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Investigating the antifungal efficacy of moringa leaf extracts against *Fusarium oxysporum*, a causal agent of fusarium dry rot

By

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A dissertation submitted in fulfilment of the requirements for the degree of

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General Abstract

Fungal diseases are amongst the most-destructive potato pathogens worldwide. Potato (Solanum tuberosum L.) is one of the most-common and -widely consumed crops in South Africa (SA). Population growth in SA is continuously putting pressure on the potato production, which has subsequently accelerated mono-cropping and chemical use, particularly in an attempt to control fungal diseases. There are currently no fusarium dry rot- resistant cultivars and no commercial biological control agents available to reduce occurrence of this disease. In an attempt to reduce agricultural pollution, and effectively control fusarium dry rot, plant extracts have been investigated for their antifungal properties. Moringa oleifera Lam. (moringa) is a tree well-known for its wide phytochemical composition and its antifungal abilities have been documented, but not on potato dry rot.

This study was, therefore, carried out to investigate the antifungal potential of moringa leaf extracts against Fusarium oxysporum, the causal agent of fusarium dry rot of potato. Various potato cultivars were used, based on seasonal availability. Since the moringa plant is containing high amounts of secondary metabolites, in this study, first, the phytochemical composition of Moringa leaf excracts (MLEs) and the effect of MLE treatment on the phenolic concentration of tubers were analysed. The Moringa leaf powder was extracted with either (30%, 50% or 80%) of acetone, ethyl acetate, methanol and the other moringa powder eas extracted with distilled water. Various MLEs were tested for their capability to improve tuber quality post- harvest by assessing potato quality parameters, such as percentage mass loss and firmness. An in vitro assay was carried out to evaluate the moringa leaf extracts' (MLEs') inhibition activity on F. oxysporum using a disc diffusion method. The ability of MLEs to prevent fusarium dry rot development and to delay disease progression was also investigated in in vivo assays.

Phytochemical analysis of MLEs revealed the presence of tannins, phenolics, flavonoids and glycosides. Tannins were, however, absent in ethyl acetate MLEs. Treatment with MLEs enhanced the concentration of free and bound phenolics in 'BP1' and 'Mondial' potatoes. Tubers coated with methanol-MLE had the highest concentration of both, free and bound phenolics.

Treatment with MLEs slowed down average percentage mass loss (seemingly water loss) in both cultivars. Treatment with 70% ethyl acetate MLE, however, accelerated this water loss in both cultivars, but particularly in 'Mondial'. This average reduction in mass was slightly less than the

average reduction in mass of control tubers. Moringa treatment also preserved the healthy appearance of tuber skin and tuber firmness more so than the control.

Macroscopic characteristics of F. oxysporum were pinkish colonies and a dark pigmentation, as observed on the PDA plates. The conodogenous cell was long and branched and the macroconidia had three to five septations. The fungus was found to be pathogenic on both cultivars used in this experiment, as tubers had dark depressions, typical for fusarium dry rot, and a white mycelium on the tuber surface. The efficacy of MLEs as antifungal agents was tested using a disc diffusion method. Three solvents were used to produce MLE stock solutions, namely acetone, ethyl acetate, ethanol, at three concentrations (30%, 50% and 70%). These solvent solutions were each further diluted to yield 2% and 4% concentrations, yielding eight MLE treatments. The dilutions of the MLEs produced with lower solvent concentrations (30% and 50%) had 100% fungal inhibition activity, while dilutions of MLEs extracted with the higher solvent concentration (70%) inhibited fungal development to a lesser degree. The 2% and 4% dilutions of 70% acetone, 70% ethyl acetate, and 70% methanol; resulted in 95.0%, 86.3% and 90.3% inhibition, respectively. Water-MLE, on the other hand, inhibited the fungus only by 87.5%. These results indicate that the extraction solvent and its concentration influence the antifungal efficiency of MLEs.

In vivo assays demonstrated that MLEs were able to prevent disease development to a certainextent. Solvent type and concentration were influential in preventing fusarium dry rot development, as tubers treated with either 30% and 50% of acetone-MLE and methanol-MLE concentrations had the smallest average lesion diameter (3.7 mm and 3.4 mm for 'Valor' and 'Mondial', respectively). The 70% Ethyl acetate-MLE, on the other hand, was ineffective in controlling fusarium dry rot in both cultivars, resulting in an average lesion diameter of 14 mm; this was not significantly different from the control, which had an average lesion diameter of 15 mm ($P>0.05$) in 'Valor' tubers. In 'Mondial', 70% ethyl acetate-MLE treatment resulted in an average lesion diameter of 11 mm.Further, tubers of both cultivars treated with 50% and 70% ethyl acetate-MLE were prone to secondary infections by bacterial soft rot.

The ability of MLE to delay and slow fusarium dry rot development is an indication of its antifungal potential. Response to MLE treatment was found to be cultivar-dependent, as following MLE treatment, 'Mondial' was more tolerant to fusarium dry rot than 'Valor'. Solvent type and concentration were also found to influence MLE antifungal activity. Tubers treated with MLEs extracted with higher solvent concentrations (70%) as well as those treated with ethyl acetate MLEs

were less tolerant to fusarium dry rot. This research, therefore, demonstrates that lower organic solvent (30% and 50%) concentrations should be used, when preparing antifungal extracts of moringa leaf powder.

Declaration

I, Carren Nonhlahla Mncube, declare that

1. The research reported in this dissertation, except where otherwise indicated, is my originalresearch

2. This dissertation has not been submitted for any degree examination at any other university

3. This dissertation does not contain other person's data, pictures, graphs or other information,unless specifically acknowledged as being sourced from other researchers.
Where other written sources have been quoted, the following measured were applied:

   a. Statements have been re-written, but the general information attributed to themhas been referenced.

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Signed

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Prof. I. Bertling (Supervisor)

Signed_____ Dr. K.S Yobo (Co-supervisor)

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Dedication

To my mother, Sikhulusini Mncube

Thank you for your prayers, love, enormous support, and encouragement

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Abbreviations

MLE	Moringa leaves extracts
Acetone-MLE	Acetone moringa leaves ectracts
Ethyl acetate-MLE	Ethyl acetae moringa leaves extracts
Methanol-MLE	Methanol moringa leaves extracts
Water-MLE	Water moringa leaves extracts
Mo-CBP3	Moringa chitin binding protein
F. oxysporum	*Fusarium oxysporum*
SM	Secondary metabolites
TBZ	Thiabendazole
SEM	Soxhlet extraction method
SAE	Solvent-assisted extraction
MAE	Microwave-assisted extraction
UAE	Ultrasound-assisted extraction
SSA	Sub-Saharan Africa
MIC	Minimum inhibitory concentration
PDA	Potato dextrose agar

General Introduction

1. Rationale for the Research

Potato is a staple crop of high economic importance worldwide. It is widely available and affordable, an important criterion for its prevalence in developing countries (Zaheer and Akhtar, 2016; Shahbandeh, 2020). Potato production is, however, limited by many phytofungal diseases, such fusarium dry rot, common scab as well as early and late blight. These diseases reduce potato production in field and greenhouse cultivation, but become particularly destructive postharvest, leading to tuber loss and tuber unmarketability. Climate change has accelerated the appearance of new pathogen strains, which are more virulent and resistant to chemical fungicides than previously known strains. Although some of these fungicides are currently very effective and widely used, they are, however, undesirable, as they can become toxic to the environment and to most animals, including humans. The need for inexpensive, effective, safe and accessible means to control plant diseases, that are neither harmful to human health nor to the environment, is becoming more and more pressing (Food and Agriculture Organization, 2008). Higher plants, such as *Moringa oleifera*, contain antimicrobial compounds that can be used to control potato diseases (Moyo, 2012). These compounds include phenolics, chitin-binding proteins, and glucosinolates (Gifoni et al., 2012; Freire et al., 2015). According to Al-Husnan and Al-Kahtani (2016), the antibacterial activity of moringa is attributed to 4-(40-O-acetyl-a-L-rhamnopyranosyloxy) benzyl isothiocyanate. In a study by Augusta and Nwakaego (2016), moringa leaf extracts (MLEs) were found to inhibit the mycelial growth of *F. oxysporum*. The moringa chitin-binding protein has been found to be the main component that inhibits fungal growth and sporulation. There is, however, insufficient research conducted to evaluate the efficacy of moringa extract as a bio-pesticide under greenhouse and field conditions. This study was, therefore, carried out to evaluate the potential of moringa as an antifungal agent *in vitro* and under greenhouse conditions.

2. Aims and objectives of the study

1. Aim

The overall aim of this research was to investigate the antifungal efficacy of various moringa leaf extracts (MLEs) against fusarium dry rot, a devastating disease of potato diseases in South Africa.

2. Objectives

The specific objectives of this study were:

a. To examine the effect of MLE treatment on the phytochemical concentration of two potato cultivars, namely, 'Mondial' and 'BP1', so as to determine whether the response to MLE treatment is cultivar-dependent.
b. To evaluate the effect of MLE treatment on physical parameters of 'BP1' and 'Mondial' tubers (percentage mass loss, firmness, and visual appearance).
c. To compare the efficacy of the type of organic solvent and the organic solvent concentration on moringa leaf powder extraction.
d. To investigate the efficacy of various MLEs (prepared using different extraction solvents) on *Fusarium oxysporum* inhibition activity *in vitro.*
e. To investigate the potential of MLEs extracted with different solvents at different concentrations as bio-fungicide against fusarium dry rot *in vivo*.

Outline of dissertation

This dissertation is structured into four sections (**1-4**), with each section addressing a specific topic.

Section I: Outlines the research rationale and significance of the study. This section further gives a brief overview of the research aim and objectives. The literature review in this section provides background information on the South African potato industry, the prevalence of fusarium dry rot disease, the chemical composition of moringa, as well as the utilization of moringa extracts in plant epidemiology (Chapter 1).

Section II: Focusses on the ability of the employed organic solvents and their concentration to extract phytocompounds from moringa powder. This section also reports on the impact of moringa leaf extract treatment on the tuber's phytochemical composition, depending on the extraction solvent used. Lastly, it reports on the impact of MLE treatment on physiological changes in tubers over a four-week storage period (Chapter 2).

Section III: Reports on the efficacy of MLEs as bio-fungicides by first reporting on inhibition activity of MLEs against *F. oxysporum* (Chapter 3), while the last chapter reports on the effectiveness of MLEs controlling fusarium dry rot, either as a preventive measure, or as a means to slow down disease progression (Chapter 4).

Section IV: In this section, the results from previous chapters are highlighted with emphasis on the South African potato production, as well on fusarium dry rot management; further, recommendations

for future studies are given.

3. **Potential impacts of the studyPoverty alleviation**

The potato production sector provides jobs to millions of South Africans. By improving potato production, unemployment might be reduced. Furthermore, many smallholder farmers cultivate potatoes for their own consumption and for commercial purposes.

Job creation

The agricultural sector is one of the biggest sectors in South Africa, providing jobs to many South Africans. This sector is largely affected by phyto-diseases that may lead to 100% production losses; such losses may result in job losses.

Zero hunger

Diseases are a major constrain, not only to potato production, but to overall vegetable production. Moringa powder is an affordable alternative to chemical fungicides for many farmers; thus, having moringa-derived pesticides will improve food production, particularly by poor people.

No poverty

Improved agricultural production will provide job opportunities to many unemployed people. This is essential, since many people recently lost their jobs due to the Covid-19 pandemic and, currently, 32.6% of South Africans are unemployed. Smallholder farmers may benefit from the postharvest use of moringa powder, reducing postharvest crop losses. Various moringa extracts could, moreover, be commercialized as fungicides, which will create more job opportunities from moringa farming to moringa processing.

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Chapter 1: Literature review

The rising problem of post-harvest fungal diseases of potatoes in South Africa

1.1 Introduction

Potato (*Solanum tuberosum* L.) is a starchy, tuberous crop in the nightshade family (Solanaceae) and is native to South America. Potato is a term that commonly refers to the edible tuber, but can also refer to the plant itself (Yasser, 2019). Potato is of economic importance and considered as a staple crop worldwide; this is due to its wide availability and affordability, particularly in developing countries (Zaheer and Khalid, 2016; Shahbandeh, 2020). According to the VB facts series (2019) potato is one of the most commonly produced food crops worldwide. Potatoes are a good source of phytochemicals essential in the human diet, such as carbohydrates, dietary fiber, various vitamins and minerals. Furthermore, potatoes are rich in antioxidants, namely vitamins, such as the provitamin A, β-carotene and polyphenols; these antioxidants play a significant role in the maintenance of the human antioxidant defence system (Zaheer and Khalid, 2016). In Sub-Saharan Africa, potato significantly contributes to food security; thus, poor production may lead to food insecurity. Several factors, however, limit the production of potato. Diseases are the leading cause of production losses in potatoes, the most common potato diseases in South Africa (SA) are fusarium dry rot and bacterial soft rot (Yasser, 2019). According to Lastochkina et al. (2020), 50-60% of the potatoes harvested world-wide are lost to postharvest diseases during storage.

Fusarium dry rot is the most devastating post-harvest disease in potatoes. Fusarium dry rot is caused by various fungal species from the *Fusarium* genus (Peters et al., 2008; Gachango et al., 2012). *Fusarium* spp. are primarily transmitted via open wounds, which normally result from mechanization during harvest and post-harvest handling; hence, fusarium dry rot epidemics have become more prevalent with the mechanization of potato production. *Fusarium* spp. have also become more virulent and weather conditions are becoming more favourable for its disease developments. The species have, unsurprisingly, developed resistance towards a number of fungicides, including benzimidazole fungicides, the most-used fungicide group to control *Fusarium* spp. in potato. Thiabendazole (TBZ) and thiophanate methyl, both benzimidazole fungicides, have been used since the 1970s to prevent decay of seed potato pieces caused by *Fusarium*, particularly in storage;

however, these fungicides are no longer as effective as they used to be (Powelson et al. 1993). Fungicide resistance has exponentially increased potato production losses (Leadbeater, 2014). These losses occur in storage and in transit of both, seed and commercial potatoes (Degebasa, 2020).

The need for inexpensive, effective, safe and accessible means to control plant diseases, while not harming human health nor the environment, is becoming increasingly pressing (Garnsey et al., 2005). This has resulted in the search for natural compounds found in higher plants, such as *Moringa oleifera* Lam., as this species contains antifungal and antibacterial compounds that can be used to control fusarium dry rot (Moyo, 2012). These compounds include phenolics, a chitin-binding protein and glucosinolates, which have all been proven to be effective fusarium inhibitors (Gifoni et al., 2012; Freire et al., 2015). The advantage of using moringa extracts, and that of other higher plant species, over manufactured fungicides, is their good disease control capacity combined with low cost, making these remedies affordable to farmers in developing countries, where fusarium dry rot and bacterial soft rot are common. Bacteria and fungi are, furthermore, less probable to develop resistance towards these extracts than to chemically produced fungicides (Babaoost et al., 2009; Das, 2010; Moyo, 2012). Phytochemicals extracted from moringa and applied to diseased/infested crops are referred to as 'natural-origin pesticides'. The use of plant extracts to reduce pathogens is not a new practice; however, recently there seems to be great interest in these extracts for use in both, agriculture and commercial products. Using plant extracts can be advantageous the use of manufactured pesticides, because the former are more likely to be easily biodegradable, environmentally friendly and to be produced in a sustainable way (Kheir et al., 2014; Zaffer et al., 2015).

1.2. Potato production and importance

Potatoes form part of staple foods in many parts of the world and are an integral part of much of the world's food supply. What has made potatoes to be considered as a staple crop is that they are fast-growing and require less input than other vegetables (Shahbandeh, 2020). Potatoes are further able to grow in almost any climate and under any condition, provided they are cultivated over frost-free periods (Haverkort and Kruit, 2015).

Potatoes are primarily produced for human consumption, as they are consumed by many cultures and almost all people of all classes, from upper class to lower class. Potatoes are consumed in different forms; they can be consumed as dehydrated or frozen food products, as commercial starch,

and as potato chips as well as fresh potatoes for boiling, baking or frying. Apart from human consumption, potatoes are also used for alcohol production (vodka), animal feed and other industrial purposes (Lisinska, 2009).

Potatoes are the fourth-most important and largely produced crops after grains. The top potato-producing countries are China, India, and Ukraine, with an average yield of 90 259155, 48 529 000, and 22 503 970 tons per year, respectively (FAOSTAT, 2019). The total world potato productionwas estimated at 368 million tons in 2018, increasing from 333.6 million tons in 2010 (Anokwuru *et al*., 2020). The production of potatoes in developed countries is higher as compared with developing countries that harvest an average of 43.26 tons per hectare, while developing countries have an average annual production of only 16.7 tons per hectare (Thomas-Sharma *et al*., 2016). In Africa, potato production has doubled over the past 20 years and has become a major source of revenue for both, commercial and smallholder farmers (DALRRD, 2020). About 22% of the total amount of potatoes produced in South Africa is stored for use as seed potato; of these, 80% is 'certified seed', while the remaining 20% is uncertified potato seed. About 53% of all potatoes harvested in SA go to the fresh produce markets and 47% is either absorbed by direct processing, the trade market, or is exported directly. Potatoes are also commonly traded in informal markets (NAMC, 2017).

