isolates on the inhibition of *B. cinerea* mycelial growth after 7 days (P= <.001). Eleven bacterial isolates inhibited the mycelial growth of *B. cinerea* by more than 50% after 7 days (Table 3.3). NG3X was the only yeast isolate that inhibited *B. cinerea* with more than 50% (Table 3.4). B23 had the highest inhibition of *B. cinerea* mycelial growth after 7 days.

Table 3.3: Secondary screening of bacterial isolates against *B. cinerea* after 7 days at 25°C. Means with the same letters are not significantly different according to Duncan's multiple range test (*P*=0.05).

Source of isolation	**Isolate name**	**Mycelial growth (mm)**	**% Inhibition**	**Group**
Solanum lycopersicum fruit	B1	40.67a	52.15	3
	B2	41.67a	50.98	2
	B4	42.00a	50.59	2
	B5	40.33a	52.55	3
	B7	40.67a	52.15	3
	B8	43.33abc	49.02	2
	B9	54.00c	36.47	2
	B10	40.00a	47.06	2
	B11	41.67a	50.98	2
	B12	41.67a	50.98	2
	B13	46.33abc	45.49	2
	B15	46.67abc	45.09	2
	B17	41.67a	50.98	2
	B18	41.33a	51.38	3
	B21	42.67ab	49.80	2
	B23	39.67 a	53.32	3
	B24	41.67 a	50.98	2
	B25	53.33 bc	37.26	2
	Control	85.00 d		
P value		<.001		
Fishers LSD		9.877		
CV%		13.1		
SED		4.870		

Table 3.4: Secondary screening of yeast isolates against *B. cinerea* after 7 days at 25°C. Means with the same letters are not significantly different according to Duncan's multiple range test (P=0.05).

Source of isolation	**Isolation name**	**Mycelial growth**	**% Inhibition**	**Group**
Ganoderma	Bb	45.00abcd	47.06	2
resinaceum	Bs	48.00de	43.53	2
	Kg4	45.33bcde	47.06	2
	Kg5	49.67e	42.35	2
	KgX	45.33bcde	46.67	2
	Kg1	45.00abcd	47.06	2
G. austroafricanum	NG1X	44.67abcd	47.45	2
	NG3X	40.67a	52.15	3
	R1Y1	43.33abc	49.02	2
	LLC22	42.33ab	50.20	2
Opuntia stricta leaves	MeRo5y	48.33de	43.14	2
	1FL	47.00cde	44.71	2
	SYB	46.00 bcde	45.88	2
	YAL	45.33 bcde	46.67	2
	Control	85.00 f		
P-value		<.001		
Fishers LSD		3.921		
CV%		4.9		
SED		1.914		

3.3.3 Molecular identification of bacterial isolates

The primers were used for the molecular identification of bacterial isolates from tomatoes that had an inhibitory effect on *B. cinerea* (Table 3.5). Both B1 and B11 were identified as *Bacillus* species where B1 was *Bacillus safensis* and B11 was *Bacillus pumilus* and B23 was identified as *Serratia marcescens* and these were the best perfoming isolates.

Table 3.5: The primers used for the identification of the bacterial biocontrol agents.

Isolate name	Species name	Primer	Accession no.
B1	*Bacillus safensis*	16S rDNA	MK785118.1
B2	*Bacillus amyloliquefaciens*	16S rDNA	GU339230.1
B3	*Bacillus thuringiensis*	16S rDNA	CP0449978.1
B4	*Bacillus cereus*	16S rDNA	MN099150.1
B5	*Bacillus thuringiensis*	16S rDNA	CP0449978.1
B6	*Bacillus cereus*	16S rDNA	MN509466.1
B7	*Bacillus sp.*	16S rDNA	MH329943.1
B8	*Bacillus cereus*	16S rDNA	KT986088.1
B9	*Bacillus velezensis*	16S rDNA	KU161296.1
B10	*Bacillus siamensis*	16S rDNA	KU605231.1
B11	*Bacillus safensis*	16S rDNA	KY595452.1
B12	*Solibacillus silverstris*	16S rDNA	KX768291.1
B13	*Bacillus cereus*	16S rDNA	MF616406.1
B14	*Serratia maecescens*	16S rDNA	CP020507.1
B15	*Bacillus sp.*	16S rDNA	JF514878.1
B16	*Bacillus megaterium*	16S rDNA	MN509795.1
B17	*Bacillus cereus*	16S rDNA	MK942520.1
B18	*Brevibacterium frigoritolerans*	16S rDNA	MG547699.1
B19	*Serratia sp.*	16S rDNA	KY935421.1
B20	*Pantoea ananatis*	16S rDNA	CP020943.2
B21	*Pseudomonas sp.*	16S rDNA	KX390639.1
B22	*Bacillus toyonensis*	16S rDNA	MN081699.1
B23	*Serratia marcescens*	16S rDNA	CP031316.1
B24	*Bacillus subtilis*	16S rDNA	MK721058.1
B25	*Bacillus sp.*	16S rDNA	KX839268.1

3.3.4 *In vivo* screening of biocontrol agents against *B. cinerea*

Fruits were observed for disease incidence and mycelial growth inhibition after 7 days of post-inoculation at 25°C (Figure 3.4). There was less disease incidence of *B. cinerea* on tomato fruits treated with B23 (17%), B1 (25%) and B11 (25%) compared to pathogen control treatment (75%). There was no significant difference between the *Bacillus* sp. and *Serratia* sp. after 7 days (P-value <.001).

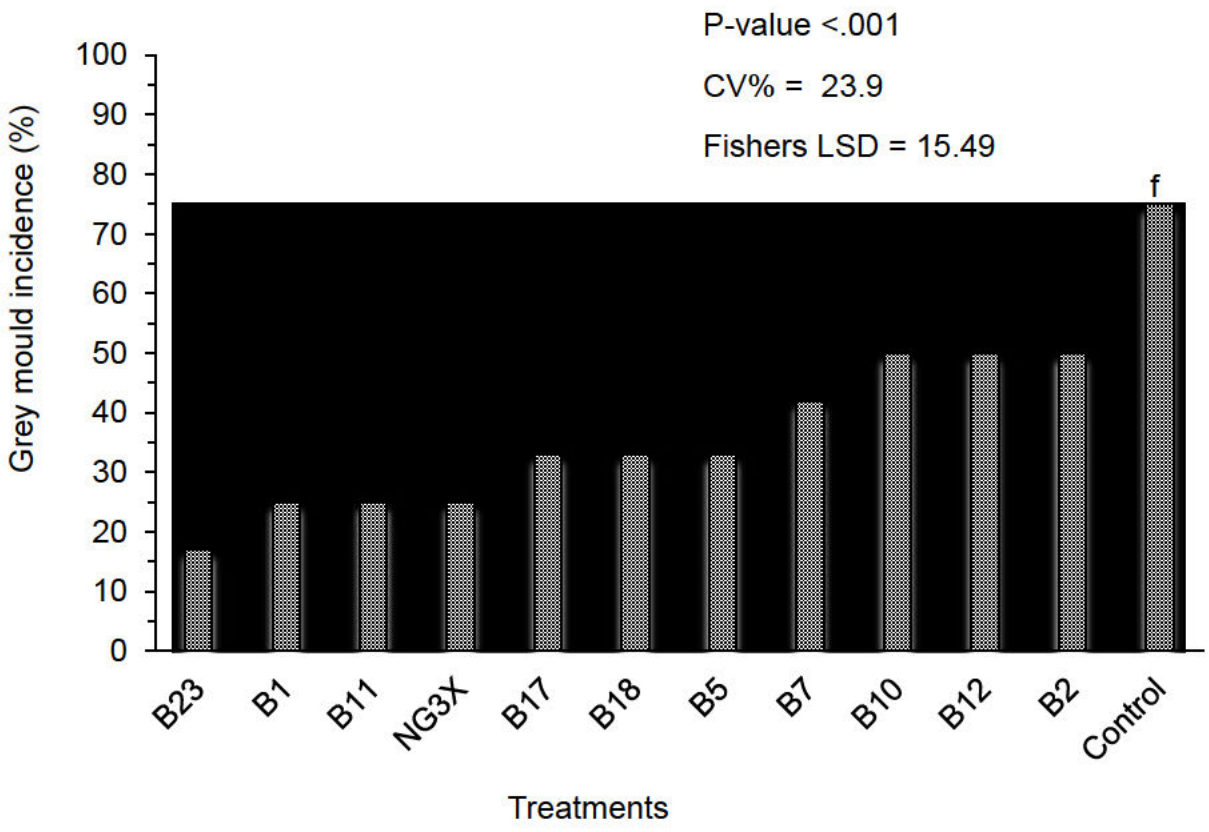


Figure 3.4: Disease incidence of *B. cinerea* on 'Jam' tomatoes after 7 days post-inoculation at 25°C. Means with the same letters are not significantly different according to Duncan's multiple range test (*P*=0.05).

Antagonistic microorganisms inhibited *B. cinerea* on tomatoes during *in vivo* screening (Figure 3.5 B. C and D) compared to control fruits (Figure 3.5 A). Fruits showed high disease incidence (75%) on tomatoes treated with *B. cinerea* only (control) (Figure 3.5 A) and low disease incidence on fruits treated with antagonistic microorganisms after 10 days of post-inoculation (Figure 3.5 B, C, and D).

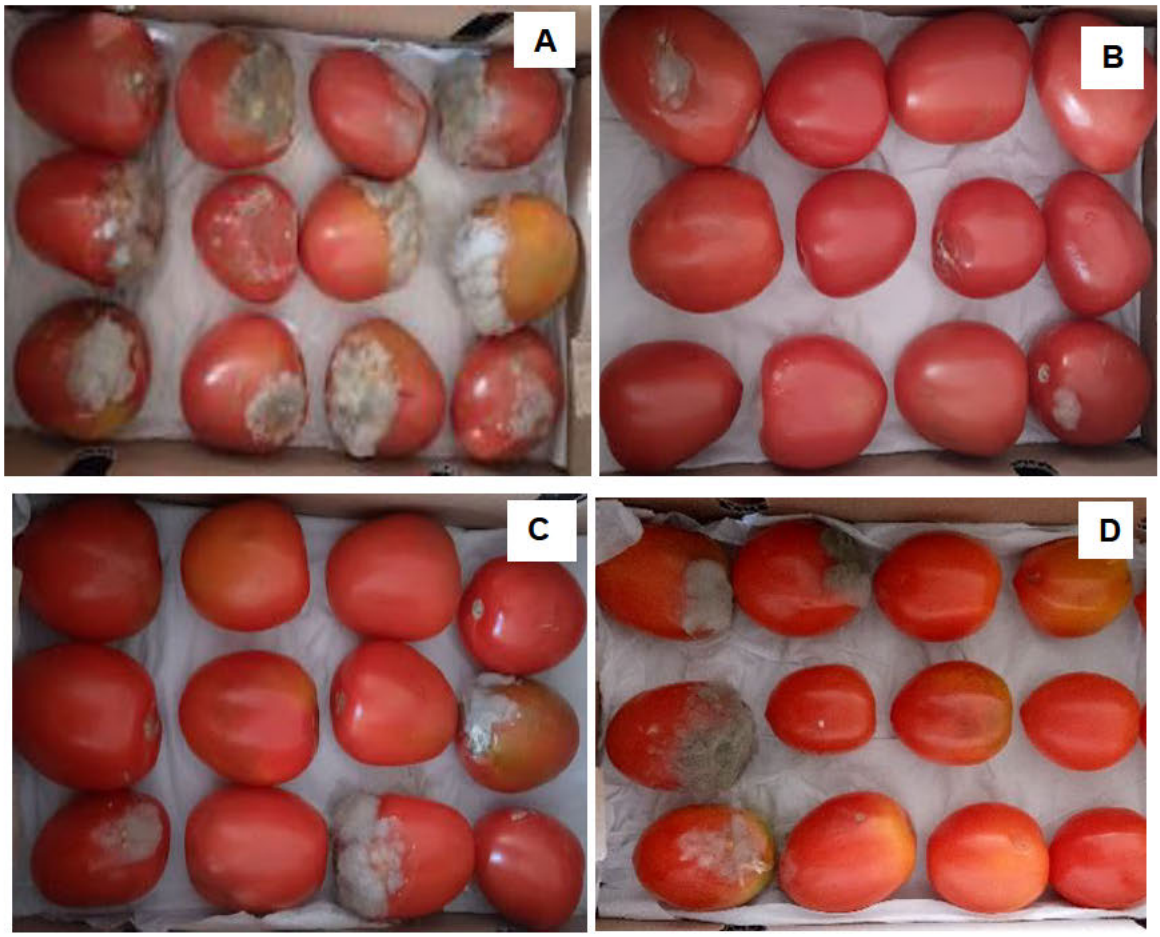


Figure 3.5: Disease incidence of grey mould on Jam tomatoes treated with the best isolates of biocontrol agents B23 (B), B1 (C) and B11 (D) after 10 days of post-inoculation at 25°C. The disease incidence of grey mould on control tomato fruits (Figure 3.5 A).

3.3.5 Scanning electron microscopy analysis of the interaction between *B. cinerea* and biocontrol agents

Mycelial growth and conidia of *B. cinerea* were observed using the SEM (Figure 3.6 A, B and C) after 7 days at 25ºC. The interaction and mode of action of biocontrol agents on *B. cinerea* were observed by the change in the mycelial and hyphal structures of *B. cinerea* treated with biocontrol agents. SEM micrographs revealed that biocontrol agents caused damage to the morphology of both mycelia and conidia (Figure 3.6 A, B and C) compared to the control (Figure 3.6 D).

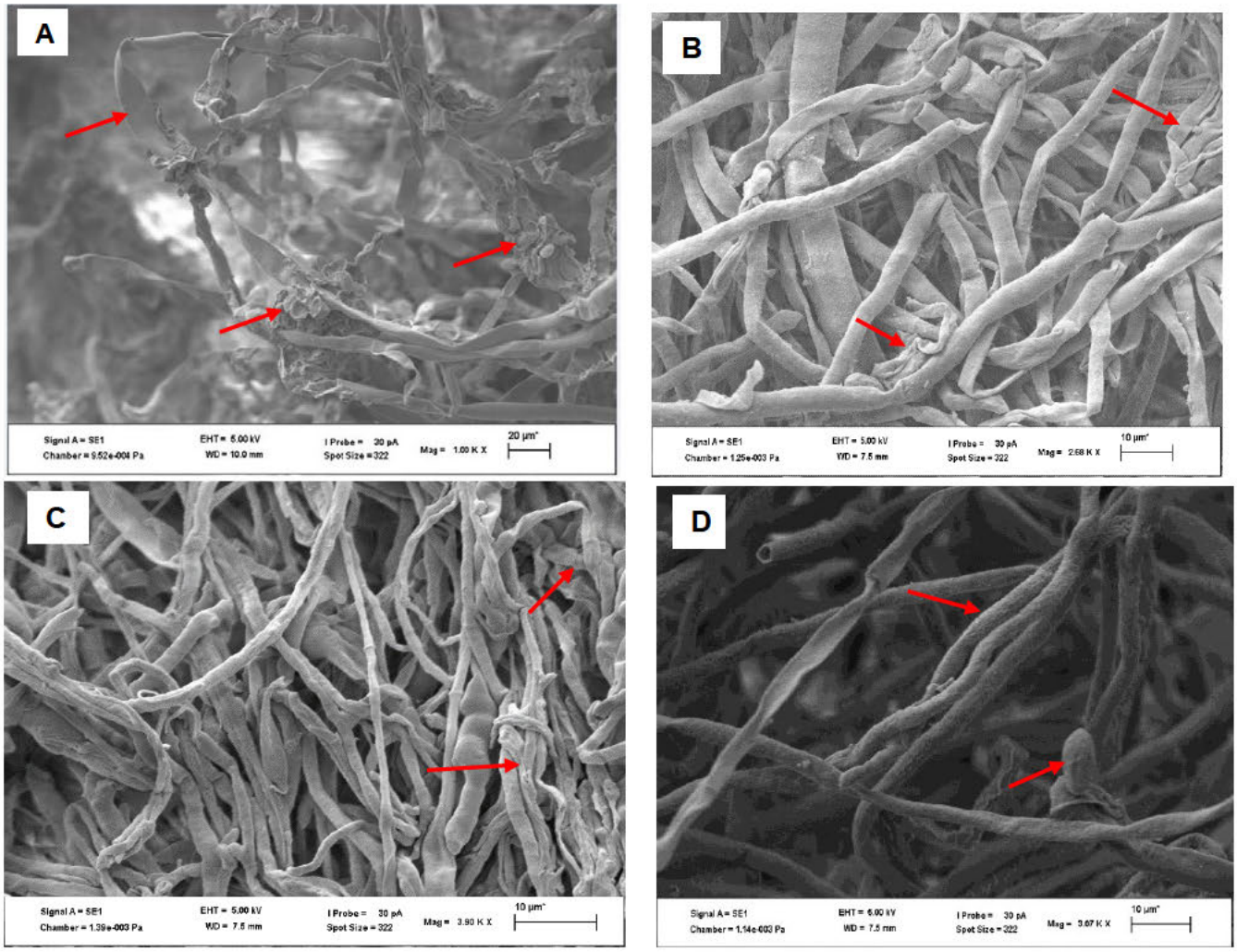


Figure 3.6: Scanning electron microscopy images showing the mode action of B23 (A), B1 (B), B11(C) and on conidia of *B. cinerea* (D) grown on potato dextrose agar after 7 days at 25ºC. Red arrows indicate damaged mycelia and spores of *B. cinerea* (A, B and C) and mycelia and spores of *B. cinerea* on PDA (D).

The interaction of bacteria isolates and *B. cinerea* on tomato surface is shown in Figure 3.7. Abundant pathogen spores were observed on the surface of control tomato fruits inoculated with the pathogen only (Figure 3.7 D) and fewer spores were found on the fruits with the interaction of bacteria and *B. cinerea* (Figure 3.7 A, B and C).