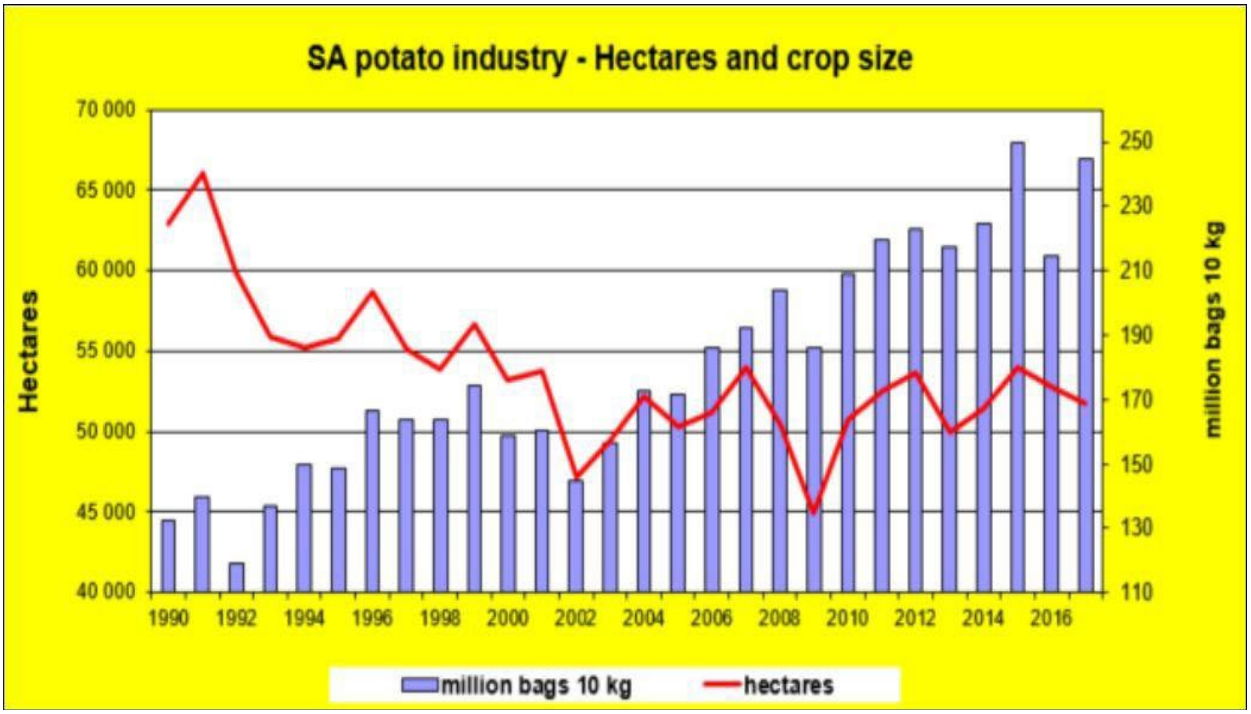

Figure 1.1: Fluctuations in potato production over the past years in South Africa (NAMC,2017)

In South Africa, potato is cultivated throughout the year in all provinces, producing about twomillion tons on over 50 000 hectares of land (Figure 1.1). The Limpopo province is the largest potato producer in South Africa contributing 40 million 10 kg bags annually. There are 101 registered potato cultivars in South Africa; however, 'Mondial' and 'Sifra' are the most-commonly produced cultivars. There are three distinctive potato market channels in SA, namely the formal market, the informal market, and the processing industry (NAMC, 2017).

1.2.1 Factors affecting production of potatoes

The production of potatoes is influenced by internal factors, such as cultivar type, seed potato age and quality; it is also influenced by external factors, such as environmental conditions, diseases, and pests. During growth stage I and growth stage II (Fig. 1.2) potatoes are most susceptible to fungal infections. Certain environmental conditions, such as a high soil moisture content and high humidity, may acceleration tuber infection rate. .

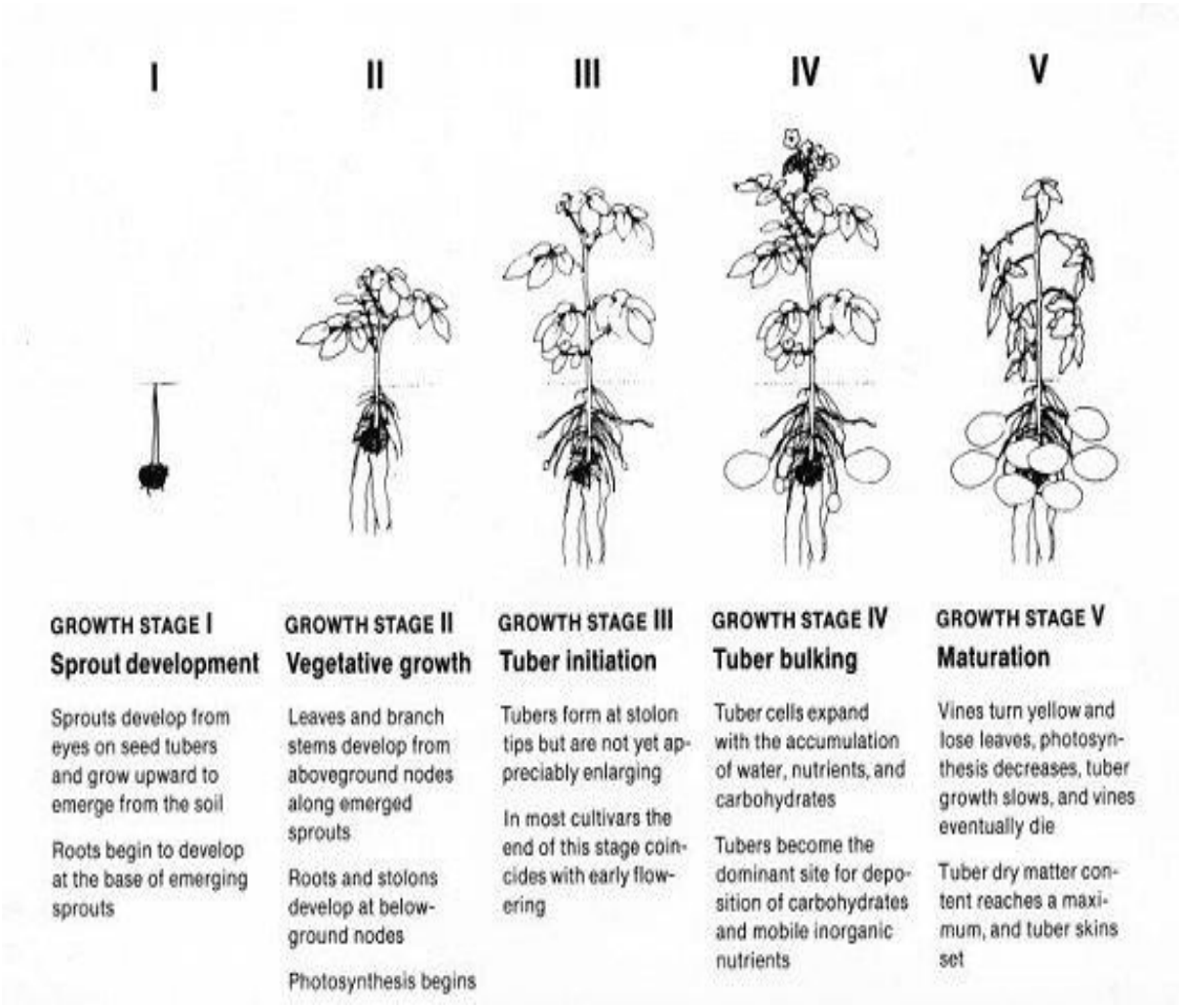

Figure 1.2: Potato comprises five growth stages, growth stage I and growth stage II are most susceptible to fungal diseases. Once potatoes reach stage V, they are ready for harvest; when exposed to wounding at this stage V, these wounds become entry points for fungal pathogens (Johnson, 2008).

1.2.2 Storage facilities

Storage facilities play a significant role in potato postharvest life; this is because poor storage facilities may severely reduce the quality of potatoes, which may subsequently reduce potato shelf-life. Poor stotage facilities they may be either too humid, too hot or cold; make potatoes more susceptible to postharvest diseases, such as bacterial soft rot and fusarium dry rot; however, good storage conditions significantly improve potato quality and postharvest life up to 12 months (Pinhero and Yada, 2016). Recommended storage facilities are required to be dark, well-ventilated, and able to maintain temperatures near 4°C for long term storage.For short-term storage temperatures need to be maintained at 7 to 10°C (Pinhero and Yada, 2016).

1.2.3 Climate change

Climate change can have a negative effect on potato production. According to literature,increased temperatures may lead to increased heat stress of potatoes (George *et al*., 2018).George *et al* (2018) further stated that genetic variability of heat-tolerant cultivars is limited and there is no hope that more heat-tolerant cultivars will be available to farmers in the near future, which puts potato production under threat of major losses including diseases infections.

Certain parts of South Africa are currently facing severe droughts. Drought does not only limit potato growth and quality, but water and heat stress also affect potato defence mechanisms negatively, resulting in resistance to pathogens, increasing infections. Climate change is, hence, likely to decrease yields in potatoes, particularly due to postharvest spoilage, due to increased potato disease presence (Obediegwu et al., 2015).

1.2.4 Potato diseases

Postharvest diseases of potatoes, such as late blight (*Phytophthora infestans*), fusarium dry rot (*Fusarium spp*.), pythium leak (*Pythium ultimum*), and pink rot (*Phytophthora erythroseptica*), are responsible for significant economic losses in the potato industry worldwide. An estimated 22% of potatoes are lost per year to viral, bacterial, and fungal diseases and pests, which is equivalent to an annual loss of over 65 million tons of potatoes (Anokwuru *et al*., 2011). Througout the world, diseases are often associated with insect pest infestations; major insect pests on potatoes include aphids, tuber moths, leaf miners and Colorado potato beetle (Molnar and Rakosy-Tican, 2021).

1.2.4.1 Potato late blight

Phytophthora infestans is an oomycete pathogen that causes late blight in potatoes and has a wide host range. Potato late blight is one of the most-destructive potato diseases. The disease affects plant growth, potato quality and quantity, resulting in enormous production losses each year (Liu *et al*., 2020). Potato late blight was first identified in the1800s and has been the most-destructive potato disease since then. Many, new strains of *P. infestans* have developed from the sexual reproduction of this pathogen; whereby an antheridium fuses with an oogonium to form a diploid oospore that later develops into a sporangium (Fig. 1.3) (Kiiker *et al*., 2018). These strains are resistant to common fungicides used to treat potato late blight (Ammour *et al*., 2017).

*Phytophthora infestan*s is characterised by an asexual life cycle divided into four phases, namely: hyphal growth, sporulation, sporangia germination and regeneration of hyphal growth (Fig 1.3,

https://www.wikiwand.com/en/Phytophthora_infestans). Late blight infections can occur at any growth stage of potato. Symptoms of late blight include dark blotches on leaf tips and stems, white mould under the leaves and darkpatches on tubers that are reddish-brown under the skin. Disease development and severityis accelerated by warm temperatures (20-24^{o}C) and moist environments. Wind and water are the main spreaders of *P. infestans*. The survival of *P. infestans* spores largely depends on the availability of a host plant. In the absence of a host plant, asexual sporangia and hyphae only survive briefly, particularly under cold and very warm temperature regimes. Several fungicides that have been used to control potato late blight are no longer effective, as *P. infestans* has developed new strains that are resistant to these fungicides (Lal *et al*., 2018).

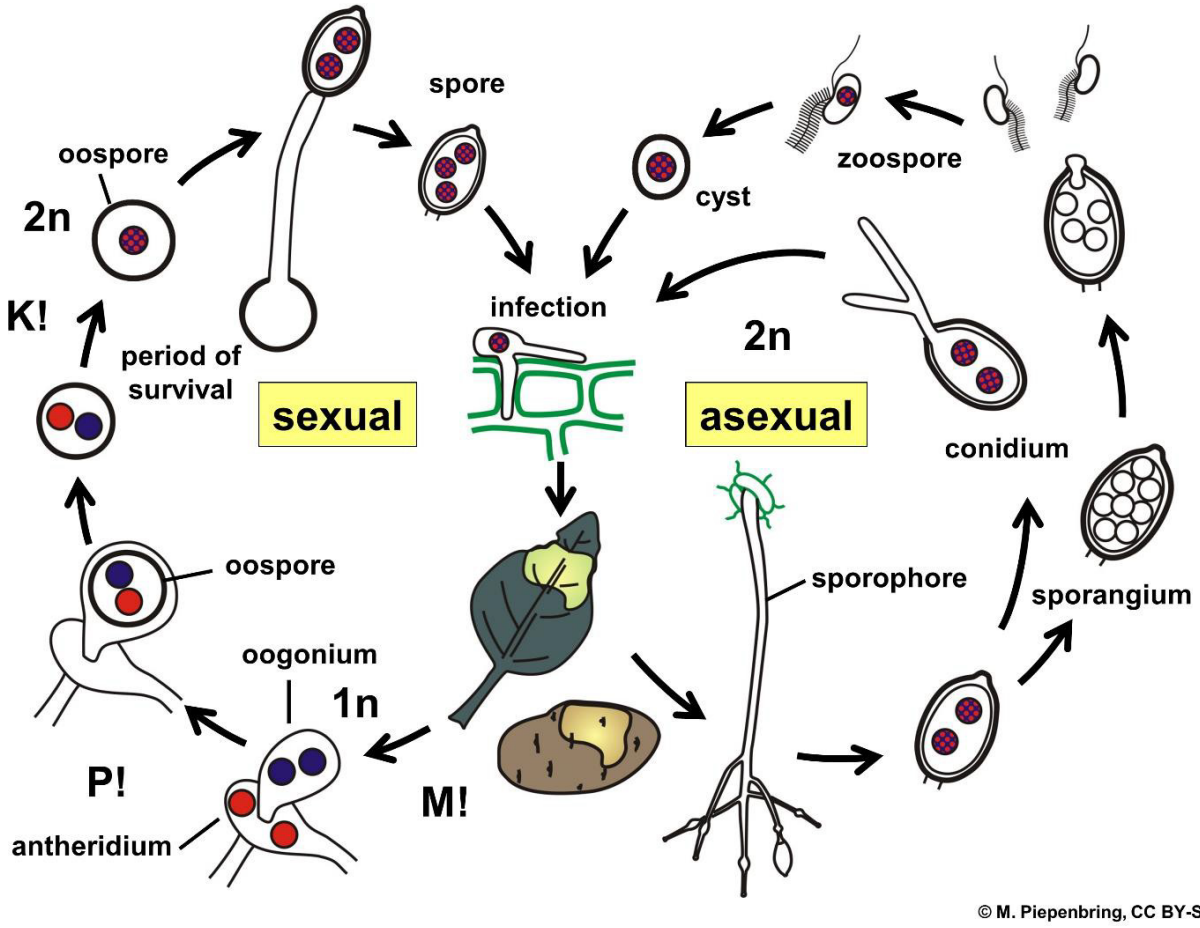

Figure 1.3: Both asexual and sexual life cycle of *P. infestans* (https://www.wikiwand.com/en/Phytophthora_infestans)

1.3 Fusarium dry rot disease

1.3.1 The genus *Fusarium:* the casual agents of fusarium dry rot

The genus *Fusarium* comprises several soil-inhibiting, filamentous species. These fusarium species are a diverse fungal group considered important in the food and drug industry, medicine, and agricultural industries. Species in the genus Fusarium have both, a sexual and an asexual form; however, due to the way they spread, these species mostly stay in their asexual form (Fig 1.4). Fusarium can infect potatoes in storage or in the field, resulting in fusarium dry rot and in seed tuber rotting after sowing, respectively (Wharton *et al*., 2007; Gachango, 2011). Certain fusarium strains have, however, been associated with mycotoxicosis in humans and animals; these mycotoxicoses are caused by fumonisins and trichothecenes, the main toxins produced by certain fusarium strains. Although most *Fusarium sp.* are harmless, some species are among the most-important fungal pathogens of plants and animals (Stefańczyk and Sobkowiak, 2017).

There are numerous *Fusarium* species found in South Africa*;* however, amongst these *F. solani* var. *coeruleum, F. roseum* var. *sambucinum* and *F. oxysporum* are the most common species, causing dry rot of potato (McLeod *et al*., 2001). Amongst these species, *F. solani* is the most common and most important causal agent of several crop diseases, resulting in root and fruit rot of *Cucurbita* spp*.*, root, and stem rot of *Pisum sativum* L., sudden death syndrome of *Glycine ma*x L, foot rot of *Phaseolus vulgari*s L. and dry rot of *Solanum tuberosum* L (McLeod *et al*., 2001).

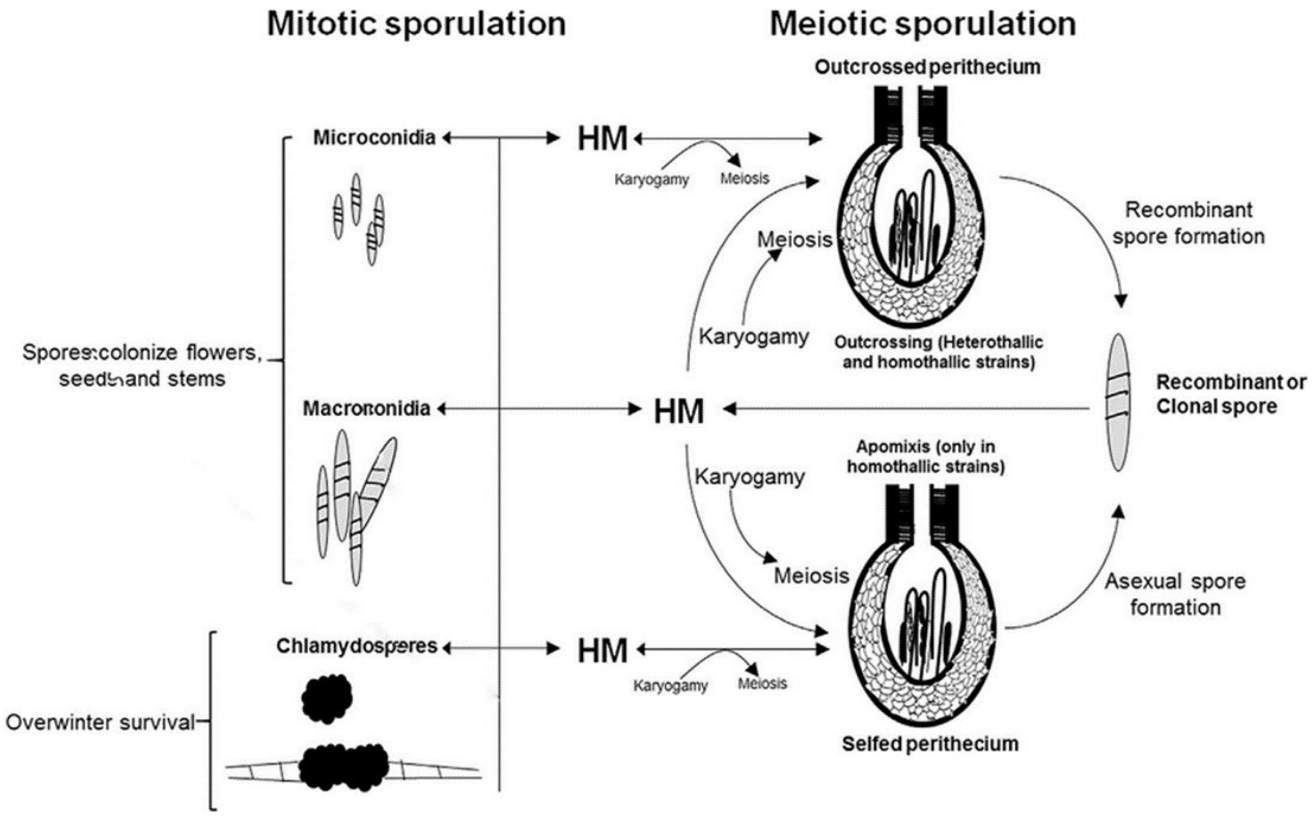

Figure 1.4: The life cycle of *Fusarium oxysporum* (Dweba *et al*., 2017)

1.3.2 Economic importance of fusarium dry rot

Fusarium dry rot reduces yield, marketability, and seed quality of several crops. Direct yield losses range from6 to 25%, but may be as high as 60%. Severe infections are accelerated by environmental conditions, and these environmental conditions are particularly conducive to dry rot development; however, losses caused by fusarium dry rot exceeded 50% in some years even in refrigerated storage. (Fagwalawa *et al*., 2014). *Fusarium sambucinum* is reported to be responsible for higher losses than *F. oxysporum*. These reported losses may become more severe in the presence of other tuber rot pathogens, such *Pythium sp*., *Pectobacterium sp*. (syn. *Erwinia sp*.), *Phytophthora erythroseptica* and other soil-borne pathogens (Daami-Remadi., 2012). These losses have a major effect on food security in South Africa and other developing countries, since potato production is associated with food security.

Fusarium dry rot mainly presents a problem in the seed industry because seed tubers are stored for prolonged periods, while potatoes destined for consumption are normally utilized soon after harvest. This fungus is also a major cause of seed piece decay after planting, resulting in reduced

plant stands (Wharton, 2007).

1.3.3 Infection process and symptom development

Fusarium requires an open wound as entry point to infect the tuber. Open wounds can also occur due to damage during harvest and post-harvest handling, and damage at harvest is likely to lead to infection, since it provides entry points for spores that are dormant in soil (Fig 1.6) (Sadfi *et al*., 2001). The fungus might also be introduced to the soil by infected seed tubers, where it can survive and contaminate progeny tubers. After infection, the fungus begins to grow inside the tuber tissue and is characterized by white or pink mycelium and colourful spores, usually pink. A dark depression on the surface in a large lesion is a typical, primary symptom of fusarium dry rot, followed by wrinkled skin in concentric rings. This appearance is due to dehydration of the underlying dead tissue, causing dry rot lesions at the point of injury. Symptoms inside the tuber are characterized by necrotic areas shaded from light to chocolate-brown or black (Fig 1.5) (Sadfi *et al*., 2001).

Figure 1.5: Potato tubers displaying fusarium dry rot. A- Whole tubers displaying external symptoms; B- Cut tubers displaying internal symptoms.

More than 50% of the sprouts developing on infected tubers may become diseased and are destroyed outright before emergence. Disease development during sprouting may cause delayed or non-emergence, which is sometimes presented by poor and uneven stands with weakened plants. Crop vigour is additionally reduced, as energy needed to supply secondary sproutsis employed to overcome the damage to primary sprouts (Wharton *et al*., 2007). *Fusarium* can overwinter as fungal propagules within the soil or can colonize living plants or crop debris (Peters *et al*., 2008).

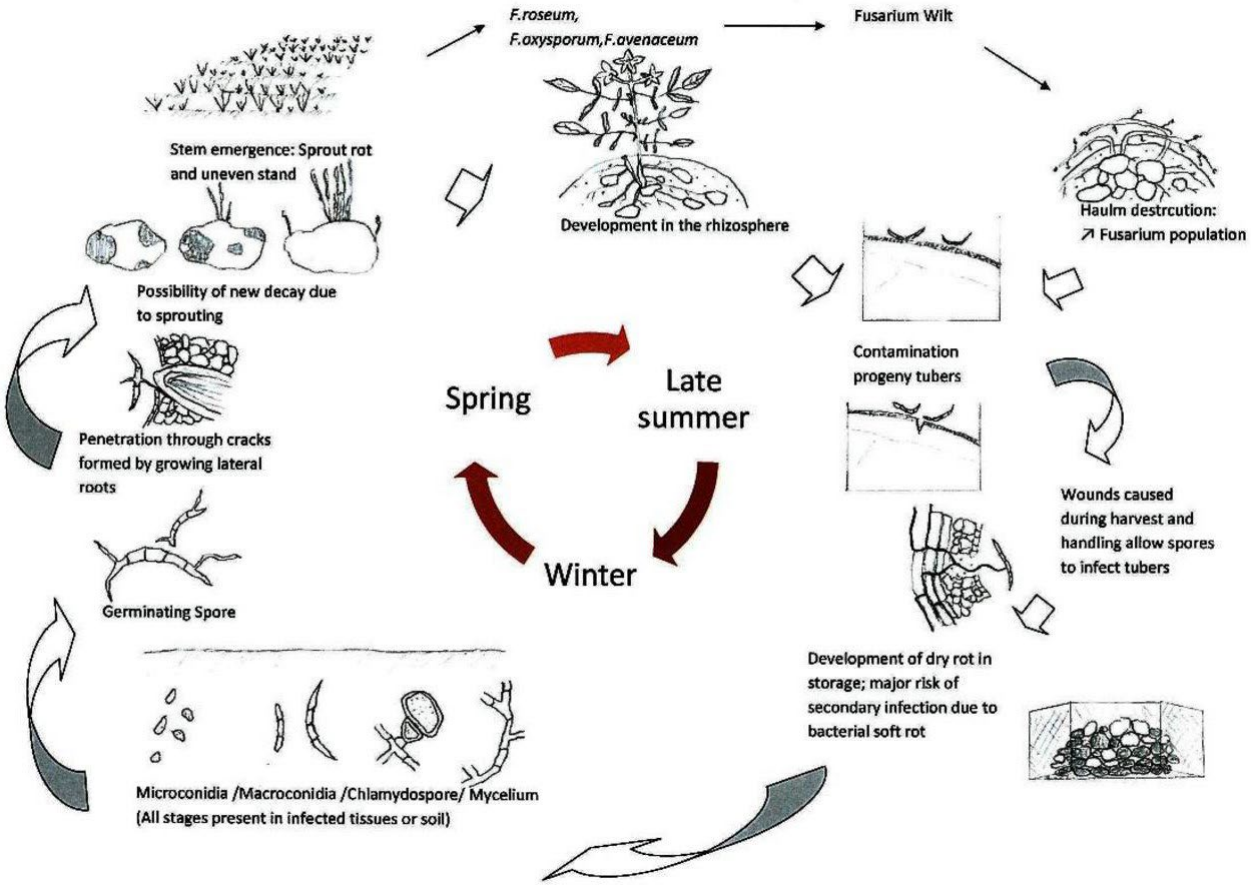

Figure 1.6: The cycle of fusarium dry rot disease (Gomez, 2012)

1.3.4 Epidemiology of fusarium dry rot

Disease development and severity of fusarium dry rot largely depend on the potato cultivar. Some potato cultivars have a certain degree of resistance towards fusarium dry rot as cultivars respond differently to fusarium infections (Isfahani *et al*., 2014). Furthermore, young tubers are more resistant to fusarium dry rot than mature tubers; it is also important to note that this resistance slows down disease progression (Gachango *et al*., 2012). Fusarium dry rot is more severe after dry and hot growing seasons and is most common in tropical regions, as the pathogen prefers warmer climates (Stefańczyk and Sobkowiak, 2017). High relative humidity (90-98%) and moderate to high temperatures (20-30°C) accelerate disease development. On the other hand, disease development is restricted under lower temperatures. Hence, cold storage restricts disease development, but the magnitude of the wound also has an impact on disease development. Fusarium dry rot damages tubers to a point where they can no longer be consumed nor marketed (Gachango *et al*., 2012).

1.3.5 Management strategies of fusarium dry rot

Cultural control, as a preventive method, is the most effective way of controlling fusarium dry rot. Cultural control involves seed selection, timely planting, and choice of suitable cultivars. Seed treatment with registered fungicides before planting is recommended as it prevents seed decay. Stored and commercial tubers can also be treated with registered fungicides, such as fludioxonil, postharvest; this is of paramount importance to reduce disease development and disease spread (Wharton *et al*., 2007). Some fusarium species, such as *F. sambucinum*, *F. oxysporum*, and *F. coeruleum*, have, however, recently developed resistance towards fludioxonil (Abbas, 2016). All infected and injured tubers need to be urgently removed as they may serve as the source of pre- and postharvest infection. During sorting, packing, and transporting, special precautions need to be undertaken to prevent mechanical damage to tubers. Dry and well-ventilated conditions are recommended for transportation and storage; temperatures need to be kept between 4 to 5°C to prevent disease development(Wharton, 2007; Stefańczyk and Sobkowiak, 2017).

1.3.5.1 Chemical control

Effective chemical control of dry rot can be achieved with several chemical fungicides such as Tops MZ®, Maxim MZ®, and Moncoat MZ® (Tab 1.1) (Wharton *et al*.,2007). These fungicides protect potatoes not only against dry rot, but also against other potato diseases, such as late blight, silver scurf,and black dot. The downside of using these fungicides is that they can retard crop emergence. These fungicides are mostly preventive measures and work best as seed treatment (Fagwalawa *et al*., 2014).

Table 1.1: Examples of some actively used fungicides in attempts of controlling fusarium dry rotof potatoes

Active Ingredient	Trade name	Application	Manufacturer name and country
Fludioxonil	Nubark Maxim®	Postharvest treatment	Syngenta® United States of America
Azoxystrobin	Quadris®	Foliar application	Syngenta® United States of America
Thiabendazole	Arbotec®	Postharvest treatment	Syngenta® United States of America
Thiophanate-Methyl	Tops MZ®	Seed treatment	Bayer CropScience LLC Belgium
Flutolanil	MonCoat MZ®	Seed treatment	Nichino America United States of America
Fludioxonil	Maxim MZ ® 4FS	Seed treatment	Syngenta® United States of America

1.4.5.1.1 Disadvantages of chemical fungicides

Although fungicides may be effective in controlling *Fusarium sp.*, they might result indamage to the surrounding ecosystem (Vatankhah *et al*., 2019). Inappropriate use of chemical fungicides can threaten the health of animals and humans. Chemical fungicides may even cause diseases, such as cancer (Fagawalwa *et al*., 2014; El– Mohamedy and Abdalla, 2014). Furthermore, fungi are developing new strains that are resistant to several fungicides and these fungal strains have better adaptability to various environmental conditions (Kiiker *et al*., 2018).

1.4.5.2 Cultural control

The most effective and inexpensive method of controlling fusarium dry rot is through cultural control (Wharton, 2007). Cultural control is a method that prevents disease development and limits disease spread. The first and foremost factor of cultural control is using certified, disease-free seeds. Cultural control can prevent the introduction of *Fusarium*sp. into a field (Nawaim *et al*., 2017). It is also important to perform physical inspections of the field by scouting for any disease symptoms that may be present on seed tubers (Aydin, 2019). This is often overlooked by subsistence farmers in South Africa, who are usually unfamiliar with fusarium dry rot disease symptoms, or any potato disease for that matter. These subsistence farmers often buy cheap seed tubers from street vendors, ending up infecting their fields with pathogens, particularly *Fusarium* spp., which have a wide host

rangeand thus infect a range of crops.

Seed tuber storage is also an important factor in fusarium dry rot prevention. The ideal seed tuber storage temperature is 4to 6^{o}C and tubers should be moderately warmed up to 10^{o}C before cutting into seed tuber pieces and planting. These slightly warmer temperatures stimulate healing of postharvest tuber wounds and cooltemperatures prevent fusarium growth (Yadav and Singh, 2021). Another important factor in cultural control is sanitation, whereby storage facilities and cutting equipment are regularly sanitized. This method minimizes fungus transmission during storage (Nawaim *et al*., 2017).

To minimize post-harvest infections, it is recommended that tubers are harvested when the periderm has matured at a temperature of 10^{o}C or higher. This process reduces the risk of injury during harvest which may subsequently create an entry point for *Fusarium* sp*.* and leadto disease development (Vatankhah *et al*., 2019).

1.4.5.3 Biological control

There are currently no effective biological or bio-fungicide controls effective against fusariumdry rot that are available commercially (Wharton *et al*., 2007). Biological control has been found to be unreliable, as its effectiveness varies with season and environment; these inconsistencies may be due to interactions, such as those of genetic, environmental - and pathogenic nature (Bubici *et al*., 2019).

1.5 Plant defence mechanisms

Potato tubers have cell walls as their first line of defence against *F. oxysporum*; however, in cases of physical injury this first line of defence is removed. The second response is the hypersensitive response (HR), which is a response that occurs at the site of infection inducedby the *F. oxysporum* (Brouwer *et al*., 2020). This HR triggers the production of secondary metabolites (SM), such as phenolics, flavonoids, terpenoids and glycoalkaloids. These compounds are known to have antimicrobial activities. Apart from SM, HR also triggers an increased production of enzymes, such as polyphenol oxidases; the aim of these enzymes is to degrade the pathogen and prevent further disease development (Vaillant, 2012). Many fungal species such as *P. infestans* and *F. oxysporum* have an ability of weakening and defeating the plant's defence response (Arie, 2019). Thus, there is an urgent need of bio-fungicidesthat can be used to treat seed tubers before planting to prevent disease occurrence.