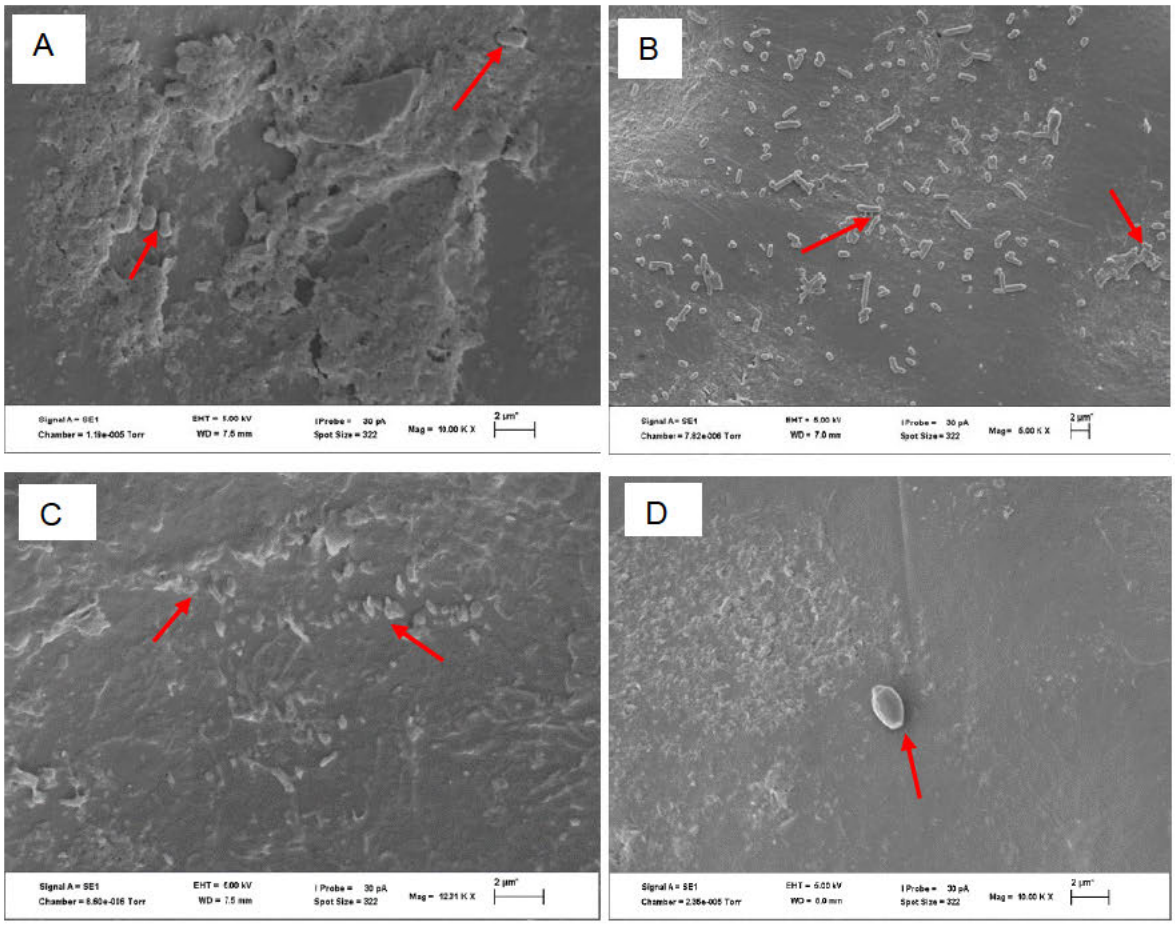


Figure 3.7: Scanning electron microscopy images showing the interaction of *B. cinerea* and B23 (A), *B. cinerea* and B1 (B), *B. cinerea* and B11 (C) and conidia of *B. cinerea* (D) on tomato surface after 10 days. Red arrows indicates bacterial cells and irregular shaped spores of *B. cinerea* (A, B and C) and spores of *B. cinerea* on tomato surface (D).

3.4 Discussion

Biological control significantly protects fruits and vegetables during pre-and post-harvest following physical and chemical control methods. Biocontrol products can decompose and result in reduced or no pollution (Gu *et al*., 2017). Studies have revealed that the application of antagonistic microorganisms as biological control is a promising method to reduce the decay of harvested fruits (Li *et al*., 2016; Wu *et al*., 2017). According to the results obtained during *in vitro* and *in vivo* screening, *Serratia* and *Bacillus* species showed significant inhibitory effects against *B. cinerea*. *Serratia* and *Bacillus* species were selected as best-performing isolates. There was no

significant difference between antagonistic organisms used against *B. cinerea* on tomato fruits although *Serratia* and *Bacillus* had higher efficacy than other isolates.

The effect on the morphology of mycelia and conidia observed include shrinkage and lysis of mycelia (Figure 3.6 A, B, and C). Normal mycelialgrowth and smooth surface were observed in the control (Figure 3.6D). The fruits treated with biocontrol agents were less damaged on the fruit surface and had fewer pathogen sporulation (Figure 3.7 A, B, and C). The inoculated fruits were injured on the fruit surface and showed spores of grey mould (Figure 3.7 D). Cell walls are rigid, dynamic protective shells that change in response to cultural conditions and environmental stresses (Gastebois *et al*., 2009; Latge', 2007). The cell wall serves many functions, including determining fungal morphology, providing osmotic protection, regulating material exchange with the external environment, adhesion to a substrate, host penetration, and cell communication (Deacon, 1997).

The surface of *B. cinerea* conidia and other *Botrytis* spp. have many short protuberances (200 to 250 nm) that are visible under SEM. Antagonistic microorganisms reported to inhibit grey mould on tomatoes include *Trichoderma*, *Bacillus*, and *Ulocladium* (Vos *et al*., 2015; Paulitz and Bélanger, 2001) and can be considered as potential biocontrol agents to minimize the use of synthetic fungicides. *Bacillus* (Gao *et al*., 2017; Zhang *et al*., 2013), *Clonostachys rosea* (Gong *et al*., 2017), and yeast strains (Parafati *et al*., 2015) have been used as postharvest treatments to suppress the development of *B. cinerea* and decay of harvested fruit and vegetables. Strains from the genus *Bacillus* such as *Bacillus subtilis*, *B. thuringiensis*, *B. amyloliquefaciens* and *B. megaterium* are significantly used in the formulation of plant growth-promoting rhizobacteria (PGPR) products (Borriss, 2011).

When *Bacillus* strains are applied to the plants, they significantly improve the plant's growth and health (Earl *et al*., 2008; Mnasri *et al*., 2017). The main known modes of action used by *Bacillus* species against *B. cinerea* are biofilm formation, competition for nutrients, cell wall-degrading enzymes and antibiosis. The results are similar to the one obtained by Mari *et al*. (1996) and Sadfi-Zouaoui *et al*. (2008) which shows that *Bacillus* spp can effectively manage *B. cinerea* on tomatoes. Besides the use of *Bacillus* species against *B. cinerea* on tomatoes, it has been used against various pathogens infecting other several crops. According to Wei *et al*. (2010) and Niu *et al*.

(2012), *Bacillus cereus* is a biocontrol agent strain with biocontrol efficacy against a variety of plant diseases, such as tomato bacterial wilt caused by *Ralstonia solanacearum* and tomato root-knot caused by *Meloidogyne incognita*.

Positive results were obtained from pilot tests using *B. subtilis* against peach brown rot (Pusey *et al*., 1988) and *Pichia guillermondii* versus *P. digitatum* of citrus fruit (Droby *et al*., 1993). *Bacillus* species produce extracellular lytic enzymes which degrade the cell walls of the pathogenic fungi resulting in reduced infection. These enzymes include chitinases, glucanases and proteases found in *B. circulans* (Rombouts *et al*., 1976), *B. subtilis* (Manjula and Podile, 2005) and *B. pumilus* (Essghaier *et al*., 2009). *Serratia marcescens* has been found to produce a chitinolytic enzyme (Akutsu *et al*., 1993; Iyozumi *et al*., 1996; Someya *et al*., 2000) and antibiotic substances (Okamoto *et al*., 1998; Someya *et al*., 2000).

Antagonistic activity of chitinolytic bacteria has been shown against pathogenic fungi by degrading the cell walls (Someya *et al*., 2011). *Serratia marcescens* produces various chitinases (ChiA, ChiB, and ChiC) (Someya *et al*., 2001), and enzymes degrade chitin in the cell wall of the fungal pathogens and the exoskeletons of insects. Certain strains of *Serratia marcescens* are reported to be active against *Rhizoctonia solani* Kühn., *Fusarium oxysporum* f.sp. lycopersici. and *Botrytis cinerea* (Someya *et al*., 2000; Someya *et al*., 2005). Most of the biocontrol strains of the genus *Serratia* belong to the *S. marcescens* species (Kobayashi *et al*., 1995). These species are reported to have the potential in controlling pathogenic fungi by producing hydrolytic enzymes such as chitinases to degrade the fungal cell walls.

3.5 Conclusion

In conclusion, this study has indicated that antagonistic microorganisms, *Serratia* and *Bacillus* species, have the potential to control grey mould on tomatoes incited by *Botrytis cinerea*. *Serratia* and *Bacillus* species can be used as alternative controls versus synthetic fungicides to control postharvest diseases of tomato fruits.

3.6 References

Akutsu, K., Hirata, A., Yamamoto, M., Hirayae, K., Okuyama, S. and Hibi, T. (1993). Growth inhibition of *Botrytis spp*. by *Serratia marcescens* B2 isolated from tomato phylloplane. *Journal Phytopathological Society of Japan*, 59, 18- 25.

An Long, C.H., Wu, Z. and Xun Deng, B. (2005) Biological control of *Penicillium italicum* of citrus and *Botrytis cinerea* of grape by strain 34–9 of *Kloeckera apiculata*. *European Food Research and Technology*, 221, 197-201.

Blakeman, J.P. and Fokkema, N.J. (1982). Potential for biological control of plant diseases on the phylloplane. *Annual Review of Phytopathology*, 120, 167-192.

Borriss, R. (2011). Use of Plant-associated *Bacillus* strains as biofertilizers and biocontrol agents in agriculture. In: Maheshwari D.K. (Ed.). Bacteria in Agrobiology: Plant Growth Responses. Springer, Berlin, Heidelberg, Germany.

Carbu, M., González-Rodríguez, V. and Garrido. C. (2016). New biocontrol strategies for strawberry fungal pathogens. In: Husaini A, Neri D (eds) *Strawberry: Growth, Development and Diseases*. Centre for Agriculture and Bioscience International, Boston, USA.

Carmona-Hernandez, S., Reyes-Pérez, J.J., Chiquito-Contreras, R.G., Rincon-Enriquez, G., Cerdan-Cabrera, C.R. and Hernandez-Montiel, L.G. (2019). Biocontrol of Postharvest Fruit Fungal Diseases by Bacterial Antagonists: A Review. *Agronomy*, *9*, 121.

Castoria, R., De Curtis, F., Lima, G. and De Cicco, V. (1997). The b-1,3-glucanase activity of two saprophytic yeasts and possible mode of action involved as biocontrol agents against postharvest diseases. *Postharvest Biology and Technology* 12, 293-300.

Chand-Goyal, T. and Spotts, R.A. (1996). Control of postharvest pear diseases using natural saprophytic yeast colonists and their combination with a low dosage of thiabendazole. *Postharvest Biology and Technology*, 7, 51-64.

Collins, E.J., Bowyer, C., Tsouza, A. and Chopra, M. (2022). Tomatoes: An extensive review of the associated health impacts of tomatoes and factors that can affect their cultivation. *Biology*, 11, 239.

Deacon JW, 1997. Modern Mycology. In: Deacon, J.W. Blackwell Science, Inc., Malden MA, United States of America.

Dean, R., van Kan, J. and Pretorius, Z.A. (2012). The Top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*, 13, 1-17.

Dik, A. and Wubben J. (2001). Biological control of *Botrytis cinerea* in greenhouse crops. Biological control of fungal and bacterial plant pathogens. IOBC WPRS Bull, 24, 49-52.

Droby, S. and Chalutz, E. (1994). Mode of action of biocontrol agents of postharvest diseases. In: Wilson, C.L., Wisniewski, M.E. (Eds.), *Biological Control of Postharvest Diseases: Theory and Practice.* CRC Press, Boca Raton, Florida

Droby, S., Hofstein, R., Wilson, C. L., Wisniewski, M., Fridlender, B., Cohen, L., Weiss, B., Daus, A., Timar, D. and Chalutz, E. (1993). Pilot testing of *Pichia guilliermondii*. A biocontrol agent of postharvest diseases of citrus fruit. *Biological Control*, 3, 47-52.

Earl, A.M., Losick, R. and Kolter, R. (2008). Ecology and genomics of *Bacillus subtilis*. *Trends in Microbiology*, 16, 269-275.

Eckert, J.W. and Ogawa, J.M. (1988). The chemical control of postharvest diseases: deciduous fruits, berries, vegetables and root/tuber crops. *Annual Review of Phytopathology*, 26, 433-469.

Eckert, J.W., Ogawa, J.M. (1985). The chemical control of postharvest diseases: subtropical and tropical fruits. *Annual Review of Phytopathology*, 23, 421-454.

Elad, Y., Köhl, J. and Fokkema, N.J. (1994). Control of infection and sporulation of Botrytis cinerea on bean and tomato by saprophytic bacteria and fungi. *Journal of Phytopathology*, 84, 1193-1200.

Essghaier, B., Bejji, M., Jijakli, H., Boudabous, A. and Sadfi Zouaoui, N. (2009). High salt-tolerant protease from a potential biocontrol agent *Bacillus pumilus* M3-16. *Annals of Microbiology*, 3, 553-558

Gao, Z., Zhang, B., Liu, H., Han, J. and Zhang, Y. (2017). Identification of endophytic *Bacillus velezensis* ZSY-1 strain and antifungal activity of its volatile compounds against *Alternaria solani* and *Botrytis cinerea*. *Biological Control*, 105, 27-39.

Gastebois, A., Clavaud, C., Aimanianda, V. and Latge'. J.P. (2009). *Aspergillus fumigatus*: cell wall polysaccharides, their biosynthesis and organization. *Future Microbiology*, 4, 583-595.

Glare, T., Caradus, J. and Gelernter, W. (2012). Have biopesticides come of age? Trends Biotechnology, 30, 250-258.

Gong, C., Liu, Y., Liu, S.Y., Cheng, M.Z., Zhang, Y., Wang, R.H., Chen, H.Y., Li, J.F., Chen, X.L. and Wang, A.X. (2017). Analysis of *Clonostachys rosea*-induced resistance to grey mould disease and identification of the key proteins induced in tomato fruit. *Postharvest Biology and Technology*, 123, 83-93.

Govindasamy, V., Senthilkumar, M., Magheshwaran, V., Kumar, U., Bose, P., Sharmaand, V. and Annapurna, K. (2010). *Bacillus* and *Paenibacillus spp*.: potential PGPR for sustainable agriculture. In: *Plant growth and health-promoting bacteria* (D.K. Maheshwari, ed.), Springer, Berlin, Germany, 333-364.

Grandillo, S., Zamir, D. and Tanksley, S.D. (1999). Genetic improvement of processing tomatoes: *A 20 year perspective. Euphytica*, 110, 85-97.

Gu, K.B., Zhang, D.J., Guan, C., Xu, J.H., Li, S.I., Shen, G.M., Luo, Y.C. and Li, Y.G. (2017). Safe antifungal lipopeptides derived from *Bacillus marinus* B-9987 against grey mould caused by *Botrytis cinerea*. *Journal of Integrative Agriculture*, 16, 1999-2008.

Haidar, R., Fermaud, M. and Calvo-Garrido, C. (2016). Modes of action for biological control of *Botrytis cinerea* by antagonistic bacteria. *Phytopathology Mediterranea*, 55, 13-34.

Helbig, J. (2002). Ability of the antagonistic yeast *Cryptococcus albidus* to control *Botrytis cinerea* in strawberry. *Biocontrol*, 47, 85-99.

Huang C.J. and Chen, C.Y. (2008). Synergistic interactions between chitinase ChicCW and fungicides against plant fungal pathogens. *Journal of Microbiology and Biotechnology*, 18, 784-787.

Iyozumi, H., Akutsu, K., Hirayae, K., Tsuchiya, K, Hibi, T. and Okuyama, S. (1996). Biological control of cyclamen grey mould (*Botrytis cinerea*) by *Serratia marcescens* B2. *Journal Phytopathological Society of Japan*, 62, 559-565.

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

Janisiewicz, W.J. and Jeffers, S. (1997). Efficacy of commercial formulation of two biofungicides for control of blue mould and grey mould of apples in cold storage. Crop Protection, 16, 629-633.

Jarvis W.R., 1977. Botryotinia and *Botrytis* species taxonomy, physiology and pathogenicity. Monograph No. 15, Canadian Department of Agriculture, Ottawa, Canada.

Kobayashi, D.Y., Guglielmoni, M. and Clarke, B.B. (1995). Isolation of the chitinolytic bacteria *Xanthomonas maltophilia* and *Serratia marcescens* as biological control agents for summer patch disease of turfgrass, *Soil Biology and Biochemistry*, 11, 1479-1487.

Köhl, J., Molhoek, W.M.L., Van der Plas, C.H. and Fokkema, N.J. (1995). Effect of *Ulocladium atrum* and other antagonists on sporulation of *Botrytis cinerea* on dead lily leaves exposed to field conditions. *Phytopathology,* 85, 393-401.

Kupferman, E.A. (1998). Postharvest chemicals applied to pears: a survey of pear packers in Washington, Oregon, and California. *Tree Fruit Postharvest Journal*,9, 3-24.

Latge', J.P. (2007). The cell wall: A carbohydrate armour for the fungal cell. *Molecular Microbiology*, 66, 279-290.

Li, W., Zhang, H., Li, P., Apaliya, M.T., Yang, Q., Peng, Y. and Zhang, X. (2016). Biocontrol of the postharvest green mould of oranges by *Hanseniaspora uvarum* Y3 in combination with phosphatidylcholine. *Biological Control,* 10, 30-38.

Lima, G., Arru, S., De Curtis, F. and Arras, G. (1999). Influence of antagonist, host fruit and pathogen on the biological control of postharvest fungal diseases by yeasts. *Journal of Industrial Microbiology and Biotechnology*, 23, 223-229.

Lima, G., Ippolito, A., Nigro, F. and Salerno, M. (1997). Effectiveness of *Aureobasidium pullulans* and *Candida oleophila* against postharvest strawberry rots. *Postharvest Biology and Technology*, 10, 169-178.

Liu, J., Wisniewski, M., Droby, S., Norelli,J., Hershkovitz, V., Tian, S. and Farrell, R. (2012). Increase in antioxidant gene transcripts, stress tolerance and biocontrol efficacy of *Candida oleophila* following sublethal oxidative stress exposure. – *FEMS Microbiology Ecology*, 80, 578-590.

Malfanova, N., Franzil, L., Lugtenberg, B., Chebotar, V. and Ongena, M. (2012). Cyclic lipopeptide profile of the plant-beneficial endophytic bacterium *Bacillus subtilis* HC8. *Archives of Microbiology*, 194, 893-899.

Manjula, K. and Podile, A. (2005). Production of fungal cell wall degrading enzymes by a biocontrol strain of *Bacillus subtilis* AF1. *Indian Journal of Experimental Biology.* 43, 892-896.

Manso, T. and Nunes, C. (2011). *Metschnikowia andauensis* as a new biocontrol agent of fruit postharvest diseases. *Postharvest Biology and Technology. 61*, 64-71.