1.6 Utilization of plant extracts in agriculture

Higher plants are known to contain phytochemicals, particularly secondary metabolites, whichare toxic to phytopathogens; this aims to serve as a defence strategy against phytopathogens. Thus, to control phytopathogens organically, these phytochemicals have been extracted fromplants and have been proven to be effective in controlling pests, when applied to pest- infested crops. A variety of phytochemicals is widely distributed from roots, leaves, barks andseeds and differs in concentration, depending on plant species (Gurjar *et al*., 2012).

1.7 Moringa plant distribution and biology

Moringa oleifera is the most-common and most-widely distributed member of the 14 species of the monogeneric Moringaceae family (Anwar *et al*., 2006). Moringa is commonly known as the 'tree of life', the 'drumstick tree', or the 'horseradish tree'. Moringa originates from Asian and African countries, but is now widely distributed in the tropical and subtropical regions of the world (Anwar *et al*., 2006; Sayeed *et al.*, 2012). Amongst these regions, India is the largest moringa producer with an estimated annual production of 1.1–1.3 million tons (Saini *et al*., 2016). In South Africa, moringa is mostly produced in Mpumalanga and in Limpopo.

Moringa is described as a perennial, angiosperm softwood tree, ranging in height from 5 to 10 m; it grows well in the humid tropics or on hot, dry lands; it can survive poor soils, and is little affected by drought owing to its tuberous roots (Padayachee and Baijnath, 2012). Moringa seeds can be planted, once they reach maturity; cuttings are, however, the recommended method of establishing moringa trees; this is to avoid genetic variation that may occur during cross-pollination. Seed propagation may, however, result in loss of important traits (Saini *et al*., 2016). The moringa plant can withstand a minimum annual rainfall of 250 mm and a maximum rainfall of 3000 mm. The pH requirements of moringa trees range between 5.0 - 9.0 (AL-Husnan and Al-Kahtani, 2016). As a tropical plant, the ideal growing temperatures of moringa are between 25-35°C and moringa trees can withstand temperatures up to 48°C. Interestingly, moringa is also frost- and drought-tolerant and can withstand various soil conditions of different alkalinity (Freire *et al*., 2015).

Most of moringa plant tissues are beneficial to humans, from its root, bark, leaves, flowers, fruit to seed; this is because extracts from moringa tissues are used for human consumption, for medical as well as industrial purposes. Thus, moringa is considered one of the most versatile trees in the world (Vongsak *et al*., 2013; Al-Husnan and Al-Kahtani, 2016). According to Kasolo *et al*. (2010), moringa

has been used in treating malnutrition in under-developed and developing countries over the past years; moringa leaves have also been used to treat various medical conditions, such as HIV/AIDS-related symptoms, bronchitis, ulcers, malaria, and fever, particularly in Africa. Moringa phytocompounds have also been used to treat hypertension, inflammation, epilepsy and diabetics in developing countries. Furthermore, moringa flowers and roots have been used by African people to treat cholera, as acertain antibiotic has been reported present in these tissues (Saini *et al.*, 2016).

Previous phytochemical analyses of moringa have shown that leaves are particularly rich in potassium, calcium, phosphorus, iron, vitamin D, essential amino acids, as well as the known antioxidants vitamin C and flavonoids. These antioxidant compounds are important, as they can be used to prolong the postharvest life of various vegetables and fruit (Amaglo *et al*., 2010; Mbikay, 2012). Moringa is also rich in compounds containing the simple, rare sugar rhamnose, as well as in glucosinolates and isothiocyanates; however, the concentration of these compounds may vary with geographic locations (Saini *etal*., 2016). Moringa contains, additionally antimicrobial compounds that are unique to the Moringaceae family (Amaglo et al., 2010).

1.6.1 The importance and application of moringa in agriculture

Moringa use is increasingly attracting attention, attributable to its unique type and composition of phytochemicals that can play important roles for human consumption, as well as have medicinal and industrial purposes. These compounds have made moringa atree of diverse uses (Tesfay *et al*., 2011; Al-Husnan and Al-Kahtani, 2016). Various phytochemicals are found in moringa roots, bark, leaves, flowers, fruit and seeds (Sayeed *et al*., 2012; Vongsak *et al*., 2013). Moringa extract has been proven to be a good fertilizer, as well as a fungicide and bactericide, amongst other agricultural uses (El-Mohamedy and Abdalla, 2014; Tesfay *etal.*, 2011). Aueous leaf extracts of moringa have been found to improve plant growth and productivity; this is due to the high concentration of certain plant growth-enhancing compounds found in moringa leaves, including ascorbates, phenolics, glucosinolates and minerals, such as Ca, K, and Fe (Sayeed *et al*., 2012; Saini *et al*., 2016). Furthermore, Tesfay *et al.* (2011) stated that moringa extracts are an effective nutrient content booster, when applied to plants and can be used to fight malnutrition, a condition which is predominant in South Africa. The authors further stated that moringa extracts can be used in edible coatings that can prolong the shelf life of fruit and vegetables. According to Vongsak *et al*. (2013), the antioxidant activity of moringa leaf extracts can be accredited to their phenolic acids and other antioxidant compounds; this makes moringa even more valuable.

1.6.2 The efficacy of moringa as a fungicide against several phytopathogenic fungi

Various studies have shown that moringa (seed, leaf, and root) extracts contain potential fungicides. Research by El-Mohamedy and Abdalla (2014) showed moringa extracts to inhibit the growth of several soil-borne fungal species, such as *F. solani, F. oxysporum* and*Rhizoctonia solani*. Moringa extracts were also found to significantly inhibit mycelial growth and spore germination of *Botrytis cinerea* (Dwivedi and Enespa, 2012; Moyo, 2012). In a study by Sayeed *et al*. (2012), moringa fruit extracts showed antibacterial and antifungalactivity against *Alternaria* spp., *Colletotrichum* spp., *Curvularia* spp. and *Fusarium* spp., as well as several bacterial species, such as *Staphylococcus aureus, Bacillus subtilis, Vibrio cholera, Bacillus cereus, Salmonella typhi,* and *Shigella dysenteriae.* Moringa leaf extracts were proven to be an effective seed treatment against *Sclerotium rolfsii*, the causal agent of root rot and damping-off in several crops (Adandonon *et al*., 2006). Results from these studies show that moringa extracts are a potential substitute of synthetic fungicides; therefore, these extracts can be effectively used in agricultural production, improving production quantity and quality of crops, acting as a plant growth enhancer (Sayeed *et al.*, 2012).

The efficacy of moringa extracts is attributed to its combination of phytochemicals; moringa extracts contain approximately 200 phytochemicals that can be categorized into hydrocarbons, ketones, fatty acids, alcohols, aldehydes, and terpenes. These phytochemicals are present in leaves, roots, seeds, and stems (Falowo *et al*., 2018). Moringa extracts are known to contain high concentrations of zeatin, quercetin, β-sitosterol, caffeoyl quinic acid and kaempferol; the combination of these antifungal compounds is unusual amongst plant species (Anjorin *et al*., 2010). Sayeed *et al*. (2012) further pointed out that moringa SMs are responsible for the antifungal activity of moringa; these compounds are synthesized by plants as a defence strategy against pathogen attack. The concentration of these phytocompounds in moringa is higher than in any other plant species, e.g. the concentration of phenolics and flavonoids in moringa leaves is two times that of cabbage (Gharekhani *et al*., 2012). There are several methods that can be used to extract moringa tissues. Soxhlet extraction and solvent-assisted extraction are the most commonly used extraction methods (Ncama *et al*., 2019). The choice of extraction method depends, however, on the plant tissue to be extracted (leaf, bark, seed, or root), and on the targeted phytochemical compounds (Gharekhani *et al*., 2012).

1.7 Methods used to extract phytochemical compounds from moringa tissues

Plant extracts are usually prepared by first grinding the plant tissue, either dry or wet, as this increases the surface area of the tissue and, thereby, improves the concnetration of phytocompounds extracted. The efficiency of plant extracts is evaluated using a number of methods, including agar disk diffusion test, diffusion test, agar well diffusion, dilution methods, agar dilution, and both macro and micro dilution assays (Gurjar *et al*., 2012). There are number of plant species that are rich in SMs and are often used in agriculture and in the pharmaceutical industry for their antimicrobial properties; however, over the past years moringa has received vast attention and has been proven to be of paramount importance in agriculture.

1.7 Extraction methods used in extracting phytochemical compounds of moringa tissues

Plant extracts are usually prepared by grinding the plant tissue either dry or wet, and this increasesthe surface area and improves the number of phytocompounds extracted. The efficiency of plants is evaluated using a number of methods including agar disk diffusion, diffusion test, agar well diffusion, dilution methods, agar dilution, and broth macro and micro dilution assays amongst others (Gurjar *et al*., 2012). There are number of plant species that are rich in secondary metabolites and are often used in agriculture and in pharmaceutical industry for their antimicrobial properties; however, over the past years moringa has received huge attention and has been proven to be of paramount importance in agriculture.

1.7.1 Solvent-assisted extraction

Solvents are the most-used means for extracting phytocompounds from moringa tissues; this is due to their ability to penetrate deep into plant tissues and dissolve phytochemicals based on polarity similarity (Anokwuru et al., 2011; Gurjar et al., 2012). The most-commonly used solvents are methanol, ethanol, acetone, hexane, and ethyl acetate. The availability and concentration of specific phytocompounds depend on the solvent type used, as phytocompounds are soluble in different solvents, depending on solvent polarity (Anokwuru et al., 2011). Solvents with higher polarity are used to extract phytocompounds, such as phenolics and other antioxidants. Saturated organic solvents, such as ethanol and methanol, are mostly used to extract antimicrobial compounds. The extraction period also plays a significant role in the phytochemical composition and concentration of the extract; furthermore, temperature, solvent pH, size of the plant tissue and solvent concentration in a solution

differ with extraction method and affect the concentration of phytochemical compounds present in an extract. Desired solvents are less toxic, easy to evaporate and stimulate fast physiologic absorption of phytochemicals (Gurjar et al., 2012). In most research studies, dried powder of plant tissue is used to reduce the effect of the water already present in the wet leaf (Altemimi et al., 2017).Soxhlet extraction method

The Soxhlet extraction method (SEM) is used to extract phytochemical compounds from plant tissues. In this method, plant tissues need to be dried and ground into fine powder; different organic solvents are then used to extract the chemical compounds. The organic solvent is suspended in the bottom of the Soxhlet extractor, while the plant powder is inserted into a porous thimble. The Soxhlet extractor is heated at the bottom, so that the organic solvent boils and evaporates in the powder. The organic solvent, containing the captured compounds, condenses as it encounters the plant powder in the extraction chamber, thereby extracting the phytocompounds (Fig 1.7). This technique is, however, not very convenient, as it takes a long time to complete the extraction, and relatively low extraction yields of the chemical compounds are achieved. As heat is used in this technique, certain chemical compounds may also be damaged (Gurjar et al., 2012; Ibrahim et al., 2017).

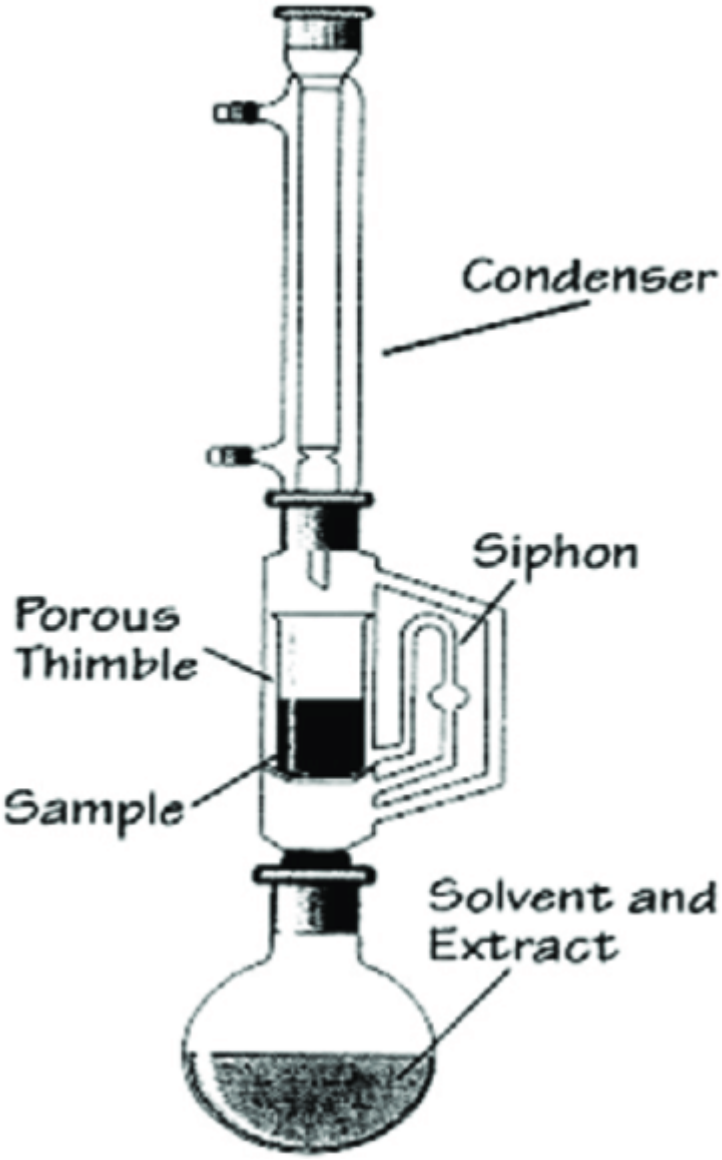

Figure 1.7: The Soxhlet apparatus used in Soxhlet Extraction (reference)

1.7.2 Microwave-assisted extraction

Microwave-assisted extraction (MAE) is a technique that employs both, microwaving and common, solvent-assisted extraction. Microwaves are used to heat solvents and plant tissuesto improve the efficacy of this technique (Delazar *et al*., 2012). In this method, solvent- containing samples are inserted into Teflon® vessels and are heated using microwaves until a specific temperature is reached (Delazar *et al*., 2012). This technique exploits the moisture content of the plant sample, as microwaves heat the sample close to the boiling point, so that pressure builds up till cell lysis occurs. Subsequently, the intracellular compounds, including the SMs, are released into the extraction solvent. The extracted chemical compounds are further dissolved by the extraction solvent, and,

thus, improve the extraction yield. The choice of extraction solvent and consistency of the microwave power, however, affect the MAE efficiency. Further, MAE is affected by the moisture content of the plant material, sample size and extraction time. This technique is advantageous, as it decreases the extraction time, requires minimal amounts of solvent, and has a better production efficiency than other extraction techniques making MAE one of the most cost-effective extraction techniques. The minimum temperature required in this technique is 150°C (Zhao *et al*., 2019). Furthermore, MAE is an environmentally friendly extraction method, since little to no solvent is evaporated into the atmosphere. The MAE method is further divided, based on advancements of the instruments used in the conduction of the experiment. The first division of MAE is pressurized microwave-assisted extraction (PMAE), whereby closed vessels are used, when conducting the experiment. The generated pressure makes it easier to reach temperatures higher than the boiling point. As a result, the extraction process becomes faster, more efficient, and loss of volatile compounds is avoided (Moret *et al*., 2019). The second subdivision of MAE is solvent-free microwave-assisted extraction (SFMAE). This is an environmentally friendly extraction method, as, instead of a solvent, this method combines dry distillation with microwave heating under atmospheric pressure and results in high quality extraction of compounds (Fig 1.8) (Benmoussa *et al*., 2016).

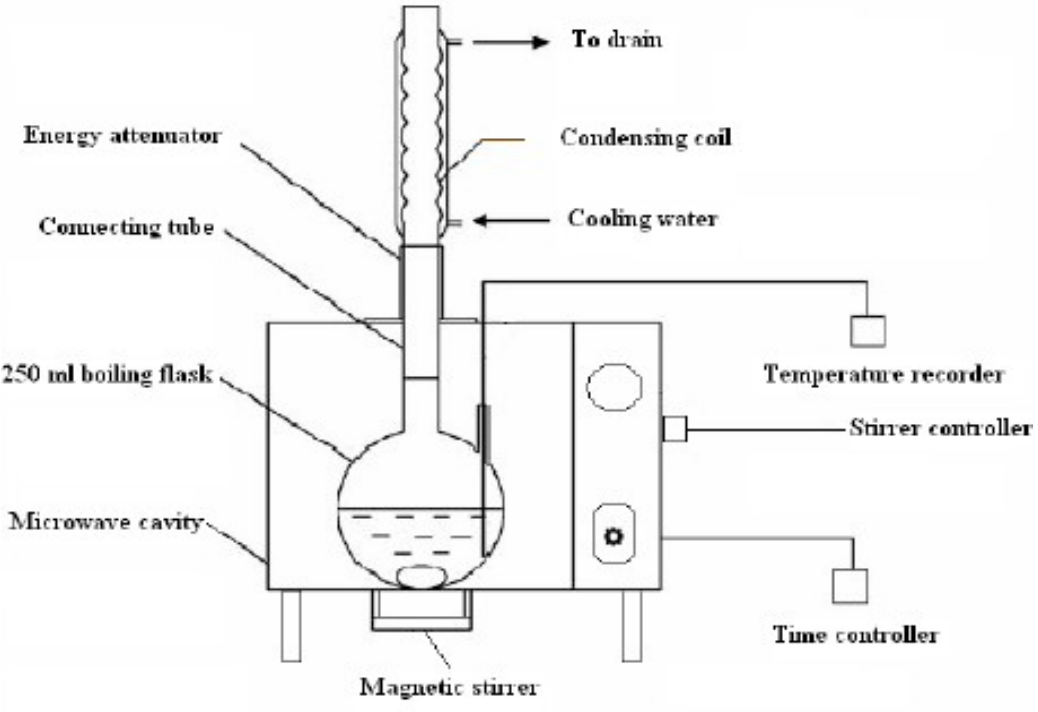

Figure 1.8: Equipment used in conducting microwave-assisted extraction (Gharekhani *et al*., 2012)

1.7.3 Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) is the most effective method of extracting phenolic compounds from plant tissues (Zhao et al., 2019). In this method, a volumetric flask containing sample-solvent solution is placed inside an ultrasonic bath, forming cavitation bubbles. These bubbles are caused by ultrasonic waves that form as the water bath is sonicated at 20-40 kHz for 10 to 60 minutes at temperatures between 60°C and approximately 100°C (Al Jitan et al., 2018; Liu et al., 2020). The ultrasonic waves go through a solvent, interacting with the sample and forming cavitation bubbles. These cavitation bubbles carry shockwaves caused by the ultrasound. When these cavitation bubbles explode at the surface of a plant sample solution; the ultrasonic shockwave raptures the cell walls (Fig 1.9) (Al Jitan et al., 2020). Large amounts of chemical compounds from the plant tissue are then released into the sample solution. The chemical compounds of interest are separated from the sample residue by filtration. The UAE technique is a simple method that requires minimum time to complete the extraction process; furthermore, low volumes of solvent are required and the method can be integrated with other extraction methods (Liu et al., 2020). The oxidation and damage of chemical compound of interest is prevented in this technique, as it is carried out under room temperature. Phenolic acids and other highly reactive hydroxyl radicals can, however, be damaged by ultrasonic waves and high temperature. This method can be used for the extraction of various compounds, but is optimal for oil extraction from plants (Tan et al., 2018).

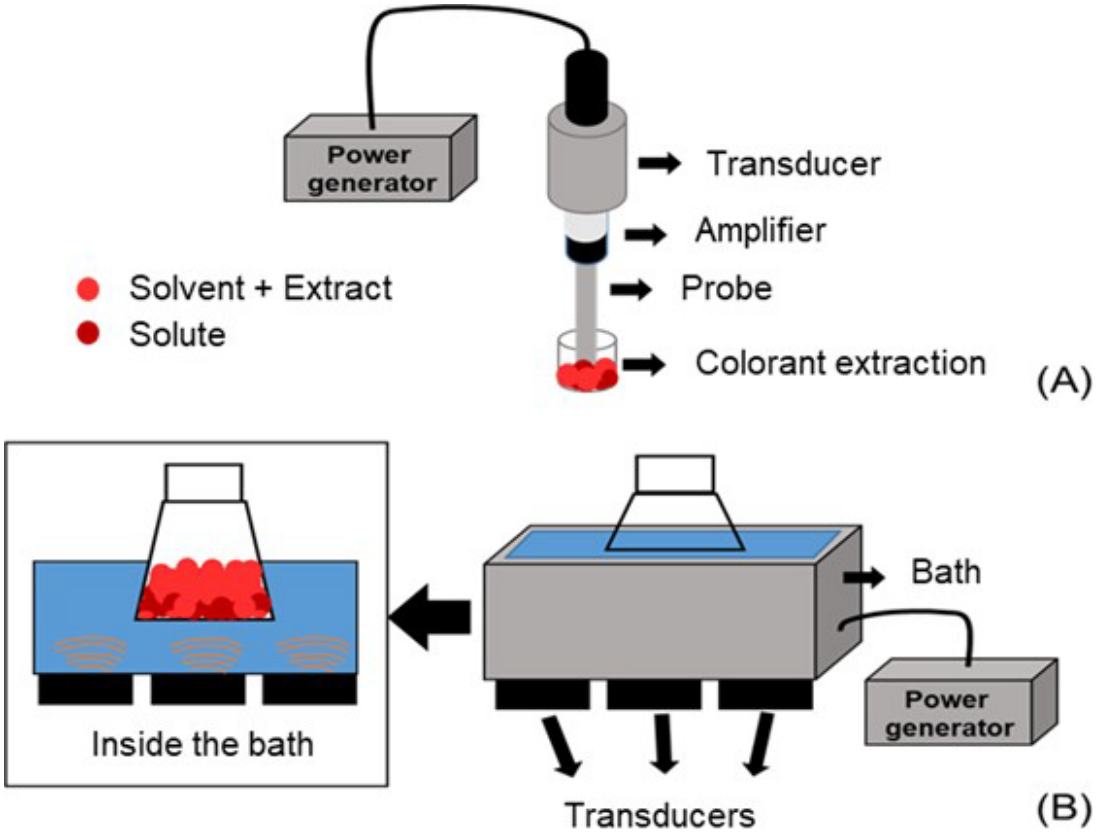

Figure 1.9: The setup of equipment used when conducting UAE (a). Ultrasound bath (b) Ultrasound probe (Strieder *et al*., 2019)

1.8 Conclusion

Potato remains the most important starch crop in Sub-Saharan Africa (SSA). This starch crop is one of the most popular crops that are consumed on a daily basis by all people in SSA. Potato is also the most-widely cultivated crop in SSA, cultivated by both commercial farmers in largequantities and by small holder farmers. Potato production is, however, limited by fungal diseases, both pre-harvest and in postharvest. Fusarium dry rot of potato, early blight, late blight, and black dot are the most common potato diseases in SSA. The severity of these diseases is enhanced by environmental conditions, such as increased temperatures and pest infestations. There are, however, limited, effective chemical control measures of fungal potato diseases. On the other hand, these fungi are quick to develop resistance towards fungicides, leaving potato production at risk. Fungicides derived from plants, including moringa; are a promising alternative to efficient fungicides. Moringa extracts are promising potential bio-fungicides that are cost-effective and readily available in SSA. Moringa is easy to cultivate as it is a self-sufficient plant. Moringa extracts have been proven, by various

researchers, to be excellent phyto-fungi inhibitors *in vitro*. The antifungal efficacy of moringa is attributed to its unique phytochemical composition. Moringa compounds include the moringa chitin-binding protein, glucosinolates, phenolic acids and zeatin. There is, however, insufficient research on the effect of moringa application on phytochemicals of crops and on the antifungal efficacy of moringa extracts on postharvest fungal diseases.

Using moringa as a bio-fungicide could rapidly improve production of many economically important crops that are susceptible to disease-causing fungi, such as *F. oxysporum, R. solani and Alternaria* sp. These crops include potato, tomato, banana, maize, andcabbage. Improving production of these crops will subsequently improve the economic statusof SSA countries by opening additional export markets, while the increased supply of these crops will ultimately drop costs of these agricultural products, thereby improving food security in these countries.

There are several effective extraction methods that can be utilized to extract chemical compounds from moringa. These extraction methods often employ organic, which are not always safe to use in agriculture, as they are not safe for consumption. Furthermore, these organic solvents are not environmentally friendly. Thus, there is a need to investigate possible bio-extraction methods that can effectively extract tightly bound chemical compounds from moringa tissues.

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Chapter 2
Enhancing secondary metabolites of potato using moringa leaf extracts

Secondary metabolites (SMs) play a crucial role in the defence system of potato plants against pathogen attacks and environmental stressors. These compounds are also significant in the phytochemical content and the quality of the tuber; however, the concentration of SM in potatoes have decreased over time due to domestication and farming practices. This study was carried out to examine the effectiveness of moringa leaf extracts (MLE) as enhancers of SM concentrations in potato and their effect on prolonging the shelf life of two potato cultivars ('Fandango', 'Mondial'). Moringa leaf powder was extracted either with acetone, ethyl acetate, and methanol, each at three concentrations of 30%, 50%, or 70% or with water. Potatoes were coated with these MLEs; seven days post-treatment, spectrophotometric analyses of alkaloids, flavonoids, glycosides and phenolics were performed. Treatment with MLEs enhanced the concentration of free and bound phenolics in both cultivars. Potatoes ofboth cultivars coated with methanol-MLE had the highest concentration of both free and bound phenolics. Moringa treatment also preserved the healthy tuber skin appearance, reduced percentage mass loss, and preserved firmness. Dormancy was delayed in tubers treated with 30% and 50% acetone, as well as with methanol-MLE and water-MLE. This study provides justification for further investigations into MLE as a potential organic treatment that could enhance potato resistance towards environmental and pathological stressors.

Keywords: Environmental stress, Phenolics, Flavonoids, Longevity

2.1 Introduction

Secondary metabolites (SMs) are essential chemical compounds produced by plants through various primary metabolic pathways, as a defence strategy against biotic and abioticstresses (Yang *et al*., 2018; Hussein and El-Anssary, 2019). These SMs are divided into several groups based on their chemical structure: phenolics, alkaloids, terpenes, saponins andcarbohydrates (Yang *et al*., 2018).

Potato (Solanum tuberosum L.) is, economically, one of the most-important crops worldwide and its popularity is increasing exponentially, particularly in the fast-food industry (Poiatti et al., 2009). Potatoes are readily available all year long and are affordable, especially to the SSA population, where potatoes are a staple crop (NAMC, 2017). Potatoes contain important SMs, such as phenolics and flavonoids, as well as primary metabolites (PMs), such as polyamines and carotenoids (Ezekiel et al., 2013). Potato has, thus, many health benefits, for instance, prevention of cardiovascular disease, diabetes type 2 and cancer (André et al., 2014). Nonetheless, in many plants, including potatoes, SMs are synthesized even more so under pathogenic attacks and due to environmental stresses, such as heat drought, and humidity stress (Yang et al., 2018).

Under favourable growing conditions, the concentration of SMs is relatively low; these favourable conditions are necessary to produce high tuber quality and large potato sizes. The low SMs concentrations might, however, result in a reduced ability to withstand environmental stresses. Low SM concentrations are a result of breeding efforts and farming methods that have focused on certain external and internal tuber quality parameters, but not on the tubers' ability to withstand environmental stresses. As SMs are synthesized by the plant as a defence strategy, potatoes are able to produce glycoalkaloids as anti-herbivore compounds; hence, potatoes with a high glycoalkaloid content are highly unpalatable or even toxic to humans and animals. This has led to selection against glycoalkaloid synthesis in the tuber during early potato domestication. Secondary metabolites are still of importance today, particularly in the pharmaceutical industry, due to their antimicrobial, anticancer and antioxidant properties (Sanchez-Maldonado *et al*., 2016 Isah, 2019).

Moringa oleifera Lam., commonly known as moringa, is an importantmedicinal plants and has gained major interest in scientific research, including pharmaceutical, industrial, and

agricultural research. Moringa is rich in minerals, proteins, vitamins, carotenes, amino acids and various phenolics; furthermore, it has a certain combination of zeatin and different types of flavonoids, particularly important in moringa's antimicrobial action. These phytochemicals are found in seeds, stems and roots; however, they are most concentrated in leaves (Dania *et al*., 2014). According to Dania *et al*. (2014) andDunsin and Odeghe (2015), moringa leaf extracts (MLEs) have the ability to speed up plant growth, improve resistance against pathogenic attacks and improve fruit quality. Abdalla (2013) further stated that moringa, as a biofertilizer, significantly improved soil quality, as it contains essential macro- and micronutrients, consequently enhancing physical and chemical attributes of the soil. Ibrahim *et al*. (2013) also pointed out that organic fertilizers tend to increase the concentration of phytochemicals in a crop, particularly of antioxidants, which are important in improving tuber longevity. Abdalla (2013) reported that application of MLE improved the antioxidant enzyme activities in Eruca (*Eruca vesicaria* subsp. *sativa*) plants; hence, it is highly likely that MLEs could also enhance potato tuber longevity.

Although moringa effects have been investigated in several solanaceous crops, such as tomatoes (*Solanum lycopersicum*) and bell pepper (*Capsicum annuum* L.), little investigations have beenmade on the moringa effect on potato, particularly its tubers. This study, hence, was carried out to investigate the effect of MLEs on the concentration of tuber phytochemicals and on tuber longevity.

2.2 Material and methods

2.2.1 Plant material

Dry moringa leaf powder was obtained from RunX-KZN, Pietermaritzburg, South Africa.

Potatoes were purchased locally from Woolworths's supermarkets based on seasonal availability, so that the initial experiment was carried out using 'Fandango' and 'Mondial', while in subsequent experiments 'Mondial' and 'BP1' were compared.

2.2.2 Preparation of moringa leaf extracts

Four extracts of moringa leaf powder were prepared using 3 g powder per 100 ml of each solvent.

2.2.2.1 Water extraction

An amount of 3 g moringa powder was boiled in 250 ml distilled water. The solution was allowed to settle overnight, before filtering the extract through Whatman No. 1 filter paper into a 250 ml conical flask. This watery extract was then stored in the refrigerator at 4°C.

2.2.2.2 Methanol extraction

Similarly, 3 g of moringa powder was extracted in 30%, 50%, or 70% aqueous methanol, before being allowed to settle overnight. The solutions were separately filtered into 250 ml conical flasks and subsequently stored in the refrigerator at 4°C until the experiment was conducted.

2.2.2.3 Acetone extraction

Moringa powder (3 g each) was also extracted in 250 ml 30%, 50% or 70% aqueous acetone, before it was allowed to settle overnight. The solutions were then filtered, and extracts were stored in a refrigerator at 4°C.

2.2.2.4 Ethyl acetate extraction

Moringa powder was extracted in 30%, 50% or 70% aqueous acetone and allowed to settle overnight. The solutions were then filtered, and extracts stored in a refrigerator at 4 °C.

2.2.3 Coating of potatoes

Solvents used to extract moringa leaves were evaporated in a Genevac evaporator (Genevac, United Scientific, Durban, SA), before 5 ml of the filtered extracts were diluted to 20 ml with distilled water. To the 20 ml a few drops of Tween-20 were added to allow even solution distribution and to facilitate potatoes to be completely submerged in and covered by these solutions. Following coating, potatoes were air-dried and incubated in the dark at ambient room temperature for seven days.

2.2.4 Spectrophotometric analysis

Following seven days of incubation at room temperature, potatoes were peeled and 1.0 g peel from each potato was weighed out and several tests conducted to determine the presence or absence of phenolics, flavonoids, glycosides and tannins.

2.2.4.1 Phenolic test

Potato peel was ground in a cold mortar using 5 ml 80% acetone and small amounts of acid-washed sand. The solutions were constantly stirred for 30 min before being filtered through Whatman No. 1 filter paper and centrifuged at 20 000 g for 10 min. Aliquots (0.1ml) of the supernatant were transferred to test tubes and 2.5 ml Folin-Ciocalteau reagent was added. The solutions were left to stand for 5 minutes; thereafter, absorbance was measuredat 765 nm using a UV-1800 spectrophotometer (Lamien-Meda, 2008).

For bound phenolics, 0.3 ml of75% sodium carbonate solution was added to the pellet to saponify the residues, before incubation at 85°C for 60 min. Spectrophotometric analysis of bound phenolics was then carried out as for free phenolics. Distilled water was measured for water-MLE while acetone, ethyl acetate and methanol were used as controls for acetone-MLE,

ethyl acetate-MLE, and methanol- MLE, respectively.

2.2.4.2 Flavonoid test

Flavonoid test was carried out according to Fachriyah et al., (2020) by adding 2ml of 2% NaOH in a test tube containing 2 ml of each MLE. Strong yellow colour formed. Few drops of diluted acid were added into each test tube, the yellow colour became colourless an indication of the presence of flavonoids.

2.2.4.3 Glycoside test

A glycoside test was carried out according to Fachriyah et al., (2020) by adding 2 ml of chloroform and 2 ml of sulphuric acid in atest tube containing 2 ml of each MLE. The test tube was shaken moderately.

Brownish coloration showed the presence of a steroidal ring which is part of the glycoside.

2.2.4.4 Tannin test

A tannin test was carried out acoording to Fachriyah et al., (2020) by adding 2 drops of ferric chloride to all the diluted moringa filtrates, diluted with 1 ml of distilled water. A transient greenish to black colour indicated thepresence of tannins.