Mari, M., Guizzardi, M. and Pratella, G.C. (1996). Biological control of grey mould in pears by antagonistic bacteria. *Biological Control*, 7, 30-37

Mnasri, N., Chennaoui, C., Gargouri, S., Mhamdi, R., Hessini, K., Elkahoui, S., Dj´ebali, N. (2017). Efficacy of some rhizospheric and endophytic bacteria *in vitro* and as a seed coating for the control of *Fusarium culmorum* infecting durum wheat in *European Journal of Plant Pathology*, 147, 501-515.

Niu, D.D., Wang, C.J., Guo, Y.H., Jiang, C.H., Zhang, W.Z., Wang, Y.P., (2012). The plant growth-promoting rhizobacterium *Bacillus cereus* AR156 induces resistance in tomato with induction and priming of defence response. *Biocontrol Science and Technology*, 22, 991-1004.

Nunes, C., Usall, J., Teixidó, N. and Viñas, I. (2001). Biological control of postharvest pear diseases using a bacterium, *Pantoea agglomerans* CPA-2. International *Journal of Food Microbiology,* 70, 53-61.

Okamoto, H., Sato, Z., Sato, M., Koiso, Y., Iwasaki, S. and Isaka, M. (1998). Identification of antibiotic red pigments of *Serratia marcescens* F- 1 - 1, a biocontrol agent of damping-off of cucumber, and antimicrobial activity against other plant pathogens. *Journal Phytopathological Society of Japan*, 64, 294-298.

Ongena, M. and Jacques, P. (2008). *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends in Microbiology,* 16, 115-125.

Parafati, L., Vitale, A., Restuccia, C. and Cirvilleri, G. (2015). Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinere*a causing post-harvest bunch rot of table grape. *Food Microbiology*, 47, 85-92

Parnell, J.J., Berka, R. and Young, H.A. (2016). From the lab to the farm: an industrial perspective of plant beneficial microorganisms. *Front Plant Science*, 7, 1-12.

Paulitz, T.C. and Belanger, R.R. (2001). Biological control in greenhouse systems. *Annual Review of Phytopathology*, 39, 103-133.

Pusey, P.L., Hotchkiss, M.W., Dulmage, H.T., Baumgardner, R.A., Zehr, E.I. and Wilson, C.L. (1988). Pilot tests for commercial production and application of *Bacillus subtilis* (B-3) for postharvest control of peach brown rot. *Plant Disease,* 72, 622-626.

Raza, W., Yang, W. and Shen, Q.R. (2008). *Paenibacillus polymyxa*: antibiotics, hydrolytic enzymes and hazard assessment. *Journal of Plant Pathology,* 90, 419-430.

Rombouts, F.M. and Phaff, H.J. (1976). Lysis of yeast cell walls. Lytic β-1, 6-glucanase from *Bacillus circulans* WL-12. *European Journal of Biochemistry,* 63, 109-120.

Sadfi-Zouaoui N., Essghaier, B., Hajlaoui, M.R., Fardeau, M.L., Cayol, J.L. and Ollivier, B. (2008). Ability of moderately halophilic bacteria to control grey mould disease on tomato fruits. *Journal of Phytopathology,* 156, 42-52.

Schena, L., Ippolito, A., Zahavi, T., Cohen, L., Nigro, F. and Droby, S. (1999). Genetic diversity and biocontrol activity of *Aureobasidium pullulans* isolates against postharvest rots. *Postharvest Biology and Technology*. 17, 189-199.

Selim, S., Negrel, J., Govaerts, C., Gianinazzi, S. and Tuinen, D.V. (2005). Isolation and partial characterization of antagonistic peptides produced by *Paenibacillus sp*. Strain B2 isolated from the sorghum mycorhizosphere. *Applied and Environmental Microbiology*, 71, 6501-6507.

Sharma, R.R., Singh, D. and Singh, R. (2009). Biological control of postharvest diseases of fruits and vegetables by microbial antagonists: a review. – *Biological Control*, 50, 205-221.

Someya, N., Kataoka, N., Komagata, T., Hirayae, K., Hibi, T. and Akutsu, K. (2000). Biological control of cyclamen soilborne diseases by *Serratia marcescens* strain B2, *Plant Disease*, 3, 334-340.

Someya, N., Nakajima, M., Hirayae, K., Hibi, T. and Akutsu, K. (2001). Synergistic antifungal activity of chitinolytic enzymes and prodigiosin produced by biocontrol bacterium, *Serratia marcescens* strain B2 against grey mould pathogen, *Botrytis cinerea*, *Journal of General Plant Pathology*, 4, 312-317.

Someya, N., Nakajima, M., Watanabe, K., Hibi, T. and Akutsu, K. (2005). Potential of *Serratia marcescens* strain B2 for biological control of rice sheath blight, *Biocontrol Science and Technology*, 1, 105-109.

Someya, N., Ikeda, S. and Morohoshi, T. (2011). Diversity of culturable chitinolytic bacteria from rhizospheres of agronomic plants in Japan, *Microbes and Environments*, 1, 7-14.

Stephen, F., Altschul, T.L., Madden, A.A., Jinghui, Z., Zheng, Z., Webb, M. and David, J.L. (1997). “Gapped BLAST and PSI-BLAST: a new genaration of protein database search programs”, *Nucleic Acids Research*, 25, 3389-3402.

Vos, C.M., De Cremer, K., Cammue, B.P., De Coninck, B. (2015). The toolbox of *Trichoderma* spp. in the biocontrol of *Botrytis cinerea* disease. *Molecular Plant Pathology*, 16, 400-412.

Walker, A.S., Micoud, A., Rémuson, F., Grosman, J., Gredt, M. and Leroux, P. (2013). French vineyards provide information that opens ways for effective resistance management of *Botrytis cinerea* (grey mould). *Pest Management Science*, 6, 667-678.

Wei, L.H., Xue, Q.Y., Wei, B.Q., Wang, Y.M., Li, S.M. and Chen, L.F. (2010). Screening of antagonistic bacterial strains against *Meloidogyne incognita* using protease activity. *Biocontrol Science and Technology*, 20, 739-750.

Williamson, B., Tudzynski, B., Tudzynnski, P. and van Kan, J.A. (2007). *Botrytis cinerea*: the cause of grey mould disease. *Molecular Plant Pathology*, 8, 561-580

Wu, Y., Lin, H., Lin, Y., Shi, J., Xue, S., Hung, Y.C., Chen, Y. and Wang, H. (2017). Effects of biocontrol bacteria *Bacillus amyloliquefaciens* LY-1 culture broth on quality

attributes and storability of harvested litchi fruit. *Postharvest Biology and Technology*, 132, 81-87.

Zhang, X., Li, B., Wang, Y., Guo, Q., Lu, X., Li, S. and Ma, P. (2013). Lipopeptides, a novel protein, and volatile compounds contribute to the antifungal activity of the biocontrol agent *Bacillus atrophaeus* CAB-1. *Applied Microbiology and Biotechnology*, 97, 9525-953

Chapter 4

Antifungal effect of ethanolic moringa leaf extract on postharvest tomatoes incited by *Botrytis cinerea*

Abstract

Botrytis cinerea is a major postharvest limiting factor for tomato production and causes economic losses. Hence the need to find solutions to control *B. cinerea* in tomatoes. *Moringa oleifera* extracts have the presence of various antioxidant compounds enabling moringa leaf extract to become a valuable source of natural antioxidants and antifungal potential. This research aimed to evaluate the efficacy of *M. oleifera* leaf extract against *B. cinerea in vitro* and *in vivo*. *In vitro* antifungal effects of moringa leaf extract (MLE) treatments MLE 1%, MLE 2%, MLE 3% and MLE 4% were evaluated by amending potato dextrose agar with moringa leaf extract and the plates were incubated at 25 °C. The MLE 3% and MLE 4% inhibited the mycelial growth of *B. cinerea* with ≥ 5% and the other concentrations of ethanolic and aqueos moringa leaf extract did not inhibit the pathogen *in vitro*. The control plates had the pathogen fully grown on the petri plate *in vitro*. 'Jam' tomato fruits were treated with moringa leaf extract concentrations (MLE 1 %, MLE 2% and MLE 3%) and control fruits were inoculated with *B. cinerea* (1 x 10^5 conidia/ml) suspension only. Fruits were incubated at 25 °C and disease incidence was evaluated after 10 days. Moringa leaf extract suppressed disease incidence of grey mould on tomato fruits treated *in vivo*. The disease incidence of tomato fruits treated MLE 2% and MLE 3% was ≤ 50% and control had a disease incidence of ≥ 85%. The results obtained from this research showed that moringa leaf extract has antifungal properties that suppress the growth of *B. cinerea in vitro* and grey mould disease incidence in tomatoes. Moringa leaf extract can be used as an alternative environmental-friendly control strategy for postharvest *B. cinerea.*

Keywords: *Moringa oleifera*, *Botrytis cinerea*, tomatoes

4.1 Introduction

Cultivating in an environment with different temperatures, irrigation management, light and carbon dioxide greatly influence tomato production and quality (Canakci and Akinci, 2006; Benghanem *et al*., 2013). Fungi, nematodes, bacteria and viruses are plant pathogens which are major limiting factors affecting tomato production (Dun-chun *et al*., 2016). *Botrytis cinerea*, the causal agent of grey mould is a catastrophic fungal disease of tomato (Benhamou *et al*., 1994). Recent studies worldwide have revealed that there are various strains of *B. cinerea* with a wide host range of species and it demonstrates genetic recombination (Johnston *et al*. 2014). The control of *B. cinerea* in tomatoes is challenged by the absence of commercial varieties resistant to *B. cinerea*. The control of *B. cinerea* is difficult due to factors such as several attack modes, diverse hosts and survival under unfavourable conditions for extended periods of up to nine months as sclerotia in crop debris and soil and the development of fungicide-resistant strains (Williamson et al., 2007).

Grey mould can be controlled by spraying systemic fungicides and the continuous use of fungicides resulting in the contamination of fruits with fungicidal residues and the development of pathogen resistance (Tripathi and Dubey, 2004). Therefore, there is an urgent need for the development of new non-chemical control measures. Generally recognized as safe (GRAS) compounds are recommended by food authorities worldwide (Palou *et al*., 2002). These compounds have the slightest adverse effects on the environment, not expensive and are accepted by consumers (Nigro *et al*., 2006). Plant extracts with fungistatic or fungicidal effects have been reported to be an alternative control for many plant diseases of fruit and vegetables (Talibi *et al*., 2012; Askarne *et al*., 2013).

There are numerous plant products including plant extracts, essential oils, gums, and resins which have been reported to have antifungal properties against several pathogens (Fawzi *et al*., 2009; Al-Askar and Rashad, 2010). Plant extract consists of natural phytochemicals which can be used as biopesticides (Satish *et al*., 2007). The crude leaf extracts of *Eucalyptus citriodora* Hook. and *Ageratum conyzoides* Linnaeus. successfully inhibited mycelia growth and spore germination of *Didymella byroniae* (Fiori *et al*., 2000). Similarly, Al-Mughrabi (2003) reported that *Verticillium dahlia* Klebahn., *Fusarium oxysporum f.sp. lycopersici*., *Rhizopus stolonifer* (Ehrenb.) Vuill.,

Penicillium italicum Wehmer., *Rhizoctonia solani* Kühn. and *Pythium* spp. were inhibited by extracts from flowers, stems and leaves of *Euphorbia macroclada* Boiss.

Moringa oleifera is the most grown species of the tropical flowering plant in the family Moringaceae consisting of thirteen diverse species (Shahzad *et al*., 2013). It is one of the significant plants identified to contain a wealth of natural antioxidants. Vongsak *et al*., (2014) and Alhakmani *et al*., (2013), reported that the phytochemical analysis of moringa leaf extract divulged the presence of several antioxidant compounds, including ascorbic acid, fatty acids and phenolic acids (Vongsak et al., 2014; Alhakmani *et al*., 2013). According to the report by Batista *et al*. (2014) and Gifoni *et al*. (2012), Moringa-Chitin Binding Protein (Mo-CBP3) isolated and purified from the seeds of *M. oleifera* had antifungal inhibitory activity against mycelial growth and spore germination of *Fusarium solani* at minimum inhibitory concentration (MIC) of 0.05 $mgmL^{-1}$).

According to Adetunji *et al*. (2013), moringa leaf extract integrated with carboxyl methylcellulose (CMC) was found to extend the shelf life and maintain the quality of oranges. Tesfay and Magwaza (2017) evaluated the efficacy of chitosan and CMC incorporated with moringa leaf extract on the postharvest quality of avocados and reported that the treatments improved quality and extended shelf-life after 21 days of cold storage at 5.5 °C. Furthermore, moringa leaf extract combined with CMC was also reported to suppress postharvest diseases of avocado and maintain the quality of avocado (Tesfay and Magwaza (2017). The aim of this study was to evaluate the efficacy of moringa leaf extract in inhibiting the mycelial growth of *B. cinerea in vitro* and their effect on the disease incidence of *B. cinerea* on 'Jam' tomato fruits *in vivo*.

4.2 Materials and methods

4.2.1. Preparation of *Moringa oleifera* leaf extract

Moringa leaf was extracted as described by Tesfay *et al*. (2017), with some modifications. Briefly, four concentrations of moringa leaf extract (MLE 1%, MLE 2%, MLE 3% and MLE 4%) were prepared by respectively weighing 100 g, 200 g, 300 g and 400 g of moringa leaf powder and mixed with 1L of 70% ethanol. The samples were constantly agitated at room temperature for 24 hours. The extracts were evaporated for 24 hours in the Genevac (Genevac® EZ 2.3; Ipswich, UK) at 37 °C. The crude extract was suspended with 1L of distilled water and integrated with 10 g of

carboxyl methylcellulose (CMC) for each concentration. The prepared plant extracts were stored at 4 °C *in vitro* and *in vivo* screening.

4.2.2 *In vitro* effect of *Moringa oleifera* leaf extract on *B. cinerea*

The effect of moringa extract on the inhibition of mycelial growth of *B. cinerea* was examined on potato dextrose agar (PDA) media. PDA was autoclaved at 121°C for 15 min and cooled in a water bath at 40°C. A volume of 90 ml PDA was amended with 10 mL of each concentration (MLE 1%, MLE 2%, MLE 3% and MLE 4%) of moringa leaf extract. The amended PDA (20 mL/ plate) was poured into sterile Petri plates and allowed to solidify on a laminar flow bench. For the control, 10 mL of sterilized distilled water was amended with PDA. Mycelial plugs (2mm x 2mm) were excised from the edges of a 3-day-old pure culture plate of *B. cinerea* and placed at the centre of PDA media amended with the different concentrations of moringa leaf extract. The inoculated plates were incubated for 7 days at 25 °C. There were three replicates per treatment. After 7 days, the mycelial growth was measured, and the inhibition percentage of mycelial growth was determined.

4.2.3 Effect of ethanolic *Moringa oleifera* extract on *B. cinerea* of tomatoes

Fresh ‘Jam’ tomato fruits were harvested at Port Shepstone, KwaZulu-Natal, South Africa. Tomato fruits were washed using 70% ethanol for 1 min and rinsed for 1 min in distilled water and allowed to dry. Fruits were dipped in MLE 1% + CMC 1%, MLE 2% + CMC 1%, MLE 3% + CMC 1% and CMC 1% for 5 min and allowed to dry for 24 hours at 25 ºC. After 24 hours, treated fruits were dipped in *B. cinerea* inoculum with a concentration of 1×10^5 conidia/mL for 5 min. For the control, tomato fruits were dipped in 1×10^5 spores/mL concentration of *B. cinerea* only. The fruits were then placed in the boxes and stored at 25°C for 14 days at 95% relative humidity (RH). The experiment was repeated twice with three replicates per treatment. The disease incidence was measured on the fruits after 7, 10 and 14 days.

4.2.4 Scanning electron microscopy analysis of the interaction between *B. cinerea* and ethanolic *Moringa oleifera* leaf extract

B. cinerea mycelial disc (2 mm x 2 mm) was placed at the centre of the PDA media amended with MLE 2% and MLE 3%. The plates were incubated for 7 days at 25°C. For *in vivo* trial, fruits were inoculated with MLE 1%, MLE 2%, MLE 3% and incubated at 25°C. After 7days, the inhibition of mycelial growth and sporulation of *B. cinerea* was observed under scanning electron microscopy (SEM) Zeiss EVO LS15, Carl Zeiss NTS Ltd., Germany conducted at the Microscopy and Microanalysis Unit, University of KwaZulu-Natal, Pietermaritzburg, South Africa. Samples were cut from the prepared plates and tissue samples were held for 2 hours in fixation of 3% buffered glutaraldehyde and washed twice in 0.05M sodium cacodylate buffer for 5 minutes. The samples were then dehydrated with approximately 2 mL aliquots of 10%; 30%; 50% and 70% ethanol for 10 minutes per concentration. The samples were rinsed three times with 100% ethanol for 10 minutes to complete the dehydration process. Following that, the samples were placed in the Quorum K850 critical drying point dryer (CPD) basket with 100% ethanol. The ethanol was replaced with liquid carbon dioxide (CO_2) during CPD. The liquid CO_2 was heated and pressurized to the critical point at which the liquid turned into a gas without damaging the samples due to surface tension, leaving the samples dry and undamaged. Using black double-sided tape, the dried samples were carefully mounted onto SEM stubs. The sample stubs were transferred to the Quorum Q150R ES sputter coater. In this step, the samples were coated twice with gold and palladium to make them conductive to the electron beam. After drying, the samples were examined under the Zeiss EVO LS15 SEM.

4.2.5 Statistical analysis

The experimental design was arranged in a completely randomized design. Data collected was subjected to analysis of variance using the ANOVA on Genstat® 20th edition. Duncan Multiple Range Test (DMRT) at $P \leq 0.05$ was used to determine differences between treatments.

4.3 Results

4.3.1 *In vitro* screening of moringa leaf extract against *B. cinerea*

Moringa leaf extracts were extracted with distilled water and ethanol and tested for their antifungal effect against *B. cinerea* on PDA (Table 4.1). Moringa ethanolic extract showed the some inhibition of mycelial growth as compared to aqueous extract (Figure 4.1). There is lack of conidia production (Figure 4.1 B and C). Higher concentrations of moringa extract were found to have higher inhibition as compared to lower concentrations of moringa leaf extract. The treatments MLE 2% and MLE 3% for ethanolic extract had an inhibitory effect on the mycelial growth of *B. cinerea* as compared to the control. There was no significant difference between MLE 1%, MLE 2% and control treatment.

Table 4.1: Inhibition of mycelium growth of *B. cinerea* by moringa leaf ethanolic extracts and aqueous extract on Potato Dextrose Agar after 7 days at 25 °C.