The remaining 40 unpeeled potatoes were further incubated for 30 days to investigate physical changes during potato storage.

2.2.3 Physical analysis of the potato peel

2.2.8.1 Percentage mass loss

The initial potato mass was recorded (day 0) and percentage mass loss determined at the end of each week (7 days) for four weeks. Total percentage mass loss was calculated by comparing the initial mass (at the beginning of the experiment) with the final mass recorded at the end of the experiment.Percentage mass loss (%) was calculated by subtracting each week's mass from the initial mass(day 0), divided by initial mass, and multiplied by 100.

$$Percentage\ mass\ loss\ (\%) = \frac{initial\ mass - final\ mass}{initial\ mass} * 100$$

2.2.2.2 Firmness

Tuber firmness was recorded using a densimeter (Bareiss, Oberdischingen, Germany) at the beginning of the experiment and continued on a weekly basis during the four-week course of the experiment.

2.2.9 Data analysis

All experiments were laid out in a factorial design. Data for physical parameters were subjected to analysis of variance (ANOVA) using Genstat (VSN International, UK, version 18th edition).

2.3 Results

2.3.1 Phytochemical analysis of MLEs

The phytochemical analysis of MLEs revealed the presence of phenolics and flavonoids in all MLEs (acetone-MLE, ethyl acetate -MLE, aqueous-MLE, and methanol MLE). Glycosides were absent in ethyl acetate-MLE but present in other MLEs (Table 2.1).

Table 2.1: Spectrophotometric evaluation of phytochemical compounds present in moringa leaf extracts (MLEs)

MLEs	Phenolis	Flavonoids	Glycosides
Acetone-MLE			
Ethyl acetate-MLE	+	+	-
Methanol MLE	+	+	+
Water-MLE	+	+	+

Plus signs (+) in the table denote presence of a phytochemical compound. Minus signs (-) in the table denote absence of a phytochemical compound.

2.3.2 Phenolic concentration in potato peels treated with MLEs

Potato tuber treatment was found to enhance the concentration of free phenolics in almost all treated tubers. Tubers treated with 30% methanol-MLE and 30%, as well as 50% acetone MLE, were found to have the highest concentration of free phenolics in both cultivars. 'Fandango' tubers treated with MLEs were found to have a higher concentration of free phenolics than non-treated 'Fandango' tubers (Fig. 2.1). The ability of MLEs to enhance the free phenolic concentration seems to depend on cultivar. The organic solvent concentration used to extract moringa powder was also found to influence the effectivity of MLE to enhance the free phenolic concentration, as tubers treated with 30% and 50% acetone and methanol-MLE had higher free phenolic concentrations than (Fig. 2.1).

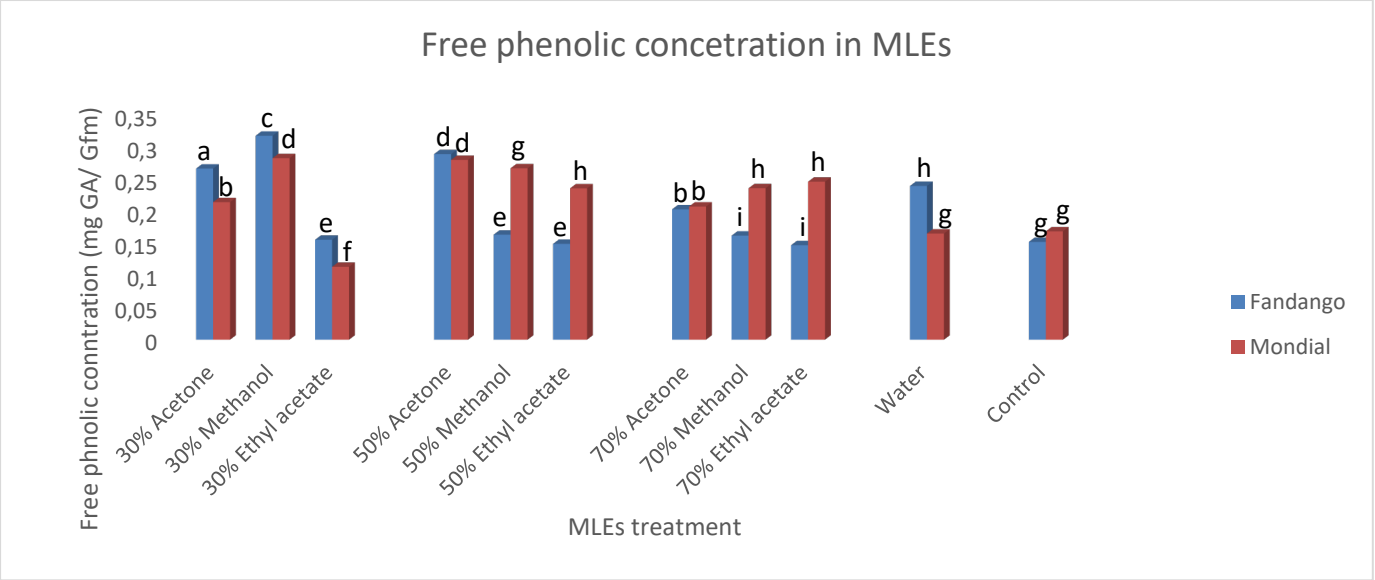


Figure 2.1: Concentration of free phenolics in potato peels 7 days after coating with various MLEs. Mean values between phenolic concentrations differed significantly (P<0.05).

The 50% and 70% methanol-MLE treatment significantly enhanced the concentration of bound phenolics in both, 'Mondial' and 'Fandango' tubers. All other MLEs enhanced the concentration of bound phenolics in tuber peels, but the fficacy differed with each treatment. 'Fandango' tubers treated with 30% methanol-MLE had a lower concentration of bound phenolics, however, it was higher than that of the control (Fig 2.2).

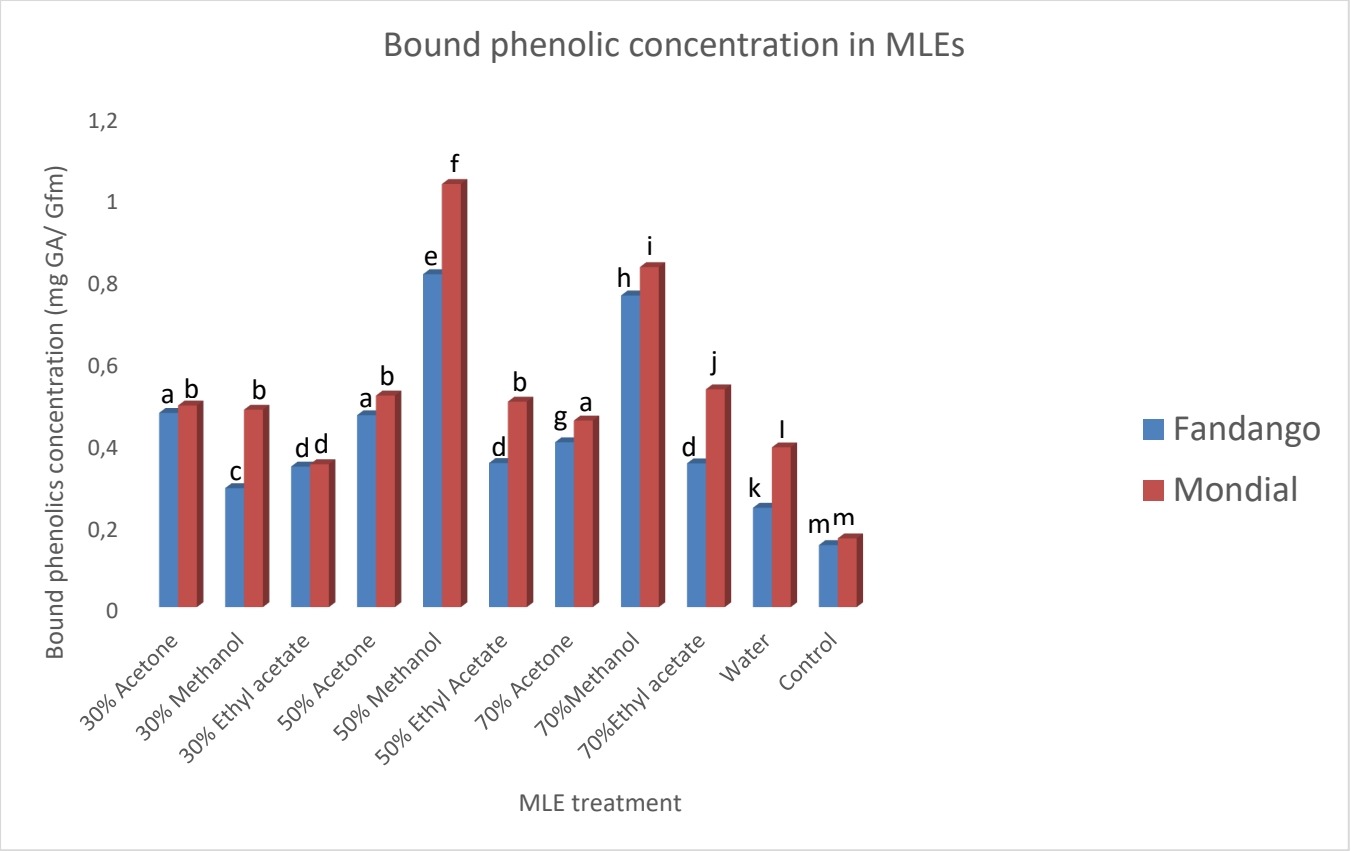


Figure 2.2: Concentration of bound phenolics in potato peels 7 days after coating with various MLEs. The mean values between the concentration of phenolics had significant difference (P<0.05)

2.3.3 Physical parameters

Potato treatment with different MLEs affected physical parameters differently; the type of solvent used as well as solvent percentage influenced physicalparameters of potato; furthermore, the cultivars ('BP1' and 'Mondial') responded differently to each treatment.

2.3.3.1 Percentage mass loss

The average percentage mass loss in 'BP1' and 'Mondial' tubers treated was affected by MLE tratment. (Figs 2.3- 2.10). While ethyl acetate MLEs were found to accelerate percentage mass loss in 'BP1' tubers, 50% methanol-MLE was found to delay percentage mass loss.

2.3.3.1.1 Percentage mass loss of 'BP1' tubers after treatment with MLE

'BP1' tubers treated with 30% ethyl acetate-MLE had the lowest percentage mass loss, while tubers coated with 80% ethyl-acetate MLE had the highest percentage mass loss. In general, tubers coated with ethyl acetate-MLE had the highest percentage mass loss, while tubers

coated with methanol-MLE had the lowest. On average, 'BP1' had higher water loss, whether treated tubers or untreated tubers (control), compared with 'Mondial' (Figs 2.3-2.10).

There was no significant difference between tubers treated with water-MLE and control tubers (untreated tubers) in the second week of storage ($P<0.05$; Fig. 2.3). From the second to the third week, differences in percentage mass loss became evident between treatments. The rate of percentage mass loss in water-MLE treated tubers was relatively lower than in control tubers, particularly in week 3.

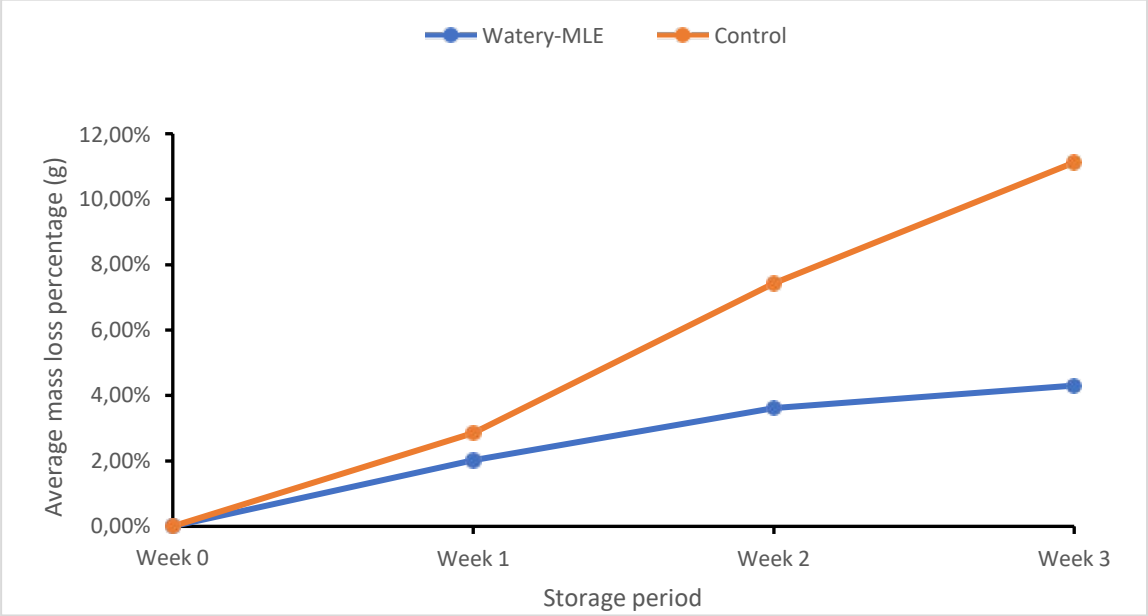


Figure. 2.3: Average percentage mass loss of 'BP1' tubers treated with water-MLE over three weeks

Tubers treated with 30% methanol-MLE had the lowest mass loss, significantly lower than control tubers ($P<0.05$). On the other hand, tubers treated with 50% methanol-MLE had a tendency towards higher mass loss than other treatments during the first two weeks, which was significantly higher than that of control tubers ($P<0.05$). From week three to week four, mass loss of tubers treated with 50% methanol-MLE significantly declined; from week three the rate of mass loss of tubers treated with 50% methanol-MLE increased at a lower rate compared with the 30 and 80% MLE treatments. At the last observation point all three methanol-MLE-treated tubers had lost significantly less mass than control tubers (Fig. 2.4).

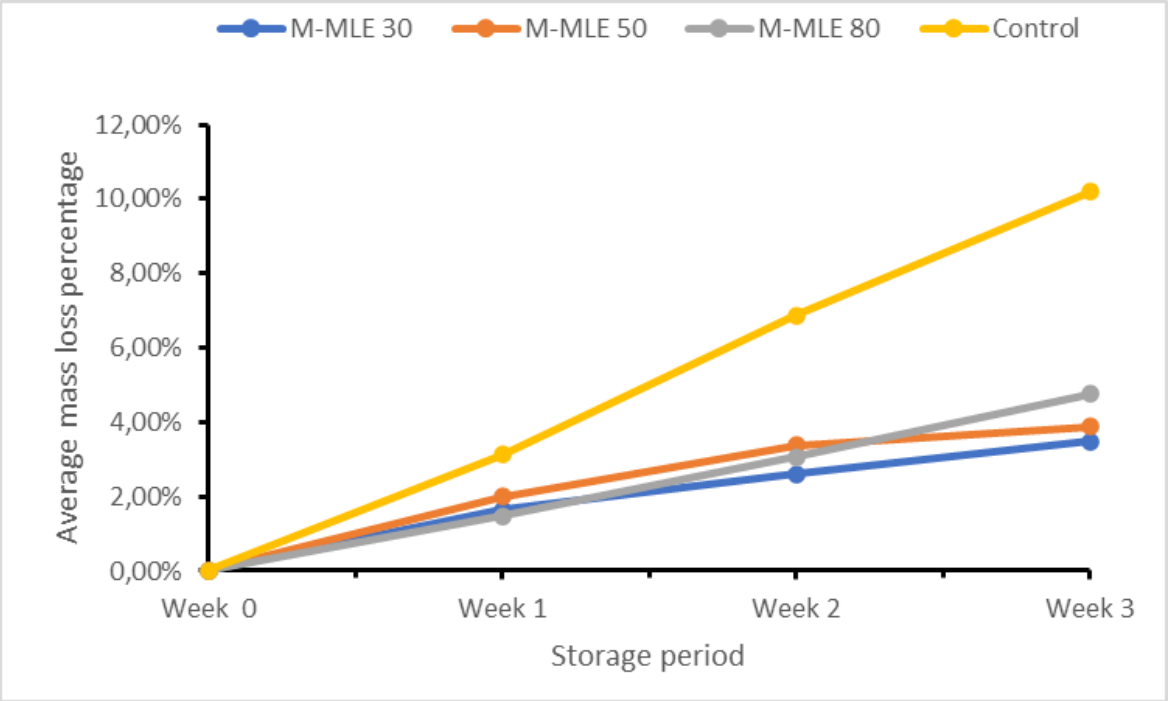


Figure 2.4: Percentage mass loss in 'BP1' tubers treated with different concentrations of methanol-MLE (M-MLE).