	Ethanol		Aqueous	
Extract	Mycelial growth (mm)	Inhibition (%)	Mycelial growth (mm)	Inhibition (%)
MLE 4%	77.00a	9.41	85.00b	0.00
MLE 3%	79.00a	7.06	85.00b	0.00
MLE 2%	85.00b	0.00	80.83a	4.91
MLE 1%	85.00b	0.00	85.00b	0.00
Control	85.00b		85.00b	
P-value	<.001		0.03	
Fishers LSD	0.84		3.01	
CV%	0.50		1.80	
SED	1.19		1.23	

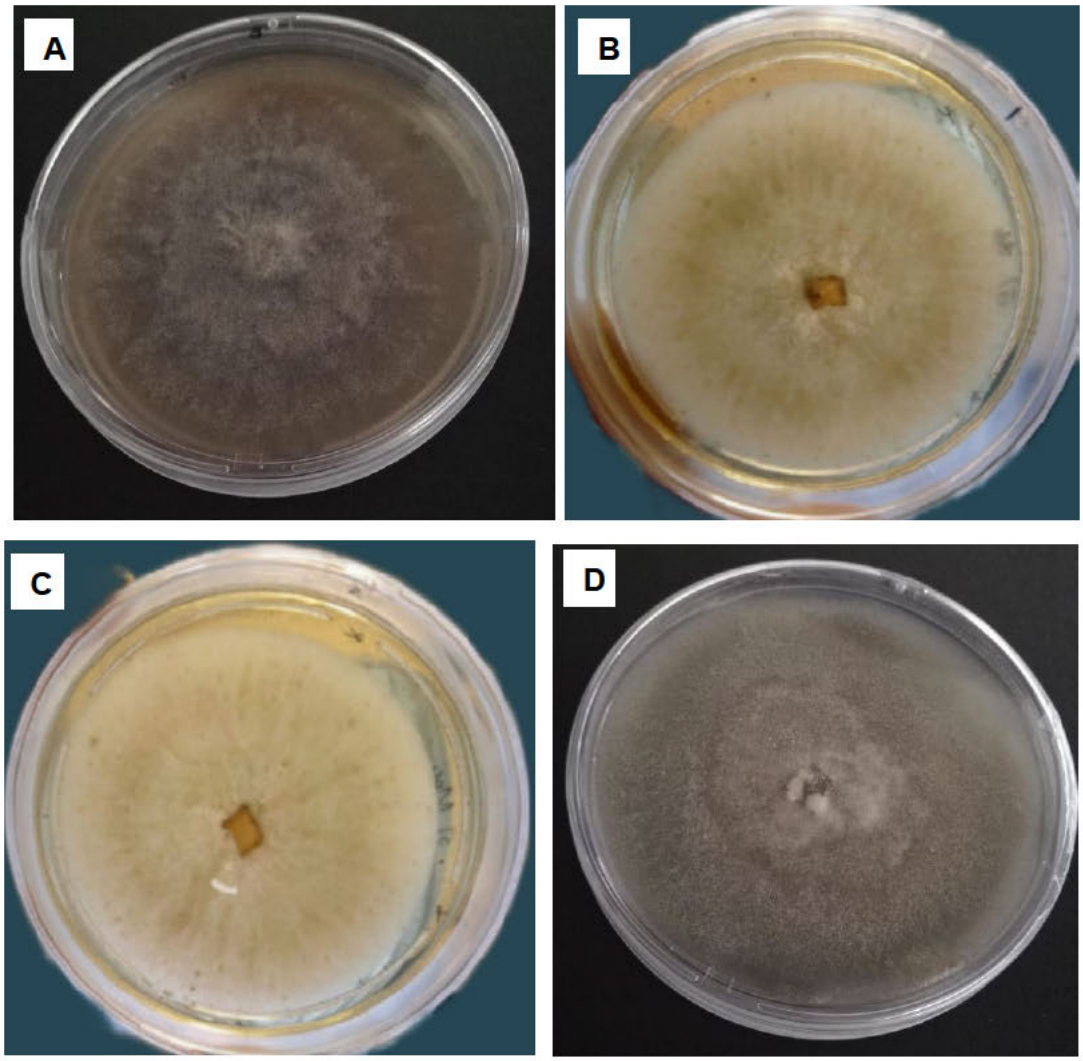


Figure 4.1: The effect of moringa leaf extract at MLE 2% (A), MLE 3% (B) and MLE 4% on the mycelial growth of *B. cinerea* and the control (D) on potato dextrose agar after 7 days at 25 °C.

4.3.2 Effect of moringa leaf extract on *B. cinerea* on tomatoes

There was significant difference observed between MLE 1% + CMC 1%, MLE 2% + CMC 1%, MLE 3% + CMC 1% and CMC 1% and control treatment. MLE 1% + CMC 1%, MLE 2% + CMC 1%, MLE 3% + CMC 1% and CMC 1% were effective in reducing the disease incidence of grey mould on tomatoes (Figure 4.2). The lowest disease incidence was observed on tomato fruits treated with MLE 3% (22.22%) and highest disease incidence was on tomato fruits treated with MLE 1% (55.55%) (Figure 4.2). The disease incidence of grey mould on tomatoes decreased with an increase in the concentration of moringa leaf extracts. Moringa leaf extract at 2% and 3%

concentrations were the most effective in reducing the disease incidence of *B. cinerea* in tomatoes (Figure 4.3 A and B). The lowest disease of grey mould on tomatoes was observed on MLE 3% + CMC 1% (33.33%) (Figure 4.2). The lowest growth inhibition of grey mould on tomatoes was observed on MLE 1% with disease incidence of more than 50%. All concentrations of moringa leaf extract inhibited disease incidence of grey mould compared to the control.

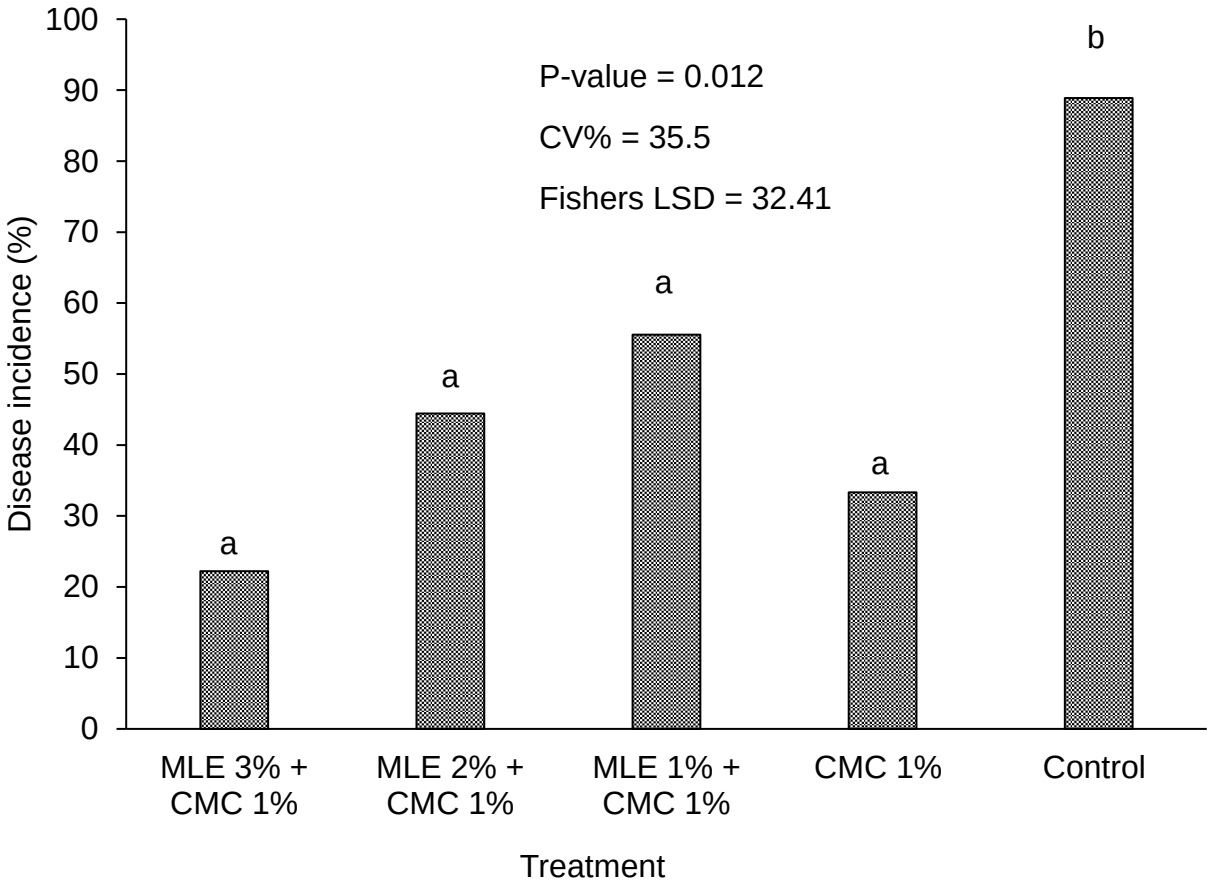


Figure 4.2**:** The disease incidence (%) of grey mould on tomatoes treated with ethanolic moringa leaf extract and CMC after 10 days at 25 °C.

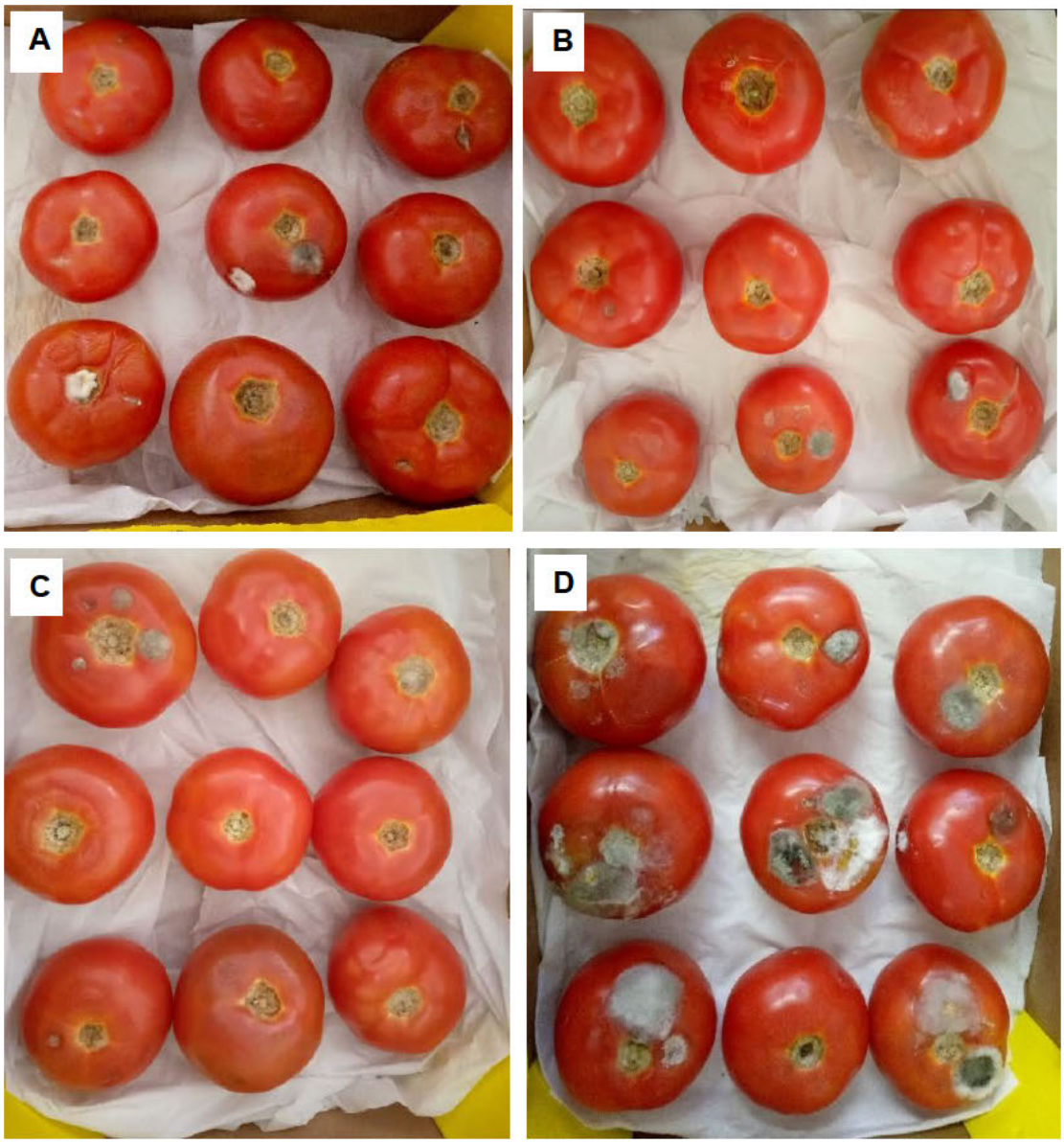


Figure 4.3: Disease incidence of grey mould on tomato fruits treated with MLE 2% + CMC 1% (A), MLE 3% + CMC 1% (B), CMC 1% (C) and control fruits (D) at 25 °C after 10 days.

4.3 .3 Scanning electron microscopy observation of the interaction between *B. cinerea* and moringa leaf extract

Moringa leaf extract suppressed the growth of *B. cinerea* after 7 days by causing breakage and shrinkage on the pathogen mycelia and conidial damaged (Figure 4.4 A, B and C). *B. cinerea* mycelia showed observable morphological changes such as reduced hyphal length and diameters, shrinkage, disruption, lysis and aggregation

(Figure 4.4 A, B and C) as compared to the control which showed the mycelial and conidia of *B. cinerea*.

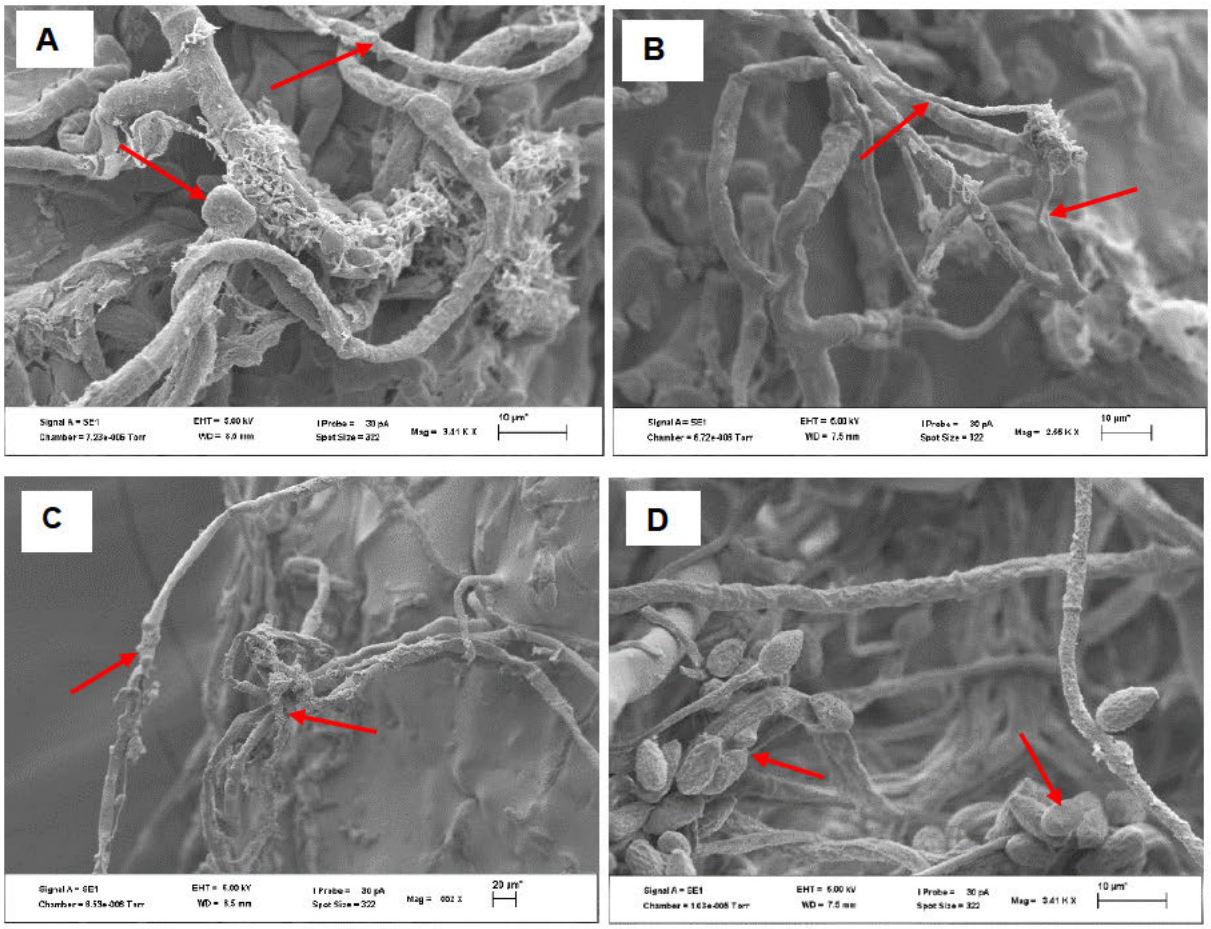


Figure 4.4: Scanning electron microscope images of mycelia and conidia of *B. cinerea* treated with MLE 1% (A), MLE (2%) and MLE (3%) and the control (D) *in vitro*. Red arrows indicates shrinking mycelia and damaged spores of *B. cinerea* (A, B and C) and mycelia and spores of *B. cinerea* on PDA (D).

The scanning electron micrographs of tomato fruit treated with ethanolic moringa leaf extract were showing damaged spores of the *B. cinerea* and moringa leaf extract deposits on the surface of tomatoes (Figure 4.5 A, B, C). The texture of the peel for the control fruit had spores of *B. cinerea* and some cracks (Figure 4.3 D).

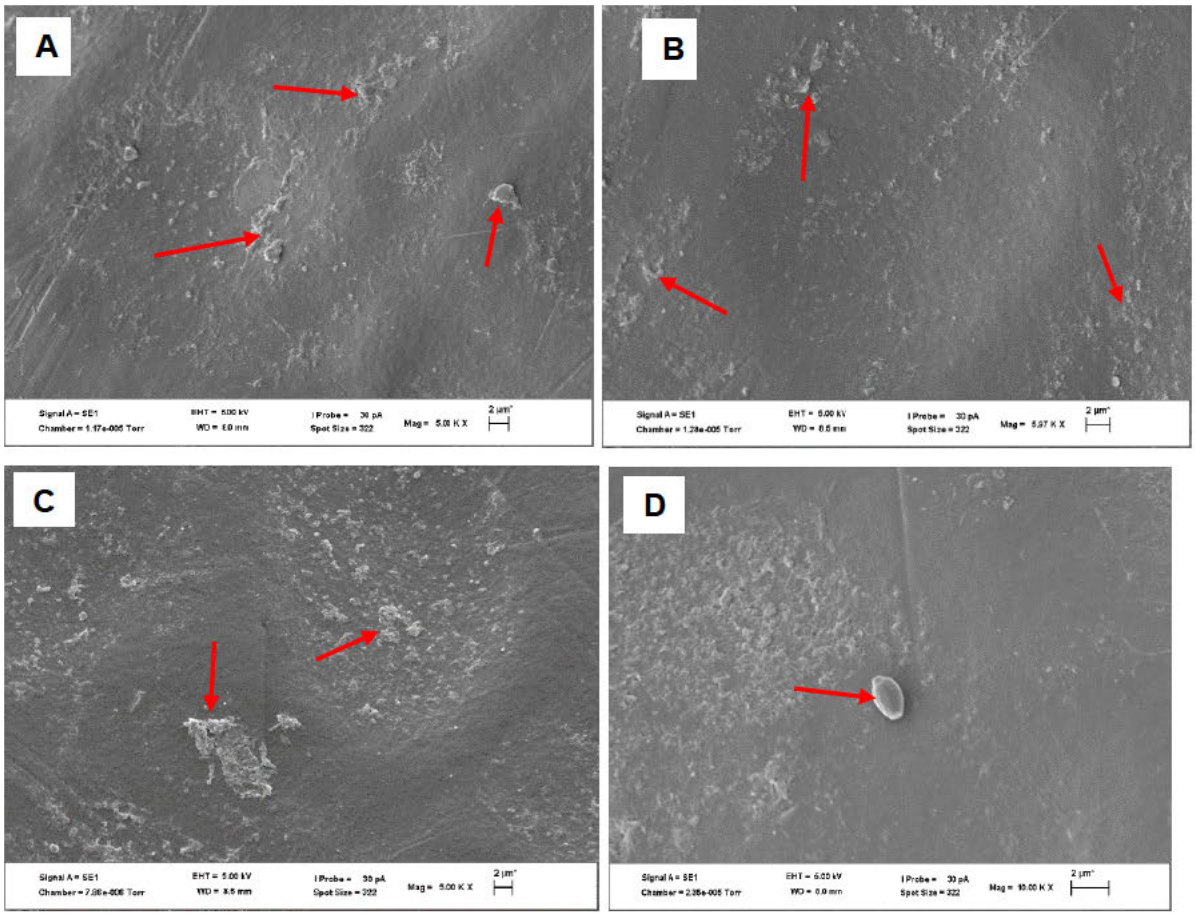


Figure 4.5: Scanning electron micrographs of the interaction of *B. cinerea* and MLE 1% (A), MLE 2% (B), MLE 3% (C) and the control tomato fruits (D) on the tomato surface. Red arrows indicate moringa leaf extract deposits and damaged spores (A, B and C) and mycelia and spores of *B. cinerea* on tomato surface (D).

4.4 Discussion

Several reports on antimicrobial compounds found in various plant parts including leaves, bark, fruit, root, and flowers have been reported (Devendra *et al*., 2011). Moringa plant has been previously reported to have antifungal and antimicrobial properties against numerous phytopathogens (Emad El-Din *et al*., 2016; Batista *et al*., 2014; Belay and Sisay, 2014; Díaz Dellavalle *et al*., 2011). This study investigated the antifungal activities of moringa leaf extract against grey mould caused by *B. cinerea* on tomatoes. The findings from *in vitro* studies showed that the diametric growth inhibition of *B. cinerea* by ethanolic and aqueous moringa leaf extracts confirmed the antifungal efficacy against fungal plant pathogens. There was no significant difference

between the treatments and control in the *in vitro* studies of aqueous moringa leaf extract because of the minimal effect observed.

It was noticed that ethanolic moringa leaf extract (MLE 3% and MLE 4%) had more antifungal efficacy as compared to aqueous moringa leaf extract *in vitro*. Ethanol is known to be one of the most efficient solvents for the extraction of plant extracts worldwide. The findings are in accordance with Emad El Din *et al*. (2016), Gurjar et al. (2012) and Das *et al*. (2010), who reported that the variation in the chemical properties of the solvent, method of extraction used, and the various structural and compositional manners of the natural plant products can result to extract solvent system with specific differences. According to Díaz Dellavalle *et al*., (2011), the variation in polarity among solvents can explicate the variation in solubility of active plant properties resulting in differences in the degree of activity.

For the *in vivo* studies, the results showed that moringa leaf extract had antifungal activities at all tested concentrations in reducing the disease incidence of *B. cinerea* on tomatoes. Antifungal activities reported in moringa leaf extract could be attributed to the presence of phenolic compounds, tannins, alkaloids, flavonoids, terpenoids, triterpenoids, and saponins (Elad *et al*., 2016; Pinto *et al*., 2015; El-Mohamedy and Abdalla, 2014; Tijjani *et al*., 2014). These compounds denature enzymes which hinder amino acids involved in the spore germination of the pathogen (Cushnie and Lamb, 2005) and this could be the reason why there were no conidia production Figure 4.1 B and C. Ethanolic moringa leaf extracts were not effective *in vitro* as compared to *in vivo*.

The scanning electron microscopy images exhibited that the morphology of the moringa treated samples changed and the mycelia was broken. The number of spores observed on the moringa treated samples were fewer as compared to the pathogen only treatment, and some spores were separated from the mycelia. According to Zaffer *et al*., (2015), the antifungal activity of moringa leaf extracts affects the pathogen hyphal growth resulting in membrane permeabilization due to the presence of lipophilic compounds that bind within or internal to the cytoplasmic membrane. Small peptides present in moringa leaf extract are significant in the antimicrobial defence system of the crop (Zaffer *et al*., 2015). These peptides are associated with defence mechanisms against phytopathogenic fungi by inhibiting the growth of micro-organisms through

diverse molecular modes (Zaffer *et al*., 2015). The binding molecular modes may include binding to increase the permeability of the fungal membranes (Zaffer *et al*., 2015).

Similar studies have been reported by different researchers on the antifungal activity of extracts of many plants (Satish *et al*., 2007; Jamil *et al*., 2010; Anwar and Rashid, 2007). The results from this study have shown that moringa plant extract can be used as a bio-fungicide to control pathogenic fungi and thus reduce the dependence on synthetic fungicides. These findings were in accordance with the earlier report of Banso *et al*. (1999) which showed that antifungal compounds present in the plant extracts were fungistatic at lower concentrations and become fungicidal at higher concentrations of the extracts. The findings presented in this chapter confirmed that moringa leaf extract has antifungal properties that suppress the growth of grey mould on tomatoes.

4.5 Conclusion

The results of this study showed that *M. oleifera* leaf extracts have antifungal and antimicrobial properties against *B. cinerea* of tomato. *M. oleifera* leaf extract can be recommended as a promising alternative method to control postharvest fungal pathogens and reduce the use of chemical fungicides. Thus, plant extracts can be well exploited in the future as an effective and environmentally friendly management strategy for various plant diseases.

4.6 References

Adetunji, C. O., Fawole, O. B., Arowora, K. A., Nwaubani, S. I., Oloke, J. K., Adepoju, A. O. and Ajani, A. O. (2013). Performance of edible coatings from carboxymethylcellulose (CMC) and corn starch (CS) incorporated with *Moringa oleifera* extract on *Citrus sinensis* stored at ambient temperature. *Agrosearch*, 13, 77-86.

Al-Askar A.A., Rashad Y.M. (2010). Efficacy of some plant extracts against Rhizoctonia solani on pea. *Journal of Plant Protection Research,* 3, 239-243.

Alhakmani, F., Kumar, S. and Khan, S. A. (2013). Estimation of total phenolic content, in–vitro antioxidant and anti–inflammatory activity of flowers of *Moringa oleifera*. *Asian Pacific journal of tropical biomedicine*, 3, 623-627.

Al-Mughrabi, K.I. (2003). Antimicrobial activity of extracts from leaves, stems and flowers of *Euphorbia macroclada* against plant pathogenic fungi. *Phytopathologia Mediterranea,* 42, 245–250.

Anwar, F. and Rashid, U. (2007). Physics-chemical characteristics of *Moringa oleifera* seeds and seed oil from a wild provenance of Pakistan Pak. *Journal Botany*, 39, 1443-1453.

Askarne, L., Talibi, I., Boubaker, H., Boudyach, E.H., Msanda, F., Saadi, B. and Ait Ben Aoumar, A. (2013). Use of Moroccan medicinal plant extracts as botanical fungicide against citrus blue mould. *Letters in Applied Microbiology*, 56, 37–43.

Banso, A., Adeyemo, S.O. and Jeremiah, P. (1999). Anti-microbial properties of Vernonia amygdalina extract. *Journal of Applied Science and Management*, 3, 9-11.

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., Lopes, J.L., Grangeiro, T.B. and Vasconcelos, I.M. (2014). New insights into the structure and mode of action of Mo-CBP3, an antifungal chitin-binding protein of *Moringa oleifera* seeds. *PLoS One*, 9, e111427.

Belay, K. and Sisay, M., (2014). Phytochemical constituents and physicochemical properties of medicinal plant (*Moringa oleifera*) around bule hora*. Chemistry and Materials Research*, 6, 61-72.

Benghanem, M., Daffallah, K. O., Joraid, A. A., Alamri, S. N. and Jaber, A. (2013). Performances of solar water pumping system using helical pump for a deep well: A case study for Madinah, Saudi Arabia. *Energy Conversion and Management*, 65, 50-56.

Benhamou, N., Lafontaine, P.J. and Nicole, M. (1994). Induction of systemic resistance to *Fusarium* crown and root rot in tomato plants by seed treatment with chitosan. *Journal of Phytopathology*, 84, 1432-1444.

Canakci, M. and Akinci I. (2006). Energy use pattern analysis of greenhouse vegetable production. *Energy*, 31, 1243-1256.

Cushnie, T. T. and Lamb, A. J. (2005). Antimicrobial activity of flavonoids. *International Journal of Antimicrobial Agents*, 2, 343-356.

Das, K., Tiwari, R.K.S. and Shrivastava, D.K. (2010). Techniques for evaluation of medicinal plant products as antimicrobial agent: current methods and future trends. *Journal of Medicinal Plants Research*, 4, 104-111.

Devendra, B. N., Srinivas, N., Prasad, V.S.S.L., and Latha, P.S. (2011). Antimicrobial activity of *Moringa oleifera* Lam., leaf extract, against selected bacterial and fungal strains. *International Journal of Pharmacy and Biological Sciences*, 2, 13-18.

Díaz Dellavalle, P., Cabrera, A., Alem, D., Larrañaga, P., Ferreira, F. and Dalla Rizza, M. (2011). Antifungal activity of medicinal plant extracts against phytopathogenic fungus Alternaria spp. *Chilean Journal of Agricultural Research*, 71, 231-239.

Dun-chun, H.E., Zhan, J. and Xie, L. (2016). Problems, challenges and future of plant disease management: from an ecological point of view. *Journal of Integrative Agriculture.* 15, 705-715.

Elad, Y., Pertot, I., Prado, A. M. C., and Stewart, A. (2016). Plant hosts of *Botrytis* spp. In: Fillinger, S.; Elad, Y (Eds). The Fungus, The Pathogen and its Management in Agricultural Systems. Springer, Cham, Switzerland.

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

Emad El Din, G.G., Esmaiel, N., Salem, M.Z. and Gomaa, S.E. (2016). *In vitro* screening for antimicrobial activity of some medicinal plant seed extracts. *Biotechnology for Wellness Industries*, 5,142-152.

Fawzi E.M., Khalil A.A. Afifi A.F. (2009). Antifungal effect of some plant extracts on *Alternaria alternata* and Fufixguscy *afeyrum*. *African Journal of Biotechnology*, 11, 2590-2597.

Fiori, A.C.G., Schwan-Estrada, K.R.F., Stangarlin, J.R, Vida, J.B, Scapim, C.A., Cruz, M.E.S. and Pascholati, S.F. (2000). Antifungal activity of leaf extracts and essential

oils of some medicinal plants against *Didymella bryoniae*. *Journal Phytopathology*, 148, 483-487.

Gifoni, J. M., Oliveira, J. T., Oliveira, H. D., Batista, A. B., Pereira, M. L., Gomes, A. S. and Vasconcelos, I. M. (2012). A novel chitin-binding protein from *Moringa oleifera* seed with potential for plant disease control. *Peptide Science*, *98*, 406-415.

Gurjar, M.S., Ali, S., Akhtar, M. and SingH, K.S. (2012). Efficacy of plant extracts in plant disease management. *Agricultural Sciences*, 3, 425-433.

Jamil, A., M. Shahid, Khan M.M. and Ashraf, M. (2010). Screening of some medicinal plants for isolation of antifungal proteins and peptides. *Pakistan Journal of Botany*, 39, 211-221.

Johnston, P.R., Hoksbergen, K., Park, D. and Beever, R.E. (2014). Genetic diversity of *Botrytis* in New Zealand vineyards and the significance of its seasonal and regional variation. *Plant Pathology*, 63, 888-898.

Nigro, F., Schena, L., Ligorio, A., Pentimone, I., Ippolito, A. and Salerno, M.G. (2006). Control of table grape storage rots by pre-harvest applications of salts. *Postharvest Biology and Technology*, 42, 142-149.

Palou, L., Usall, J., Smilanick, J.L., Aguilar, M.J. and Vinas, I. (2002). Evaluation of food additives and low-toxicity compounds as alternative chemicals for the control of *Penicillium digitatum* and *Penicillium italicum* on citrus fruit. *Pest Management Science*, 58, 459-466.

Pinto, C.E., Farias, D.F., Carvalho, A.F., Oliveira, J.T., Pereira, M.L., Grangeiro, T.B., Freire, J.E., Viana, D.A. and Vasconcelos, I.M. (2015). Food safety assessment of an antifungal protein from *Moringa oleifera* seeds in an agricultural biotechnology perspective. *Food and Chemical Toxicology*, 83, 1-9.

Satish, S., Mohana, D.C., Ranhavendra, M.P. and Raveesha, K.A. (2007). Antifungal activity of some plant extracts against seed borne pathogens of *Aspergillus sp*. *An International Journal of Agricultural Technology*, 3, 109-119.

Shahzad, U., Khan, M.A., Jaskani, M.J., Khan, I.A. and Korban, S.S. (2013). Genetic diversity and population structure of *Moringa oleifera*. *Conservation Genetics*, 14, 1161-1172.

Talibi, I., Askarne, L., Boubaker, H., Boudyach, E.H., Msanda, F., Saadi, B. and Ben Aoumar, A.A. (2012). Antifungal activity of Moroccan medicinal plants against citrus sour rot agent *Geotrichum candidum. Letters in Applied Microbiology*, 55,155-161.

Tesfay, S. Z. and Magwaza, L. S. (2017). Evaluating the efficacy of moringa leaf extract, chitosan and carboxymethyl cellulose as edible coatings for enhancing the quality and extending postharvest life of avocado (*Persea americana* Mill.) fruit. *Food Packaging and Shelf Life*, 11, 40-48.

Tijjani, A., Adebitan, S.A., Gurama, A.U., Haruna, S.G. and Safiya, T. (2014). Effect of some selected plant extracts on *Aspergillus flavus*, a causal agent of fruit rot disease of tomato in Bauchi State. *International Journal of Biosciences*, 4, 244-252.

Tripathi, P. and Dubey, N.K. (2004). Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. *Postharvest Biology and Technology*, 32, 235-245.

Vongsak, B., Sithisarn, P. and Gritsanapan, W. (2014). Simultaneous HPLC quantitative analysis of active compounds in leaves of *Moringa oleifera* Lam. *Journal of chromatographic science*, *52*(7), 641-645.

Williamson, B., Tudzynski, B., Tudzynski, P. and van Kan, J. (2007). *Botrytis cinerea*: the cause of grey mould disease. *Molecular Plant Pathology*, 8, 561-580.

Zaffer, M., Ganie, S. A., Gulia, S. S., Yadav, S. S., Singh, R. and Ganguly, S. (2015). Antifungal efficacy of *Moringa oleifera* Lam. *AJPCT*, *3*, 28-33.

Chapter 5

Integrated control of postharvest grey mould on tomatoes using ethanolic moringa leaf extract, *Serratia marcescens, Bacillus safensis and Bacillus pumilus*

Abstract

Grey mould caused by *B. cinerea* is one of the most destructive diseases of tomatoes in the greenhouse and causes great losses during the pre-harvest and postharvest periods of tomatoes. The research study aimed to evaluate the compatibility of moringa leaf extract when combined with *Serratia marcescens*, *Bacillus safensis* and *Bacillus pumilus* and; to evaluate the effect of integrating moringa leaf extracts and *Serratia marcescens*, *Bacillus safensis* and *Bacillus pumilus* against *B. cinerea* both *in vitro* and *in vivo*. Two concentrations (MLE 2% and MLE 3%) were integrated with *Serratia marcescens*, *Bacillus safensis* and *Bacillus pumilus* with three replicates per treatment. Potato dextrose agar (PDA) was amended with moringa leaf extract and allowed to solidify, then streaked the *Serratia marcescens, Bacillus safensis* and *Bacillus pumilus* to test their compatibility with moringa leaf extract. PDA media was amended with moringa leaf extract and biocontrol agent suspensions, the mycelial plug of *B. cinerea* was placed at the centre of the amended PDA media *in vitro*. *Serratia marcescens, Bacillus safensis* and *Bacillus pumilus* were compatible with MLE 2% and MLE 3%. The combination of *Serratia marcescens* and MLE 3% was the most effective treatment in inhibiting mycelia growth (≥ 50%) of *B. cinerea in vitro.* Tomato fruits were treated with *Serratia marcescens*, *Bacillus safensis* and *Bacillus pumilus* integrated with MLE 2% and MLE 3% and stored at 25 °C for 24 hrs. After 24 hours, tomato fruits were inoculated with the pathogen suspension of 1 x 10^5 spore/mL. Inoculated fruits were incubated at 25°C and the disease incidence was measured after 5, 7 and 10 days. The combination of *Bacillus safensis* and MLE 3% was the most effective treatment in inhibiting grey mould with ≥80% on tomatoes *in vivo*. The scanning electron microscopy observations were done *in vitro* and *in vivo* for the interaction of *B. cinerea* and biocontrol agents integrated with moringa leaf extract. The SEM images showed the shrinkage and lysis on the morphology of the *B. cinerea* mycelia. The integration of moringa leaf extract and biocontrol agents had

strong antifungal efficacy on mycelial growth and disease incidence of *B. cinerea* and can be used as potential alternative control.