There was a significant difference between acetone-MLE treated tubers and control tubers (P>0.05), particularly in week 2 and week 4. The rate of percentage mass loss in all acetone-MLE treated tubers was linear (Fig. 2.5). Tubers treated with 30% acetone-MLE had the least rate of percentage mass loss over the three-week storage period.

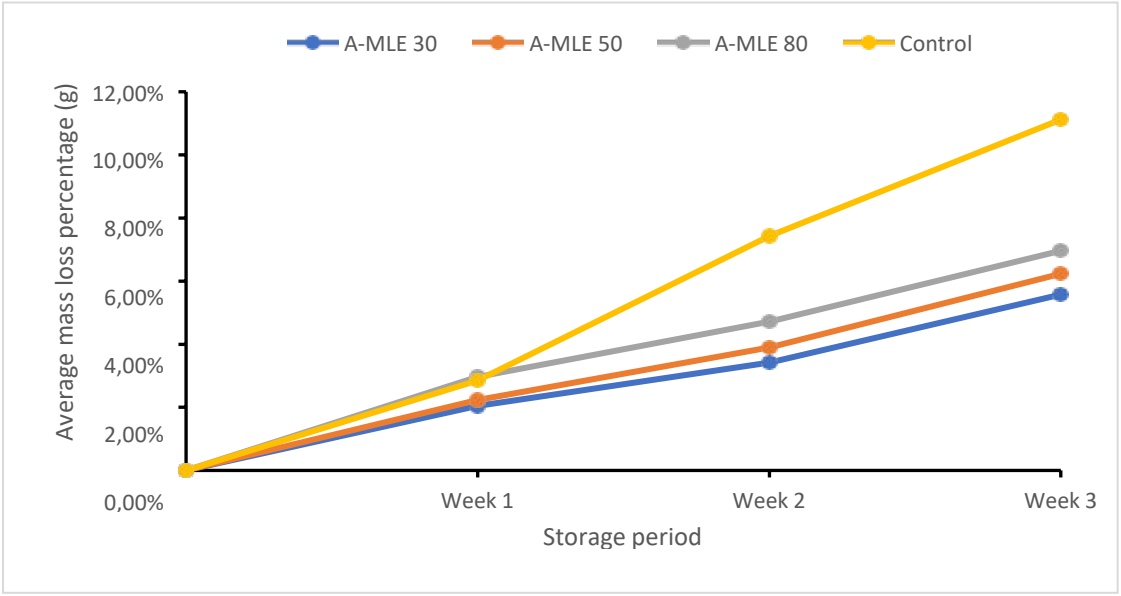


Figure 2.5: Average percentage mass loss in 'BP1' tubers treated with various concentrations of acetone-MLE (A-MLE).

In the first week, there was no significant difference between control tubers and 80% ethyl acetate treated tubers; and between 30% and 50% ethyl acetate treated tubers. Nevertheless, the rate of percentage mass loss of control tubers increased rapidly in the second week and became significantly different from the treated tubers (Fig. 2.6).

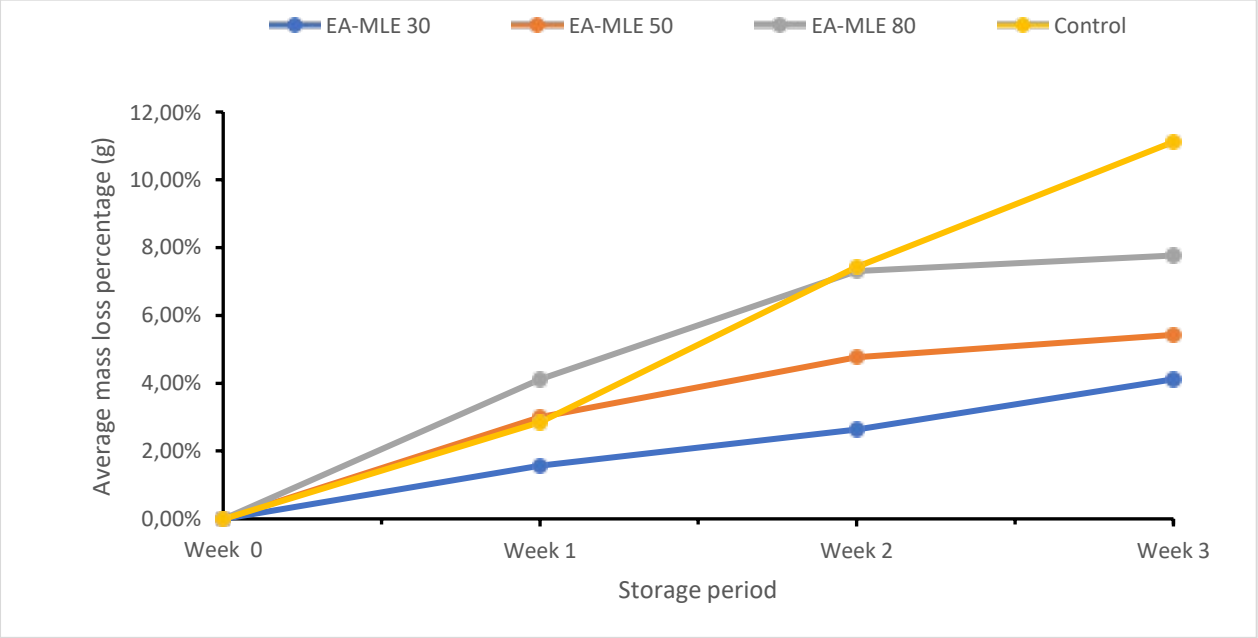


Figure 2.6: Percentage mass loss of 'BP1' tubers coated with various ethyl acetate-MLE solutions after three weeks of storage

2.3.3.1 Percentage mass loss of 'Mondial' tubers treated with various MLEs over a four-week period

'Mondial' tubers treated with water-MLE had a significantly lower mass loss compared with control 'Mondial' tubers (untreated) ($P<0.05$). The average mass in tubers treated with water-MLE was particularly low during the first two weeks of storage. The rate of mass loss, however, increased in these treated tubers from week 3 onwards (Fig. 2.7)

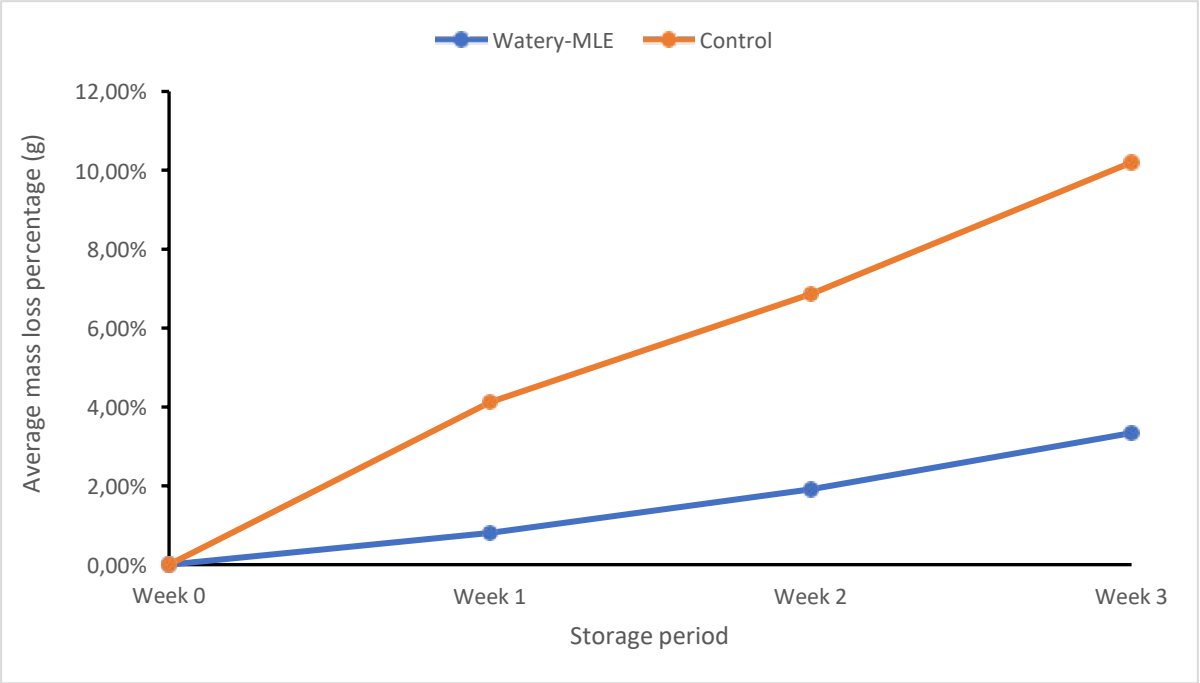


Fig. 2.7: Percentage mass loss of control 'Mondial' tubers and tubers treated with water-MLE incubated for a three-week period

Methanol-MLE significantly decreased percentage mass loss in 'Mondial' tubers, maintaining the average mass loss below 5% throughout storage period. There were no significant differences amongst the methanol-MLE treated tubers and control tubers ($P>0.05$) (Fig. 2.8). Methanol-MLE significantly decreased rate of percentage mass loss in tubers, maintaining average percentage mass loss rate below 5% throughout storage period (Fig. 2.8).

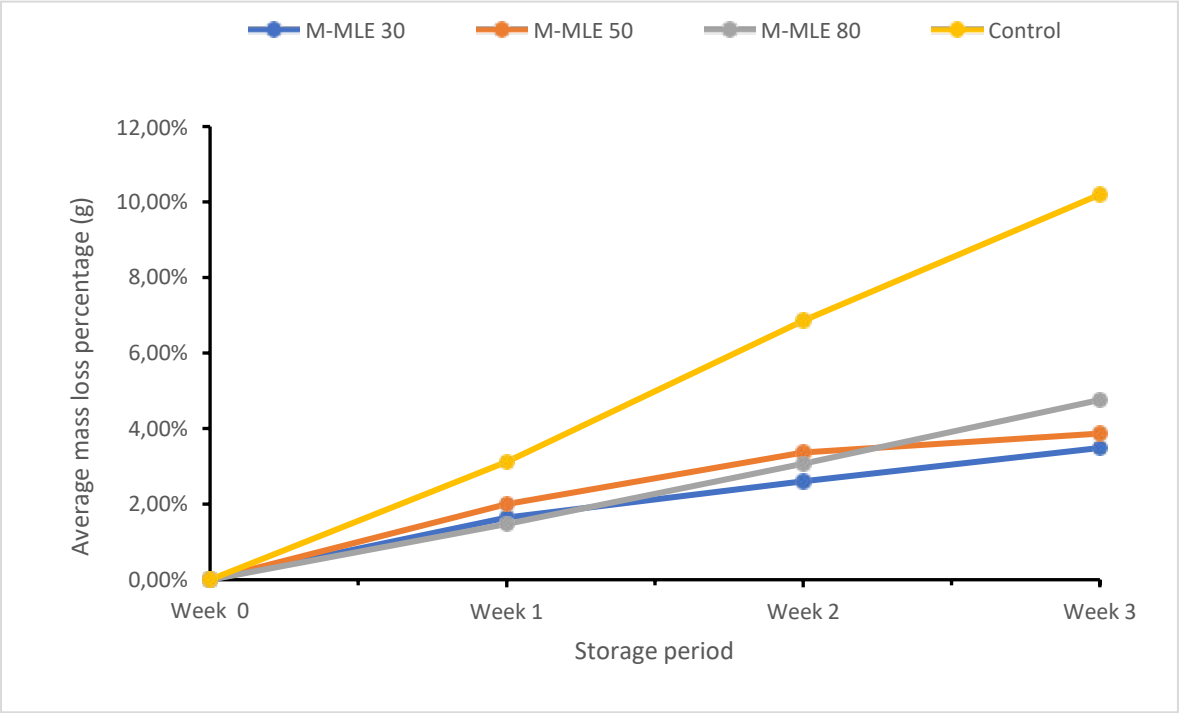


Figure 2.8: Percentage mass loss in 'Mondial' tubers coated with methanol-MLE over the three-week storage period (M-MLE)

There was no significant difference in percentage mass loss between control tubers and tubers treated with 30% acetone-MLE after the first week. During the third week, mass loss in tubers treated with 80% acetone-MLE seemed to slow down; therefore, at the end of week 3, acetone-MLE treatments had all lost significantly less mass than control tubers (Fig. 2.9).

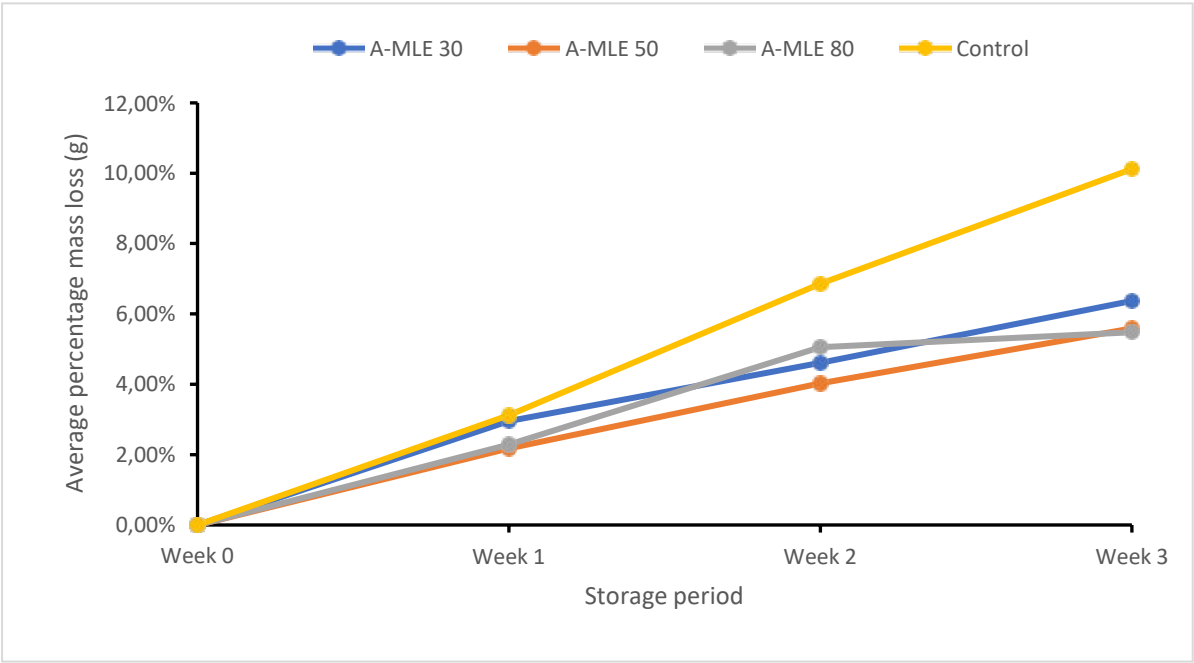


Fig. 2.9: Percentage mass loss of 'Mondial' tubers treated with acetone-MLE and control

tubers (A-MLE)

Similar to methanol MLE, all ethyl acetate MLE treatments reduced percentage mass loss; however,the better performance of the 30% treatment became clear, when comparing the three ethyl acetate treatments.

All MLEs resulted in significantly lower mass loss than in the control (Fig. 2.10).