Keywords: Grey mould, *Serratia marcescens*, *Bacillus safensis*, *Bacillus pumilus*, *Moringa oleifera*, integrated control, tomatoes.

5.1 Introduction

Grey mould caused by *Botrytis cinerea* Pers. is one of the most destructive diseases of tomato and causes great losses during the pre-harvest and postharvest of tomatoes (Elad *et al*., 2007; Williamson *et al*., 2007; Jones *et al*., 2014). Tomatoes are vulnerable to *B. cinerea* through injury during pruning, harvesting or direct penetration (Elad *et al*., 2007). The susceptible growth stages of tomatoes to *B. cinerea* are the flowering and fruit enlargement periods (Hong, 2012). The fungus cause infections on the leaves, stem and fruits leading to great losses in crop yield (Menzies and Jarvis, 1994). The significant environmental factors that promote infection by *B. cinerea* are high relative humidity, moisture, and favourable temperatures (O'Neill *et al*., 1997). Temperature from 10 to 25 °C coupled with relative humidity (90 to 100%) results in characteristic symptoms of *B. cinerea* (Ciliberti *et al*., 2015). *B. cinerea* can tolerate low temperatures (-1°C) when the relative humidity is high and produce high spore yields. The effect of grey mould is more severe after tomato harvest and hence prevention of *B. cinerea* is a key approach for the protection and development of tomato products worldwide (Dik and Elad, 1999).

Antagonistic microorganisms such as fungi, bacteria and yeast can be used to control preharvest and postharvest plant diseases through antibiosis (Aqueveque *et al*.,2017; Kasfi *et al*.,2018; Calvo-Garrido *et al*., 2019), competition for places and nutrients, volatile compounds (Mulero-Aparicio *et al*., 2019; Chen *et al*., 2019), parasitism (Köhl *et al*., 2019) and initiation of plant defence (Mhlongo *et al*., 2018). Moreover, there are plenty of advantages to the use of biological control including low toxicity, low or no residual levels, low pollution, safety, and efficiency. Several research discloses information on the application of antagonistic microorganisms to control tomato grey mould and leaf mould diseases (Smith, 1996; Yuan *et al*., 2017). Xi and Tian (2005), reported on the efficacy of *Cryptococcus laurentii* (Kufferath) Skinner in suppressing *B. cinerea* in post-harvest tomato and post-harvest decay caused by *Pythium*.

Phytopathologists are very interested in the use of fungal and bacterial antagonists for the control of various plant diseases.

Bacillus subtilis Cohn., *Pseudomonas fluorescens* Migula. and *Trichoderma harzianum* Rifai. are the commonly used fungal and bacterial biocontrol agents against various plant diseases (Nakkeeran *et al*., 2005; Saravanakumar *et al*., 2007). Soil bacteria such as *Bacillus* species including *B. subtilis*, *Bacillus pumilus*, *Bacillus amyloliquefaciens*, and *Bacillus licheniformis* possess fungicide and biofertilizer activity as they promote plant growth and health, (Pérez-García *et al*., 2011; Dimkić *et al*., 2013). *Moringa oleifera* is a medicinal plant that contains a variety of bioactive compounds and is used to treat a variety of diseases. Moringa extracts contain antibacterial alkaloids, steroids, triterpenes, flavonoids, and polyphenols. Furthermore, *M. oleifera* contains several antimicrobial peptides (Gomes *et.al*., 2018). Alkaloids, tannins, quinones, coumarins, phenolic compounds, and phytoalexins are all antifungal compounds found in moringa extracts (Fawcett and Spencer, 1970; Kagale *et al*., 2005).

According to Adandonon *et al*., (2006), moringa leaf extract has been combined with *Trichoderma* as an integrated biocontrol agent against *Sclerotium* damping-off and stem rot disease of cowpea in the field condition. However, limited information has been published on the effect of plant products when integrated with antagonistic microorganisms such as bacteria and yeast. Considering this, it is vital to investigate new and effective plant extracts and to test their compatibility with antagonistic microorganisms as the use of plant bio-fungicide treatments represents an environmentally friendly alternative to the use of chemical fertilizers and synthetic fungicides. The objectives of the study are (1) to evaluate the compatibility of plant extract when combined with antagonistic microorganisms and (2) to evaluate the effect of integrating plant extracts and antagonistic microorganisms against *B. cinerea* in both *in vitro* and *in vivo* screening.

5.2 Materials and methods

5.2.1 Compatibility of ethanolic moringa leaf extract and biocontrol agents

Potato dextrose agar (PDA) was prepared and autoclaved at 121 °C for 15 minutes. A volume of 10 mL of prepared ethanolic moringa leaf extract with the concentrations of MLE1%, MLE2%, MLE2.5% and MLE3% was amended with cooled PDA media

and poured on Petri dishes to solidify. *Serratia marcescens, Bacillus safensis* and *Bacillus pumilus* were streaked using the three-way steak method and the plates were sealed using parafilm. The plates were stored at 25°C for 48-72 hours. After 48 to 72 hours, the plates which showed the growth of biocontrol agents were selected as compatible with moringa leaf extract and were further used for *in vitro* screening against *B. cinerea.*

5.2.2 Effect of integrating *Serratia marcescens*, *Bacillus safensis*, *Bacillus pumilus* and ethanolic moringa leaf extract against *B. cinerea.*

The concentrations of moringa extract MLE 2% and MLE 3% were found to be compatible with biocontrol agents. A volume of 5 mL of prepared MLE 2% and MLE 3% and 5 mL of *Serratia marcescens*, *Bacillus safensis* and *Bacillus pumilus* and with a concentration of 1×10^8 cells/mL each were amended with PDA media and agitated to allow the proper mixing and media was poured into petri dishes to solidify. Once the amended media solidified in the laminar flow bench, the Petri dishes were inoculated with mycelial plugs (2mm x 2mm) excised from the 3-day old culture of *B. cinerea* and placed at the centre of each plate. Pure cultures of *Serratia marcescens*, *Bacillus safensis* and *Bacillus pumilus* that were grown on PDA were streaked on the amended PDA media using the inoculating loop on the two opposite sides about 3 cm from the pathogen mycelial plug. For the control, the mycelial plug was placed at the centre of the unamended fresh PDA. The trial was repeated twice with three replicates for each concentration. The mycelial growth was measured and recorded after 7 days and inhibition of *B. cinerea* by the integration of MLE 2% and MLE 3% and *Serratia marcescens, Bacillus safensis* and *Bacillus pumilus* was calculated.

5.2.3 *In vivo* screening of *Serratia marcescens, Bacillus safensis*, *Bacillus pumilus* and ethanolic moringa leaf extract against *B. cinerea* on tomato fruits.

Tomato ‘Jam’ fruits were harvested at Port Shepstone, KwaZulu-Natal, South Africa. Tomato fruits were sterilised with 70% ethanol and used distilled water to rinse and allowed to dry out at room temperature. Fruits were dipped into the following treatments: Pathogen control, 1×10^8 cells/mL of *Serratia marcescens* + MLE 2%, 1×10^8 cells/mL of *Bacillus safensis* + MLE 2%, 1×10^8 cells/mL of *Serratia marcescens* + MLE 3%, 1×10^8 cells/mL of *Bacillus safensis* + MLE 3% for five minutes and stored

at 25 °C for 24 hours. After 24 hours, fruits were dipped into the conidial suspension of *B. cinerea* (1×10^5 spores/mL) and placed in boxes, then stored at 25 °C and relative humidity of 95%. The experiments were repeated twice with 3 replicates per treatment. The results of disease incidence were observed and recorded after 5,7 and 10 days.

5.2.4 Scanning electron microscopy analysis of the interaction of integrating *Serratia* marcescens, *Bacillus safensis*, *Bacillus pumilus* and ethanolic moringa leaf extract against *B. cinerea*

B. cinerea mycelial disc (2 mm x 2 mm) was placed at the centre of the PDA media amended with *Serratia* marcescens, *Bacillus safensis*, and *Bacillus pumilus* and MLE 2% and MLE 3%. The plates were incubated for 7 days at 25°C. After seven days, the inhibition of mycelial growth and sporulation of *B. cinerea* was observed under scanning electron microscopy (SEM) Zeiss EVO LS15, Carl Zeiss NTS Ltd., Germany conducted at the Microscopy and Microanalysis Unit, University of KwaZulu-Natal, Pietermaritzburg, South Africa. Samples were cut from the prepared plates and tissue samples were held for 2 hours in fixation of 3% buffered glutaraldehyde and washed twice in 0.05M sodium cacodylate buffer for 5 minutes. The samples were then dehydrated with approximately 2 mL aliquots of 10%; 30%; 50% and 70% ethanol for 10 minutes per concentration. The samples were rinsed three times with 100% ethanol for 10 minutes to complete the dehydration process. Following that, the samples were placed in the Quorum K850 critical drying point dryer (CPD) basket with 100% ethanol. The ethanol was replaced with liquid carbon dioxide (CO_2) during CPD. The liquid CO_2 was heated and pressurized to the critical point at which the liquid turned into a gas without damaging the samples due to surface tension, leaving the samples dry and undamaged. Using black double-sided tape, the dried samples were carefully mounted onto SEM stubs. The sample stubs were transferred to the Quorum Q150R ES sputter coater. In this step, the samples were coated twice with gold and palladium to make them conductive to the electron beam. After drying, the samples were examined under the Zeiss EVO LS15 SEM.

5.2.5 Statistical analysis

Data obtained from the experiments was analysed using the analysis of variance (ANOVA) procedure using Genstat 20th Edition. The experiments were arranged in a completely randomised design with seven treatments and three replicates. Treatment

means were separated at a 5% significance level using Duncan's multiple range test at $P \leq 0.05$.

5.3 Results

5.3.1 Compatibility of ethanolic moringa leaf extract and *Serratia* marcescens, *Bacillus safensis,* and *Bacillus pumilus*.

Moringa leaf extract was found to be compatible with *Serratia marcescens*, *Bacillus safensis,* and *Bacillus pumilus* at all concentrations (Figure 5.1 A, B and C). The colonies of *S. marcescens*, *B. safensis,* and *B. pumilus* were observed on PDA media amended with ethanolic moringa leaf extract (Figure 5.1 A, B and C).

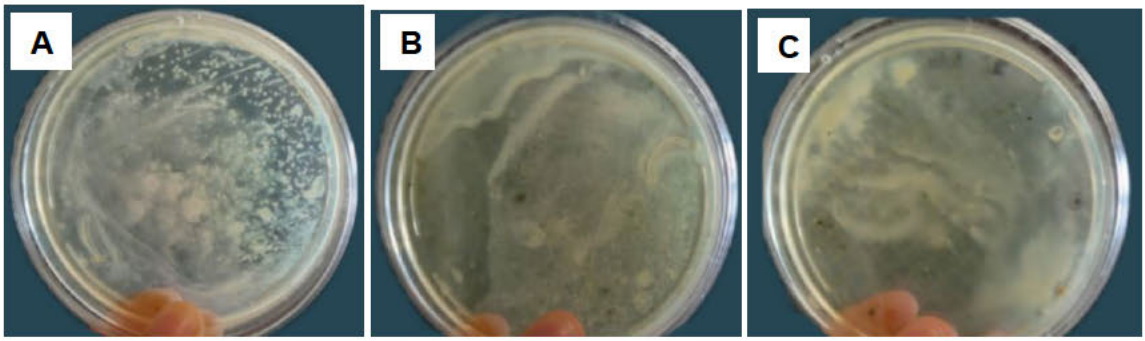


Figure 5.1: The bacterial growth of *Serratia marcescens* (A), *Bacillus safensis* (B) and *Bacillus pumilus* (C) on PDA media amended with moringa ethanolic leaf extract after 5 days at 25 °C.

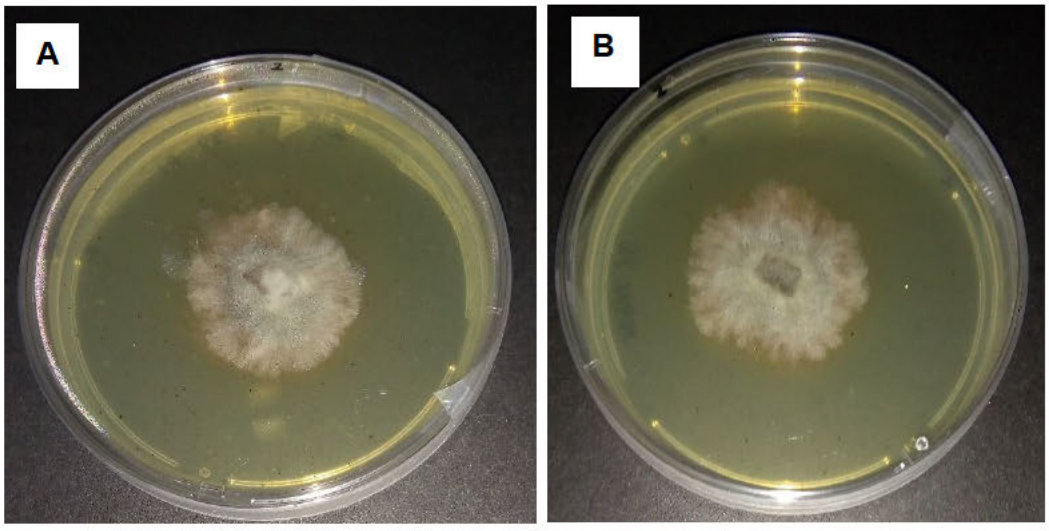
5.3.2 Effect of integrating *Serratia marcescens*, *Bacillus safensis, Bacillus pumilus* and moringa leaf extract against *B. cinerea in vitro.*

Two concentrations of moringa leaf extract (MLE 3% and MLE 2%) were integrated with three isolates of biocontrol agents. The integration of *Serratia marcescens* and MLE 3% inhibited the mycelial growth of *B. cinerea* with ≥ 50% (Table 5.2). There was a significant difference between the in inhibition of *B. cinerea* by the treatments compared to the control. The combination of *S. marcescens* and MLE 3% had the highest mycelial inhibition of *B. cinerea* which was above 50% as compared to the mycelial growth inhibition by *Bacillus safensis* and MLE 2%, and *Bacillus pumilus* and MLE 3% which had the reduced growth of *B. cinerea* (Figure 5.2 A, B and C). All the

treatments showed efficacy in inhibiting *B. cinerea* compared to the control (Figure 5.2 D).

Table 5.1: Inhibition of mycelial growth of *B. cinerea* by the combination of *Serratia marcescens*, *Bacillus safensis, Bacillus pumilus* and moringa ethanolic leaf extract on PDA after 7 days at 25 °C.

Integration	**Mycelial growth (mm)**	**Inhibition (%)**
Serratia marcescens and MLE 3%	38.00 a	55.29
Bacillus safensis and MLE 3%	49.67 b	41.56
Bacillus pumilus and MLE 3%	61.17 c	28.04
Bacillus pumilus and MLE 2%	72.83 d	14.32
Serratia marcescens and MLE 2%	76.00 de	10.59
Bacillus pumilus and MLE 2%	80.17 ef	5.68
Control	85.00 f	
P-value	<.001	
Fishers LSD	5.90	
CV%	5.1	



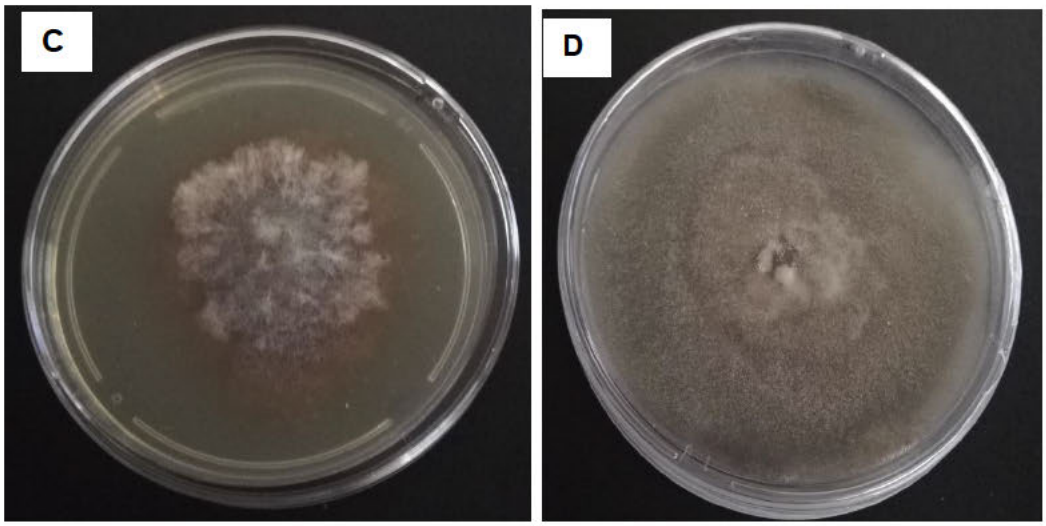


Figure 5.2: Mycelial growth of *B. cinerea* on PDA media amended with the integration of *Serratia* marcescens and MLE 3% (A), *Bacillus safensis* and MLE 3% (B) and *Bacillus pumilus* and MLE 3% (C) and the *B. cinerea* (D) after 7 days at 25 °C

5.3.3 *In vivo* screening of integrated *Serratia marcescens*, *Bacillus safensis*, *Bacillus pumilus* and moringa leaf extract against *B. cinerea* on tomato fruits.

The integration of moringa leaf extracts with *Bacillus safensis* and MLE 3%, *Serratia marcescens* and MLE 3% and *Bacillus safensis* and MLE 3% showed an antifungal effect against *B. cinerea* on tomatoes (Figure 5.4 A, B and C). All the treatments inhibited grey mould incidence in tomatoes by <50% (Table 5.4). The treatment *Bacillus safensis* and MLE 3% was more effective in suppressing the *B. cinerea* as compared to other treatments.

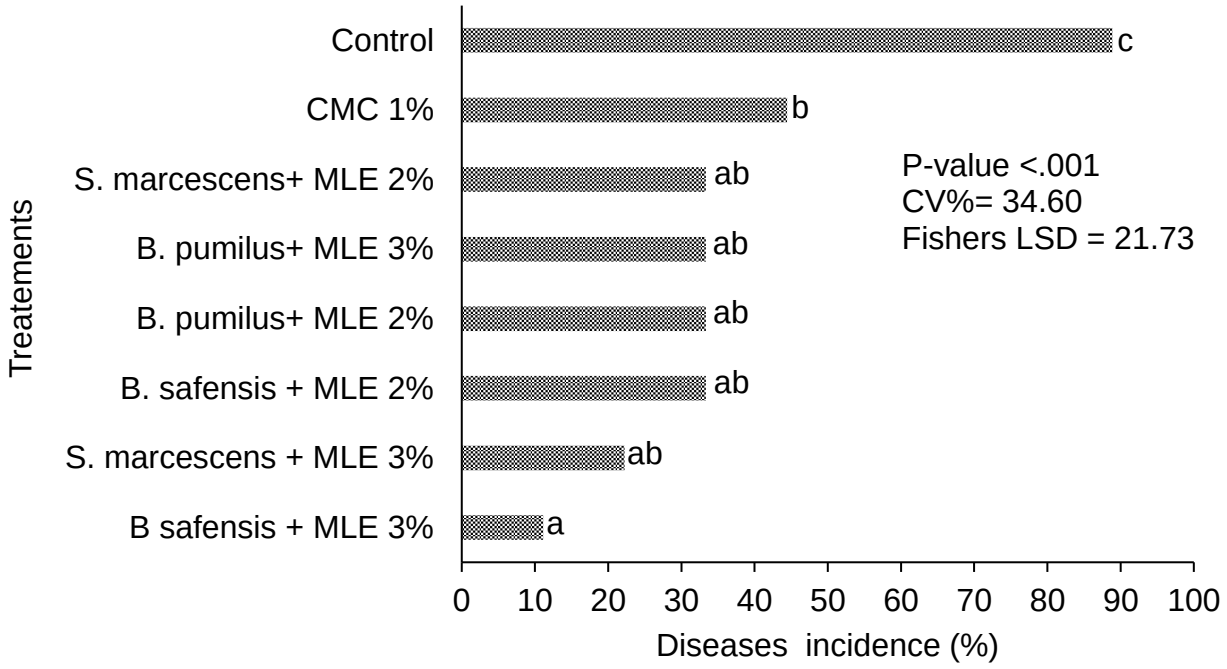


Figure 5.3: The disease incidence of grey mould incidence on tomatoes treated with integrated biocontrol agents and moringa leaf extract after 10 days at 25°C.

Figure 5.4: Grey mould on tomatoes inoculated with *B. cinerea* and treated with *Bacillus safensis* and MLE 3% (A), *Serratia* marcescens and MLE 3% (B) and *Bacillus pumilus* and MLE 3% (C) and untreated control fruits (D) after 10 days at 25 °C.

5.3.4 Scanning electron microscopy analysis of the integration of *Serratia marcescens*, *Bacillus safensis*, *Bacillus pumilus* and moringa leaf extract against *B. cinerea*.

The *B. cinerea* mycelia viewed under scanning electron microscope showed abnormal growth, shrinkage, and breakage (Figure 5.5 A, B and C) of mycelia and conidia (Figure 5.5 D). The damaged spores of *B. cinerea* caused by *Serratia* marcescens, *Bacillus safensis,* and *Bacillus pumilus* integrated with MLE 3% (Figure 5.6 A, B and

C). The control treatment micrograph for mycelium and spores was observed with normal growth (Figure 5.6 D)

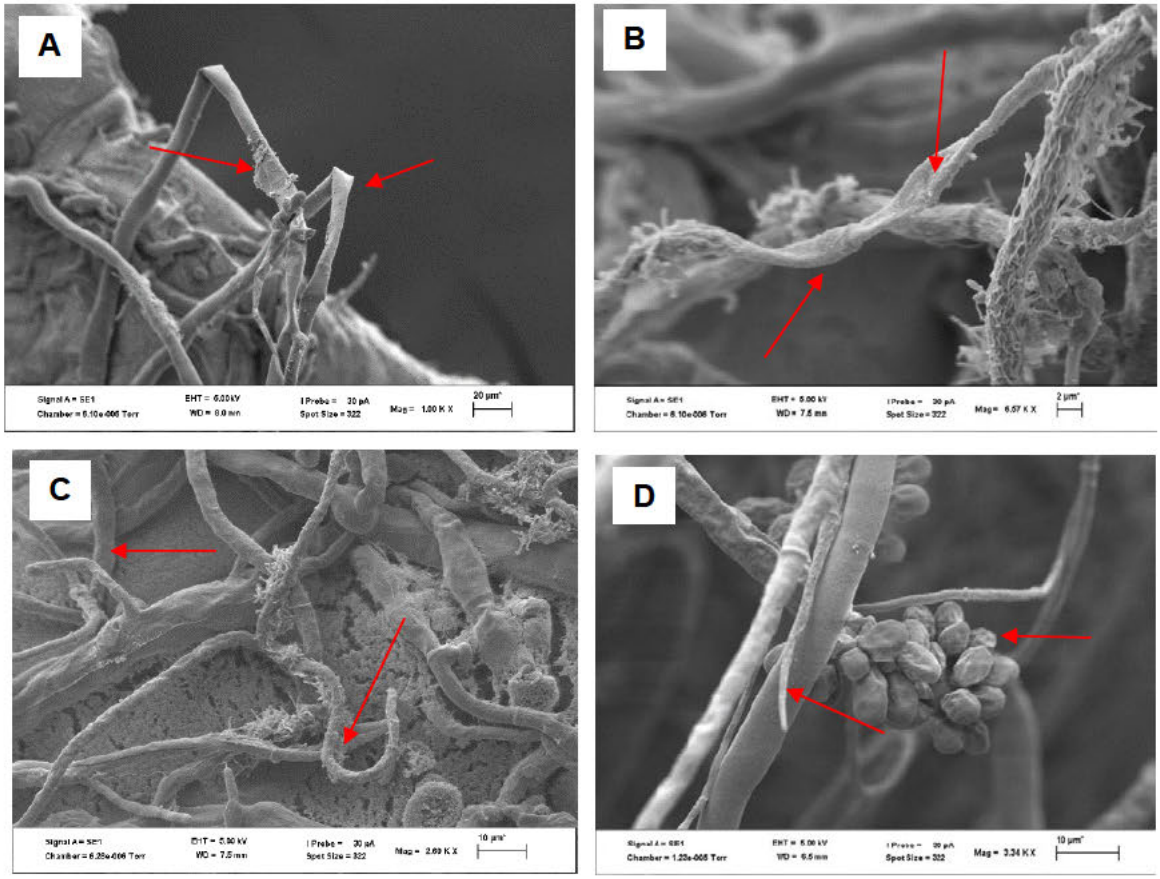


Figure 5.5: The scanning electron microscope images of *B. cinerea* mycelia treated with *Serratia* marcescens and MLE 3% (A), *Bacillus safensis* and MLE 3% (B) and *Bacillus pumilus* and MLE 3% (C) and conidia of *B. cinerea* (D). Red arrows indicates breaking and shrinkage mycelia of *B. cinerea* (A, B and C) and mycelia and spores of *B. cinerea* on PDA (D).

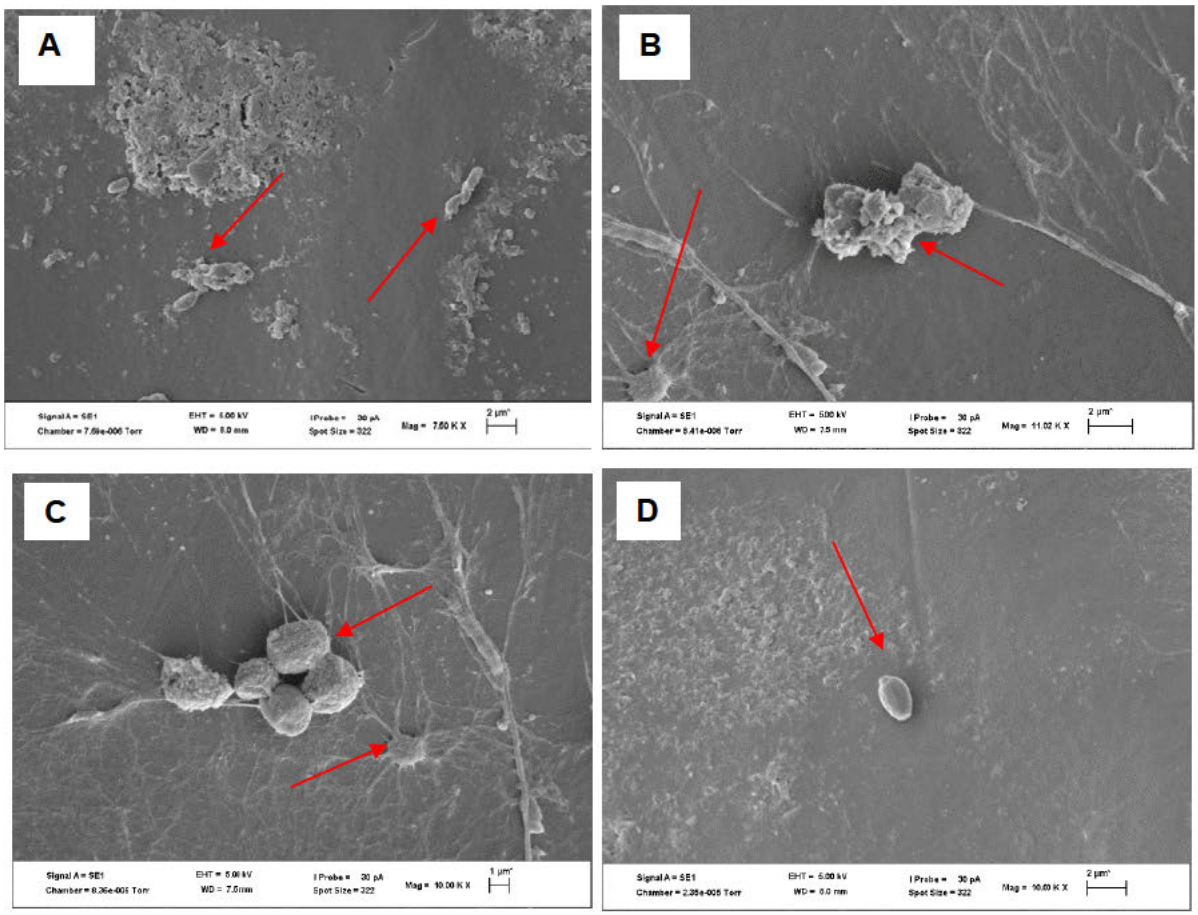


Figure 5.6: The scanning electron microscope images showing the interaction of *Serratia* marcescens and MLE 3% (A), *Bacillus safensis* and MLE 3% (B) and *Bacillus pumilus* and MLE 3% (C) and *B. cinerea* on tomato surface and control fruit with conidia of *B. cinerea* (D). Red arrows indicates damaged spores of *B. cinerea* (A, B and C) and mycelia and spores of *B. cinerea* on tomato surface (D).

5.4 Discussion

Tomato grey mould is a devastating disease caused by *B. cinerea*. Many studies have shown that certain beneficial microorganisms, such as biocontrol agents, can inhibit tomato grey mould, such as *Streptomyces* spp, *Bacillus* spp, and some fungi such as *Trichoderma* spp. (Barakat, 2008; Bardin *et al*., 2008; Abro *et al*., 2014). *Moringa oleifera* is one of the most valuable trees in the world because almost every part of the moringa tree can be used for food or has curative properties (Mehta *et al*., 2003; Khawaja *et al*., 2010). In this study, moringa leaf extracts integrated with *Serratia*

marcescens, *Bacillus safensis,* and *Bacillus pumilus* have shown efficacy in suppressing grey mould on tomatoes

The results obtained showed that the integration of moringa leaf extract with *Serratia marcescens*, *Bacillus safensis* and *Bacillus pumilus* successfully suppressed the incidence of grey mould and this is evident by lower disease incidence on the treated fruits as compared to the control. SEM micrographs of *B. cinerea* mycelium and conidia treated with the integration of *Serratia marcescens, Bacillus safensis,* and *Bacillus pumilus* with MLE 3% showed their negative impact on the pathogen morphology of both mycelia and conidia. The mycelia of *B. cinerea* had abnormal growth and reduced length and diameter. Moringa contains provides zeatin, quercetin, b-sitsterol, caffeoylquinic acid and kaempferol which are rich in antifungal and antibacterial activities (El-Mohamedy and Abdalla, 2014).

Most of these phytochemicals are secondary metabolites and compounds such as flavonoids and tannins (Mamphiswana *et al*., 2010; Shafighi *et al*., 2012), which are the main antifungal components linked to disease suppression. Furthermore, secondary metabolites form complexes with polysaccharides and proteins associated with the outer layer of fungal cells, which may result in pathogen death. Although plant extracts are high in secondary metabolites, their efficacy is not solely dependent on the presence of secondary metabolites but also on their concentration and integration with other components that influence plant extract activity against pathogens (Dzotam *et al*., 2016).

Plant extracts have been shown to support microbial growth because the nutrients needed by microorganisms for growth are directly supplied when they are combined with phyto-extracts. The mechanisms used by *Bacillus* spp. to inhibit pathogens include competition for nutrients and space, production of antibiotics, hydrolytic enzymes, siderophores, and/or inducing systemic resistance (Beneduzi *et al*., 2012). The commercial *Bacillus*-based products used worldwide contain beneficial strains of *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus*, *B. licheniformis*, *B. megaterium*, *B. velezensis*, *B. cereus* and *B. thuringiensis* (Mazzola and Freilich, 2017).

According to De Melo *et al*. (2009), *B. pumilus* had antifungal activity against *Rhizoctonia solani* Kühn, *Pythium aphanidermatum* (Edson) Fitzp*.*, and *Sclerotium rolfsii,* Curzi because of the production of lipopeptide pumilacidin from the surfactin

family. Moreover, *Bacillus* spp. relies on the proportion and diversity in the production of antibiotics for their antimicrobial activities (Meena and Kanwar, 2015). Zalila-Kolsi et al., (2016) and Bjelić *et al*., (2018) reported that *B. subtilis* effectively inhibited *Fusarium* clove rot of garlic and the head blight of wheat. The integration of moringa extract and biocontrol agent in all treatments was found to have an inhibitory effect on *B. cinerea*, as evidenced by a decrease in mycelial growth as moringa leaf extract concentration increased. The inhibition of mycelial growth observed by moringa leaf extracts and biocontrol agents was most likely caused by a disruption in pathogen membrane functions.

5.5 Conclusion

The study evaluated the antifungal activity of moringa plant extracts and biocontrol agents against *B. cinerea* which causes postharvest grey mould on tomato fruits. The integration of moringa leaf extract with biocontrol agents revealed that they significantly inhibit tomato grey mould. The plant extracts tested are easily accessible to smallholder farmers, easy to process, and environmentally friendly; they can thus be used as potential components of an integrated control measure against postharvest grey mould on tomatoes.

5.6 References

Abro, M.A., F. Lecompte, Bardin, M. and Nicot, P. C. (2014) “Nitrogen fertilization impacts biocontrol of tomato grey mould,” *Agronomy for Sustainable Development*, 34, 641-648.

Adandonon, A, Aveling, T.A.S., Labuschagne, N. and Tamo, M. (2006). Biocontrol agents in combination with *Moringa oleifera* extract for integrated control of *Sclerotium* caused cowpea damping-off and stem rot. *European Journal of Plant Pathology*, *115*, 409-418.

Aqueveque, P., Céspedes, C.L., Becerra, J., Aranda, M. and Sterner, O. (2017). Antifungal activities of secondary metabolites isolated from liquid fermentations of *Stereum hirsutum* (Sh134-11) against *Botrytis cinerea* (grey mould agent). *Food and Chemical Toxicology*, 109, 1048-1054.

Barakat, R. M. (2008). “The effect of *Trichoderma harzianum* in combination with organic amendment on soil suppressiveness to *Rhizoctonia solani*,” *Phytopathologia Mediterranea*, 47,11-19.

Bardin, M., Fargues, J. and Nicot, P. C. (2008). “Compatibility between biopesticides used to control grey mould, powdery mildew and whitefly on tomato,” *Biological Control*, 46, 476-483.

Beneduzi, A., Ambrosini, A. and Passaglia L.M.P. (2012). Plant growth-promoting rhizobacteria (PGPR): Their potential as antagonists and biocontrol agents. *Genetic Molecular Biology.* 4, 1044-1051.

Bjelić D., Ignjatov M., Marinković J., Milošević D., Nikolić Z., Gvozdanović-Varga J. and Karaman M. (2018). *Bacillus* isolates as potential biocontrol agents of *Fusarium* clove rot of garlic. *Zemdirbyste,* 105, 369-376.

Calvo-Garrido, C., Roudet, J., Aveline, N.; Davidou, L., Dupin, S. and Fermaud, M. (2019). Microbial antagonism toward *Botrytis* bunch rot of grapes in multiple field tests using one *Bacillus ginsengihumi* strain and formulated biological control products. *Frontiers in Plant Science*, 10, 105.

Chen, X., Wang, Y., Gao, Y., Gao, T. and Zhang, D. (2019). Inhibitory abilities of *Bacillus* isolates and their culture filtrates against the grey mould caused by *Botrytis cinerea* on postharvest fruit. *Plant Pathology Journal*, 35, 425.

Ciliberti, N., Fermaud, M., Roudet, J. and Rossi, V. (2015). Environmental conditions affect *Botrytis cinerea* infection of mature grape berries more than the strain or transposon genotype. *Phytopathology*, 105, 1090-1096.

De Melo, F.M.P.D., Fiore, M.F., Moraes, L.A.B.D., Silva-Stenico, M.E., Scramin, S., Teixeira, M.D.A. and Meloa I.S.D. (2009). Antifungal compound produced by the cassava endophyte *Bacillus pumilus* MAIIIM4A. *Scientia Agricola,* 66, 583–592.

Dik, A. and Elad, Y. (1999). Comparison of antagonists of *Botrytis cinerea* in greenhouse-grown cucumber and tomato under different climatic conditions. *European Journal of Plant Pathology*, 105, 123-137

Dimkic, I., Zivkovic, S., Beric, T., Ivanovic, Z., Gavrilovic, V. and Stankovic, S. (2013). Characterization and evaluation of two *Bacillus* strains, SS-12.6 and SS-13.1, as

potential agents for the control of phytopathogenic bacteria and fungi. *Biological Control,* 65, 312-321.

Dzotam J.K., Touani F.K., Kuete V. (2016). Antibacterial and antibiotic – modifying activities of three food plants (Xanthosoma mafaffa Lam., *Moringa oleifera* (L.) Schott and Passiflora edulis Sims) against multidrug-resistant (MDR) Gram-negative bacteria. *BMC complementary and alternative medicine,* 16, 1-8.

Elad, Y., Williamson, B., Tudzynski, P. and Delen, N. (2007). *Botrytis* spp. and diseases they cause in agricultural systems -an introduction. In Botrytis: Biology, Pathology and Control. Springer, Berlin/Heidelberg, Germany.

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.

Fawcett, G.H. and Spencer, D.M. (1970). Plant chemotherapy with natural products. *Annual Review of Phytopathology*,8, 403-418

Gomes, F., Martins, N., Barros, L., Rodrigues, M.E., Oliveira, M.B., Henriques, M. and Ferreira, I.C. (2018). Plant phenolic extracts as an effective strategy to control Staphylococcus aureus, the dairy industry pathogen. *Journal of Industrial Crops and Products*, 112, 515-520.

Hong, S.J. (2012). National Academy of Agricultural Science, RDA, Suwon, Republic of Korea, Study on the Control of Leaf Mould, Powdery Mildew and Grey Mould for Organic Tomato Cultivation. *Korean Journal of Organic Agriculture*, 20, 655-668.

Jones, J.B., Zitter, T.A., Momol, T.M. and Miller, S.A. (2014). Compendium of Tomato Diseases and Pests. APS Press, St. Paul MN, USA.

Kagale, S., Marimuthu. T., Thayumanavan, B., Nandakumar, R. and Samiyappan, R. (2005). Antimicrobial activity and induction of systemic resistance in rice by leaf extract of Datura metel against *Rhizoctonia solani* and *Xanthomonas oryzae* pv oryzae. *Physiological and Molecular Plant Pathology*, 65, 91-100.

Kasfi, K., Taheri, P., Jafarpour, B. and Tarighi, S. (2018). Identification of epiphytic yeasts and bacteria with potential for biocontrol of grey mould disease on table grapes caused by *Botrytis cinerea*. *Spanish Journal of Agricultural Research*, 16, e1002.

Khawaja, T.M., Tahira, M. and Ikram, U.K. (2010). *Moringa oleifera*: a natural gift - A review. *Journal Pharmacy Science Research,* 2, 775-81.

Köhl, J., Kolnaar, R. and Ravensberg, W.J. (2019). Mode of action of microbial biological control agents against plant diseases: Relevance beyond efficacy *Frontiers in Plant Science*, 10, 845.

Mamphiswana, N.D., Mashela, P.W. and Mdee, L.K. (2010). Distribution of total phenolic and antioxidant activity in fruit, leaf, stem and root of *Monsonia burkeana. African Journal of Agricultural Research*, 5, 2570-2575.

Mazzola, M. and Freilich, S. (2017). Prospects for biological soilborne disease control: Application of indigenous versus synthetic microbiomes. *Phytopathology,* 107, 256-263.

Meena, K.R. and Kanwar, S.S. (2015). Lipopeptides as the antifungal and antibacterial agents: Applications in food safety and therapeutics. *BioMed Research International.* 2015, 473050.

Mehta, L.K, Balaraman, R, Amin, A.H, Bafna, P.A. and Gulati, O.D. (2003). Effects of fruits of *Moringa oleifera* on the lipid profile of normal and hypo-cholesterolemic rabbits. *Journal of Ethnopharmacology,* 86, 191-195.

Menzies, J. and Jarvis, W. (1994). Grey Mould. Diseases and Pests of Vegetable Crops In: Howard, R., Garland, J. and Seaman, W. (Eds). The Canadian Phytopathological Society and Entomological Society, Ottawa, Canada

Mhlongo, M.I., Piater, L.A., Madala, N.E., Labuschagne, N. and Dubery, I.A. (2018). The chemistry of plant–microbe interactions in the rhizosphere and the potential for metabolomics to reveal signalling related to defence priming and induced systemic resistance. *Frontiers in Plant Science*, 9, 112.

Mulero-Aparicio, A., Cernava, T., Turra, D., Schaefer, A., Pietro, A.D., Escudero, F.J.L., Trapero, A. and Berg, G. (2019). The role of volatile organic compounds and rhizosphere competence in the mode of action of the non-pathogenic *Fusarium oxysporum* FO12 towards Verticillium wilt. *Frontiers in Microbiology*, 10, 1808.

Nakkeeran, S., Renukadevi, P. and Marimuthu, T. (2005). Antagonistic potentiality of Trichoderma viride and assessment of its efficacy for the management of cotton root rot. *Archives of Phytopathology and Plant Protection*, 38, 209-225

O'Neill, T. M., Shtienberg, D. and Elad, Y. (1997). "Effect of some host and microclimate factors on infection of tomato stems by *Botrytis cinerea*," Plant Disease, 81, 36-40.

Pérez-García, A., Romero, D., and de Vicente, A. (2011). Plant protection and growth stimulation by microorganism: biotechnology applications of *Bacilli* in agriculture. *Current Opinion in Biotechnology*, 22, 187-193.

Saravanakumar, D., Vijayakumar, C., Kumar, N. and Samiyappan, R. (2007). PGPR-induced defense responses in the tea plant against blister blight disease. *Crop Protection*, 26, 556-565

Shafighi, M., Amjad, L. and, Madaniin, M. (2012). *In vitro* antifungal activity of methanolic extract of various parts of *Punica granatum* (L.). *International Journal of Engineering Science*,3, 2229-5518.

Smith, S.M. (1996). Biological control with Trichogramma: Advances, successes, and potential of their use. *Annual Review of Entomology*, 41, 375-406.

Williamson, B., Tudzynski, B., Tudzynnski, P. and van Kan, J.A. (2007). *Botrytis cinerea*: the cause of grey mould disease. *Molecular Plant Pathology,* 8, 561-580.

Xi L. and Tian S.P. (2005) Control of postharvest diseases of tomato fruit by combining antagonistic yeast with sodium bicarbonate. *Scientia Agricultura Sinica.* 38, 950-955

Yuan, Y., Feng, H., Wang, L., Li, Z., Shi, Y., Zhao, L., Feng, Z. and Zhu, H. (2017). Potential of Endophytic Fungi Isolated from Cotton Roots for Biological Control against Verticillium Wilt Disease. *PLoS ONE*, 12, e0170557.

Zalila-Kolsi, I., Mahmoud, A.B., Ali H., Sellami, S., Nasfi, Z., Tounsi, S. and Jamoussi, K. (2016). Antagonist effects of *Bacillus* spp. strains against *Fusarium graminearum* for protection of durum wheat (*Triticum turgidum* L. subsp. *durum). Microbiological Research,* 192,148-158.

Chapter 6

Thesis overview

6.1 Introduction

Tomato is one of the most vegetables produced globally. It is grown commercially, by subsistence, resource-poor farmers and home gardeners in South Africa. Tomato is primarily grown in open fields in South Africa, with a small number of farmers using soilless systems under controlled conditions (Maboko *et al,* 2009). However, postharvest diseases that affect fruits and vegetables result in significant losses in food production. Fruit rots are typically caused by opportunistic pathogens, which cannot infect fruit tissues directly unless the tissues are stressed. Grey mould is caused by *Botrytis cinerea* and is known as one of the most catastrophic postharvest diseases of tomatoes. Infection of tomatoes by *B. cinerea* results in great economic losses at the pre-and post-harvest stages of tomato fruits. *B. cinerea* can affect more than 200 crop species including tomatoes (Elad *et al*., 2004). Grey mould causes substantial financial losses worldwide (Wang *et al*., 2013; Hua *et al*., 2018) and crops can be infected by injury after pruning and harvesting or direct penetration.

Postharvest storage is not only affected by diseases but can be affected by various factors such as tomato variety, climatic conditions, cultivation conditions, degree of ripeness during harvest, and storage conditions (Raheem *et al*., 2019; Ben-Arie and Lurie, 1986., Tolesa and Workneh *et al*., 2017). Controlling temperature between the period of harvest and consumption is an important factor in maintaining the quality of fruits and vegetables (Kitinoja and Kader, 2015; Kabir et al., 2019). Meticulous research has been conducted worldwide with great intention to find bacteria possessing antagonistic microorganisms against *B. cinerea* (Elmer and Reglinski, 2006; Compant *et al*., 2013). The significance of these studies was to combine all possible and available methods to control diseases in a way to optimise their benefits and minimize their risks for producers, consumers, and the environment.

6.2 Research objectives and major findings

This study aimed to investigate the effect of integrating biocontrol agents and *Moringa oleifera* leaf extract to control *B. cinerea* of tomatoes *in vitro* and *in vivo*. The specific objectives were as follows: (1) Evaluate the *in vitro* and *in vivo* screening of antagonistic microorganisms against *B. cinerea* of tomato. (2) Evaluate the *in vitro* and *in vivo* screening of moringa leaf extracts against *B. cinerea* of tomato. (3) Determine the mode of action of biocontrol agents and moringa extract against *B. cinerea* on tomato fruits. (4) Evaluate the integration of biocontrol agents and moringa extract on of tomato fruit. The major research findings are highlighted below.

Chapter 3: *In vitro* and *in vivo* screening of antagonistic microorganisms against *Botrytis cinerea* of tomato.

- B23 (*Serratia marcescens*), B1 *(Bacillus safensis*) and B11 (*Bacillus pumilus*) isolates successfully suppressed the pathogen and showed higher efficacy against *B. cinerea* both *in vitro* and *in vivo*.
- B23 showed chitinolytic activities on the SEM images *in vitro* and it was evidenced by the breakage of the hyphal cell wall.
- B1 and B11 revealed antifungal properties which were evidenced by the shrinking and breaking of the mycelial of the pathogen.
- SEM images have shown the presence of bacteria on the tomato peel and very few spores were produced.
- The bacteria are competing for space essential nutrients resulting in reduced growth of the pathogen.

Chapter 4: Antifungal effect of moringa leaf extract on postharvest tomatoes incited by *Botrytis cinerea*.

- All concentrations of moringa leaf extract (MLE1%, MLE2%, MLE3%, MLE4%) showed the antifungal effect against the mycelial growth of *B. cinerea*.
- Both MLE 2% and MLE 3% showed higher efficacy in inhibiting fungal growth of *B. cinerea* both *in vitro* and *in vivo*.

- The SEM images showed that moringa leaf extract was affecting the morphology of the pathogen hyphae *in vitro*.
- The SEM images showed that plant extract deposits were found on the tomato peel which reduced the spread of the pathogen on the fruit resulting in a reduced disease incidence of grey mould *in vivo*.

Chapter 5: Integrated control of postharvest grey mould on tomatoes using moringa leaf extract, *Serratia* and *Bacillus* species.

- Biocontrol agents were compatible with moringa leaf extract on the amended PDA plate.
- *Serratia marcescens*, *Bacillus safensis* and *Bacillus pumilus* inhibited the growth of grey mould alone. However, their efficacy was improved when combined with MLE 2% and MLE 3%.
- Scanning electron microscope showed damaged and lysed mycelia of *B. cinerea* and reduced production of pathogen spores.
- The SEM images showed tomato peel with a combination of bacteria and moringa leaf extract which was reducing the production of spores on the fruits.

6.3 Recommendations and conclusion

To minimise postharvest grey mould on tomato fruit, alternative economically viable methods are required. Antifungal and antimicrobial properties found in numerous plant extracts can be used for crop protection. Biocontrol agents and moringa leaf extract used in this study have shown an inhibitory and antifungal effect on grey mould of tomato. Integrated control of grey mould on tomato using moringa leaf extract and biocontrol agents can be considered as a potential method postharvest. To further this study, investigating the effect of edible coatings incorporated with moringa leaf extract and biocontrol agents (*Serratia marcescens* and *Bacillus safensis*) on the phytochemicals, antioxidant activitiesand defence mechanism, quality of tomatoes such as weight loss, fruit firmness and colour is crucial. This study is showing

significant outcome of the effectiveness and potential use of the combination of biocontrol agents and moringa leaf extract as an alternative to synthetic fungicides.

6.4 References

Ben-Arie, R. and Lurie, S. (1986). Prolongation of fruit life after harvest. In: Monselise, S.P. (Ed.). In Handbook of Fruit Set and Development; CRC Press, Boca Raton, USA.

Calvo-Garrido, C., Roudet, J., Aveline, N., Davidou, L., Dupin, S. and Fermaud, M. (2019). Microbial antagonism toward Botrytis bunch rot of grapes in multiple field tests using one *Bacillus ginsengihumi* strain and formulated biological control products. *Frontiers in Plant Science*, 10, 105.

Compant, S., Brader, G., Muzammil, S., Sessitsch, A., Lebrihi, A. and Mathieu, F. (2013). Use of beneficial bacteria and their secondary metabolites to control grapevine pathogen diseases. *Biocontrol*, 58, 435-455.

Elad, Y., Williamson, B., Tudzinski, P., Delen, N. (2004). Botrytis spp. and diseases they cause in agricultural systems – an introduction. In: Elad, Y.; Williamson, B.; Tudzinski, P.; Delen, N. (Eds). Botrytis: Biology, Pathology and Control.The, Kluwer Academic Publishers, Dordrecht, Netherlands.

Elmer, P.A.G. and Reglinski, T. (2006). Biosuppression of *Botrytis cinerea* in grapes. *Plant Pathology*, 55, 155-177.

Hua L., Yong C., Zhanquan Z., Boqiang L., Guozheng Q., Shiping T. (2018). Pathogenic mechanisms and control strategies of *Botrytis cinerea* causing post-harvest decay in fruits and vegetables. *Food Quality and Safety.* 2, 111-119.

Kabir, M.S.N., Chowdhury, M., Lee, W.H., Hwang, Y.S., Cho, S.I. and Chung, S.O. (2019). Influence of delayed cooling on quality of bell pepper (*Capsicum annuum* L.) stored in a controlled chamber. *Emirates Journal of Food and Agriculture*. 31, 271-280.

Kitinoja, L. and Kader, A.A. (2015). Small-Scale Postharvest Handling Practices: A Manual for Horticultural Crops, 5th ed.; Postharvest Horticulture Series No. 8E; University of California, Postharvest Technology Research and Information Center, Davis, CA, USA.

Maboko M.M., du Plooy, C.P. and Bertling, I. (2009). Comparative performance of tomato on soilless vs in-soil production systems. *Acta Horticulturae*, 843, 319-326.

Raheem, D., Shishaev, M. and Dikovitsky, V. (2019). Food system digitalization as a means to promote food and nutrition security in the Barents region. *Agriculture*, 9, 168.

Tolesa, G.N. and Workneh, T.S. (2017). Influence of storage environment, maturity stage and pre-storage disinfection treatments on tomato fruit quality during winter in KwaZulu-Natal, South Africa. *Journal of Food Science and Technology*, 54, 3230–3242.

Wang J., Xia X.M., Wang H.Y., Li P.P., Wang K.Y. (2013). Inhibitory effect of lactoferrin against grey mould on tomato plants caused by *Botrytis cinerea* and possible mechanisms of action. *International Journal Food Microbiology,* 161, 151-157.

Appendices

Table 1: Primary screening of bacterial isolates against *B. cinerea in vitro*.

Source of isolation	**Treatment**	Mean±SE	%Inhibition	Group
	Control	85.00±0.00	0.00	0
Solanum lycopersicum fruit	B1	44.67±0.88	47.45	2
Solanum lycopersicum fruit	B2	42.67±1.20	49.80	2
Solanum lycopersicum fruit	B3	85.00±0.00	0.00	1
Solanum lycopersicum fruit	B4	50.00±6.11	41.18	2
Solanum lycopersicum fruit	B5	39.67±0.33	53.33	3
Solanum lycopersicum fruit	B6	85.00±0.00	0.00	1
Solanum lycopersicum fruit	B7	45.33±2.03	46.67	2
Solanum lycopersicum fruit	B8	44.67±0.33	47.85	2

Solanum lycopersicum fruit	B9	47.33±2.85	44.12	2
Solanum lycopersicum fruit	B10	41.00±1.00	51.76	3
Solanum lycopersicum fruit	B11	42.67±1.20	49.80	2
Solanum lycopersicum fruit	B12	41.67±1.20	50.98	2
Solanum lycopersicum fruit	B13	41.67±1.33	50.98	2
Solanum lycopersicum fruit	B14	85.00±0.00	0.00	1
Solanum lycopersicum fruit	B15	38.67±2.40	54.51	3
Solanum lycopersicum fruit	B16	85.00±0.00	0.00	1
Solanum lycopersicum fruit	B17	40.33±2.60	52.55	3
Solanum lycopersicum fruit	B18	45.00±0.58	47.06	2
Solanum lycopersicum fruit	B19	71.67±13.33	15.68	1
Solanum lycopersicum fruit	B20	71.00±1.53	16.08	1
Solanum lycopersicum fruit	B21	41.33±0.00	51.38	2
Solanum lycopersicum fruit	B22	85.00±0.00	0.00	1
Solanum lycopersicum fruit	B23	44.33±0.67	47.85	2
Solanum lycopersicum fruit	B24	45.00±2.65	47.06	2
Solanum lycopersicum fruit	B25	55.67±8.29	34.51	2
Opuntia stricta leaves	1FL	32.33±1.45	61.96	3
Opuntia stricta leaves	7X5	85.00±0.00	0.00	1

Table 2: The primary screening of *B. cinerea* inhibited by yeast isolates *in vitro.*

Source of isolation	**Treatment**	**Mean±SE**	**%Inhibition**	**Group**
	Control	85.00±0.00	0.00	0
Opuntia stricta leaves	MeRo5y	44.00±2.08	48.24	2
Opuntia stricta leaves	MeRo2y	85.00±0.00	0.00	1
Opuntia stricta leaves	MeRo4y	76.33±8.67	10.20	1
Opuntia stricta leaves	YAL	50.00±1.00	41.18	2
Opuntia stricta leaves	YMO	85.00±0.00	0.00	1
Opuntia stricta leaves	R1Y1	44.33±2.33	47.85	2
Opuntia stricta leaves	SYB	50.33±1.20	40.79	2
Opuntia stricta leaves	3YL	85.00±0.00	0.00	1

Opuntia stricta leaves	OLC21	85.00±0.00	0.00	1
Opuntia stricta leaves	OLC23	84.67±0.33	0.39	1
Opuntia stricta leaves	WDB41	85.00±0.00	0.00	1
Opuntia stricta leaves	LLC22	44.33±5.33	47.85	2
Opuntia stricta leaves	WDS2	76.33±4.67	10.20	1
Opuntia stricta leaves	WD31	78.00±1.15	8.24	1
Opuntia stricta leaves	BJB14	71.33±9.21	16.08	1
Opuntia stricta leaves	LLB3/34	81.00±0.00	5.09	1
Ganoderma resinaceum	Kg1	30.00±1.00	64.71	3
Ganoderma resinaceum	Kg4	30.67±1.20	63.92	3
Ganoderma resinaceum	Kg5	38.00±1.73	55.29	3
Ganoderma resinaceum	Bb	35.67±1.20	58.04	3
Ganoderma resinaceum	Bs	37.67±0.88	55.68	3
Ganoderma austroafricanum	NG1X	44.67±2.40	40.33	2
Ganoderma austroafricanum	NG3X	40.67±2.96	52.15	3
Ganoderma austroafricanum	NG5	73.33±6.01	13.73	1
Ganoderma resinaceum	KgX	45.33±0.88	46.67	2
Ganoderma resinaceum	KgX1	68.00±10.44	20.00	1