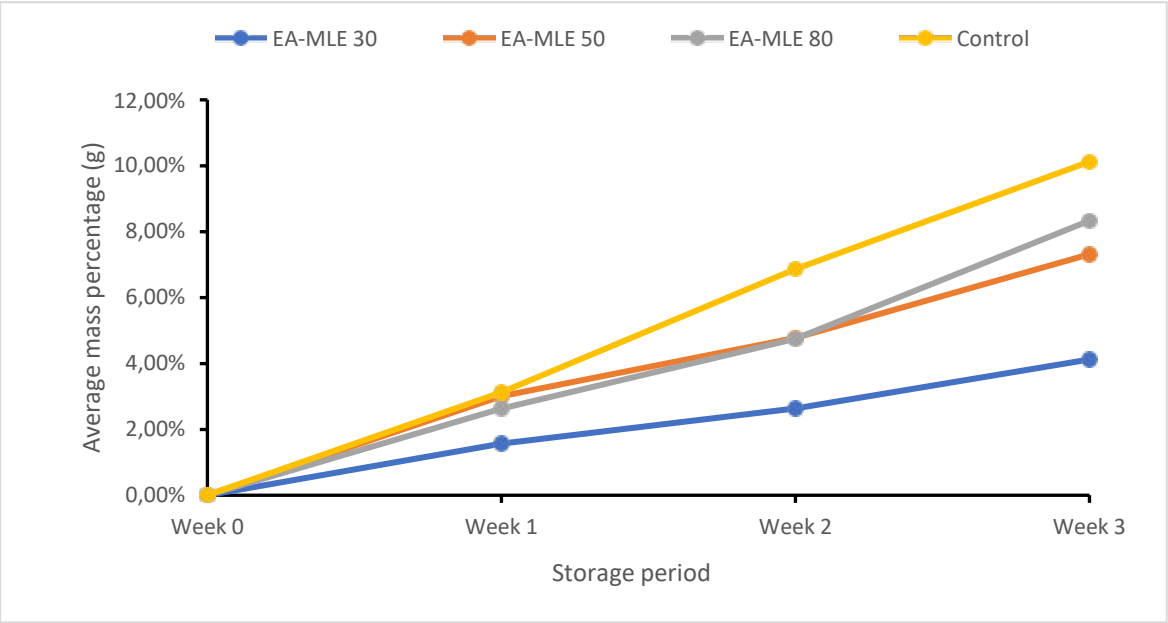
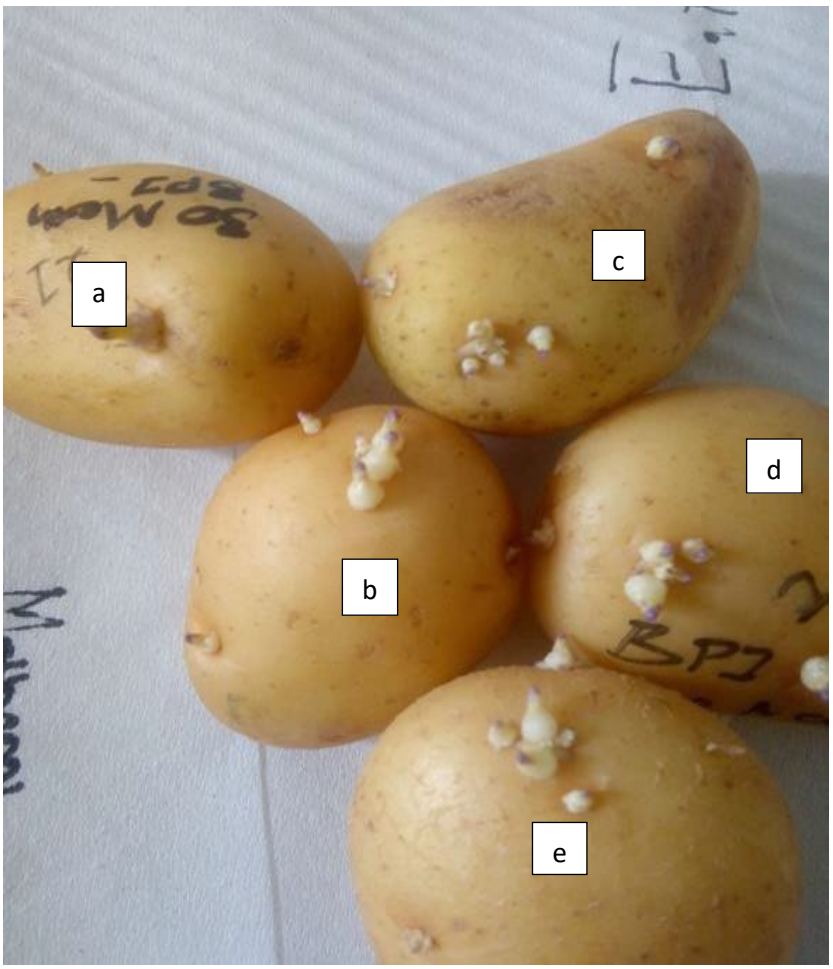


Figure 2.10: Percentage mass loss of 'Mondial' tubers treated with ethyl acetate compared with untreated tubers over thethree week storage period

2.3.4 Physical appearance

Extracts of moringa powder prepared using lower solvent concentrations were found to betterpreserve tuber appearance. High solvent concentration seemingly influenced tuber skin appearance negatively, as tubers treated with 50% and 70% methanol-MLE, as well as with 70% ethyl acetate showed russeting in both cultivars (Fig. 2.11e). In tubers treated with water-MLE (Fig 2.12) and MLEs extracted with lower solvent concentrations (30% and 50%) (Fig 2.11) sprouting was delayed, as the shoots appeared a week later after other tuber had developed sprouts. Sprouts of tubers treated with lower solvent concentrations had purplish tips (Fig. 2.11b, d&e), indicating presence of anthocyanins; this colouring was absent in



tubers treated with water-MLE.

a=30% methanol-MLE, b=50% acetone-MLE, c= 30% ethyl acetate-MLE, d=30% acetone-MLE, e= 50% methanol-MLE.

Figure 2.11: Tubers coated with various MLEs produced anthocyanin-containing sprout tips, visible as purplish colour; these sprouts were absent on the control tubers and water-MLE treated tubers (Fig. 2.12).

Treatments with 30% methanol MLE, water-MLE and 30% acetone-MLE (Fig. 2.11) were found to maintain healthy tuber appearance. Tubers treated with 50% methanol MLE showed skin russeting. Tubers treated with 30% ethyl acetate were visually less appealing as they had russeting. Tubers treated with water-MLE produced sprouts that seemed to have relatively low concentrations of anthocyanins, as the sprout tips did not have a purplish colour (Fig. 2.12).).

Figure 2.12: Water-MLE treated tubers did not show purple colour on the sprout tips indicating absence or low concentration of anthocyanins

2.4 Discussion

Potato production forms a pivotal component of food security in South Africa (Alamar et al., 2017). As a widely consumed crop, it is vital that its phytochemical constituents are improved to fight malnutrition, predominant in South African villages and townships.Phenolics, flavonoids, tannins, and alkaloids are amongst the important phytochemicals found in potato tubers; they are crucial in tuber quality and tuber disease resistance, factors essential in the processing and seed potato sectors (Alamar et al., 2017). During storage, large volumes of tubers can be lost due to poor tuber quality. To prevent tuber loss and improve potato tuber phytonutritients, tuber treatment with moringa extracts was explored in this study, as the multitude of phytochemicals found in moringa have exceptional antioxidant activity (Yasmeen et al., 2014) and, therefore, are likely to reduce oxidative stress on tubers.

Spectrophotometric analysis revealed the presence of phenolics, flavonoids and glycosides in all MLEs, except for absence of glycoside in ethyl acetate extracts. This finding is in accordance with Zaffer et al. (2015), who also reported the absence of glycosides in ethyl acetate-MLEs. Coating with MLEs enhanced the concentration of both, free and bound phenolics (Figs 2.1 and

2.2) in potato peels Treatment with 30% ethyl acetate-MLE reduced the free phenolic concentration in 'Mondial', with no notable effect on 'BP1' (Fig. 1), an important finding, as phenolics are vital component of a plant's defence mechanism (Agrawal, 2007). Even though potatoes naturally contain phenolic compounds all MLE applications enhanced the phenolic concentration in potato peels (Figs 2.1 and 2.2). Coating with MLEs can, therefore, not only enhance the concentration of phenolics, but also of other phytochemical compounds. Together, these phytochemicals might play an important role in antimicrobial and antioxidant activity of the tuber. Presented results are in accordance with those obtained by Ibrahim et al. (2018), who reported that organic fertilizer applied 'in-field' to the herb *Labisia pumila* improved total phenolic and flavonoid concentrations in its leaves.

Solvent type and solvent concentration used to prepare MLEs was found to influence tuber response to MLE treatment. The concentration of both, free and bound tuber phenolics, was also found to be affected by solvent concentration and organic solvent type. This is likely to be due to the solvent composition, whereby higher organic solvent concentrations (e.g. 70%) resulted in lower tuber phenolics. Further, moringa leaf powder extracted with methanol resultedin higher phenolic concentrations in the MLE than extraction with ethyl acetate (Fig. 2.2). This is similar to results obtained by Nobossé et al. (2018), who found that methanol-MLEs contained higher phenolic concentrations than extracts prepared with ethyl acetate, acetone or water. This may be caused by the polarity, diffusion strength and/ or structural complexity of the solvent used to extract moringa leaves (Munatel et al., 2017; Nobossé et al., 2018). The reason for higher phenolic concentrations in tuber peels treated with methanol-MLE could be that methanol triggered a defence mechanism in tubers, so that tubers released more phenolics from bound forms as a defence strategy. The phenolics might have been soluble in methanol, but insoluble in acetone, ethyl acetate and water; these phenolics might have antifungal activity. Enhanced phenolics in potato tubers may also result in benefits to humans upon consumption of such potatoes; Nevertheless, the phenolic concentration of the whole tuber would have to be analyzed. Methanol is, however, a toxic substance and cannot be recommended for human consumption, as it may cause illnesses that can result in poisoning or even death (https://www.cdc.gov/niosh/ershdb/emergencyresponsecard_29750029.html); thus, the methanol residue from the tuber would have to be reduced. Alternatively, A bio-extract that is safe for consumption and has similar polarity will have to be used during the moringa powder extraction process.

According to Altemimi et al. (2017) solvents with higher polarity tend to be more effective in extracting antioxidants. This may be the reason why the MLE extracts obtained using methanol (polarity 11) as a solvent, had higher phenolic concentrations than those obtained using other solvents. Moringa leaf extracts prepared using ethyl acetate (polarity 4.4) had the lowest phenolic concentrations (Figs 2.1 and 2.2). Given that phenolics are antioxidant compounds, these results may also explain the variation in physical changes observed amongst tubers treated with MLEs. Tubers treated with ethyl acetate-MLE had the highest rate of water loss, particularly those of the 'BP1' cultivar, while methanol-MLE treated tubers showed the lowest water loss rate in both cultivars.

Tubers coated with methanol-MLE had the least russeting amongst all tubers (Fig. 2.11e). Control tubers showed most russeting and were less firm compared with treated tubers. This could be attributed to the antioxidant activity of the phenolic compounds present in methanol-MLE. Phenolics have an ability to scavenge free radicals, which are commonly produced under stress. Free radical damage is caused by reactive oxygen species, which, when present in high concentrations, can cause tissue damage in plant cells (Tesfay et al., 2011). This cell damage results from a chain reaction that leads to cell death. Antioxidants act, hence, as a defence system by scavenging free radicals, ultimately reducing the concentration of reactive oxygen species and, subsequently, preventing lipid peroxidation. Antioxidants derived from plants also have minimal side effects, in contrast with synthetic antioxidants (Vats and Gupta, 2017).

Percentage mass loss in tubers is associated with tuber quality degradation, which results in unappealing tubers, thereby often negatively influencing consumer buying decision. Russeting often appears on tubers after a percentage mass loss of more than 10% (Ngceni, 2019). One method of maintaining potato postharvest quality would, hence, be to minimize mass loss in any way possible. In this study all treatments were able to reduce mass loss, however, treatments of higher solvent concentration (80%) led to higher mass loss, while lower solvent concentrations (50% and 30%) reduced mass loss. In this case, it is also important to note that cultivars responded differently, as 'BP1' tubers treated with 30% ethyl acetate-MLE had a lower percentage mass loss than control tubers, while 'Mondial' tubers treated with 50% methanol-MLE had the lowest percentage mass loss (Fig. 2.3-2.10). This may be due to the thicker periderm of certain potato cultivars compared with tubers with a thinner periderm, the latter will lose more mass postharvest. Cultivars which lose less mass postharvest are preferred by consumer and retailer alike, because they remain firmer for longer and maintain visual

attractiveness to the consumer. According to Azad et al. (2017), tuber water loss due to respiration and transpiration leads to percentage mass loss. Furthermore, external factors, such as postharvest storage, and internal factors, such as cultivar, genotype, and maturity, have a major impact on tuber mass loss (Azad et al., 2017).

2.5 Conclusion

Extraction of moringa leaf powder with 50% methanol and 30% acetone is recommended over other methanol and acetone concentrations as well as other organic solvents, as methanol-MLEs had the highest concentration of phenolic compounds. It is, however, also important to note that methanol has toxic effects and, thus, cannot be used in the food industry. These experiments need, therefore, to be extended to 'safer' extraction solvents that have similar polarity to methanol, the organic extraction solvent that significantly enhanced total phenolic concentration, and reduced percentage mass loss according to these experiments. Acetone-MLE and aqueous-MLE were also effective in enhancing SMs in potato. Results also indicate that potato cultivars respond differently to MLE treatment. Hot water could also be used to prepare moringa extracts, as, in this study cold water was found to be less effective in extracting SMs from moringa leaves as compared with 30% acetone and 50% methanol. The results from this study indicate that MLEs arer promising postharvest treatments that could be used to enhance the concentration of SMs and, thus, improve phytonutrient concentrations present in potato tubers.

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Chapter 3
In vitro analysis of the fungal inhibition activity of moringaleaf extracts against *Fusarium oxysporum*

Fusarium oxysporum (F. oxysporum) is a soil-borne plant pathogen that causes serious diseases in many economically important crops worldwide. In this study, moringa leaf powder was extracted with acetone, ethyl acetate, methanol, or water, at three concentrations (30 (w/v), 50 (w/v) or 80 (w/v), to investigate the antifungal efficacy of these moringa leaf extracts (MLEs) against *F. oxysporum*. It was further attempted to evaluate the effect of solvent type and solvent concentration on the efficacy of MLE on F. oxysporum inhibition. Following incubation for three days at ambient temperature, symptom development was evaluated. The disc diffusion method was used to examine MLE's inhibition activity on *F. oxysporum.* Various prepared MLE stock solutions (3 g moringa leaf powder in 250 mL acetone, ethyl acetate, methanol, or water) were further diluted with distilled water to 2% (w/v) and 4% (w/v) solutions, to determine the minimum inhibitory concentration (MIC). All MLEs using 30% and 50% solvent had 100% inhibition activity, while water MLE had an inhibition activity of only 87.5%. The MLEs extracted with 80% acetone, ethyl acetate or methanol, had an inhibition activity of 95.0%, 86.4% and 90.3%, respectively, significantly lower than the 30 and 50% solvent extracts. The MIC was 2% (v/v) for all stock solutions. Thus, MLEs showed high antifungal efficacy, particularly extracts with lower and medium solvent concentrations. These findings indicate the potential of MLEs as biofungicides potentially used to prevent crop losses due to *F. oxysporum*.

Keywords: Secondary metabolites, potato, acetone, methanol, ethyl acetate

3.1. Introduction

The genus *Fusarium* comprises various species that are distributed all over the world. *Fusarium oxysporum* is a common species of the *Fusarium* genus. It is a soil-borne fungus adapted to different soil types; therefore, it is prevalent in many countries (Lombard *et al*., 2019). The fungus is differentiated from other *Fusarium* spp. by morphological characteristics,such as shape and size of both macroconidia and microconidia, colour of colony, chlamydospore absence or presence and the growth rate of cultured species on growth media. *Fusarium oxysporum* has an ability to infect and cause disease in animals and plants (Lombard *et al*., 2019). All strains of *F. oxysporum* are asexual saprophytes that have an abilityto endure unfavourable conditions for extended periods in soils, water bodies, plant debris, as well as on harvesting equipment and in storage facilities without a host plant (Sandoval- Denis *et al.*, 2018; Lombard *et al*., 2019).

Fusarium oxysporum is an economically important species and has been listed in the top 10 most important pathogens in the world; this is because the species has a wide host range, including economically important crops such as maize (*Zea mays* L.), sugarcane (*Saccharum officinarum*), bean (*Phaseolus vulgaris* L.), banana (*Musa* spp.), potato (*Solanum tuberosum* L., tomato (*Solanum lycopersicum* L.), and various ornamentals (Michielse and Rep, 2009). This fungus is a causal agent of several plant diseases such as vascular wilts, damping-off, corn rot and root rots. According to Hafizi *et al*. (2013), *F. oxysporum* is becoming a human pathogen that is expected to cause serious illness in individuals with compromised immune systems. For *F. oxysporum* to induce an infection, it needs an open wound to infiltrate the tissue. From there on, it colonizes the vascular system of the plant, which results in pre- harvest diseases characterized by damping-off, wilting and reduced crop vigour (Michielse and Rep, 2009). Some *F. oxysporum* strains are endophytes and, thus, do not induce visible symptoms (Michielse and Rep, 2009; Sandoval-Denis et al., 2018).

Diseases caused by *F. oxysporum* are mainly controlled by preventive measures, such as integrated pest control, including crop rotation, biological control, and fungicides. There are currently no commercially available biological control measures, nor any crop cultivars resistant to *F. oxysporum* (Wharton *et al*., 2007). Although some fungicides are efficient at controlling *F. oxysporum* diseases, the fungi continue to develop resistance towards these fungicides. Furthermore, there are many concerns considering the use of chemical fungicides,

due to the fact that their residues are detrimental to the environment; additionally, chemicalcontrol can only prevent around 7% of such diseases (Gifoni *et al*., 2012). Thus, there is high pressure for the development of ecosystem-friendly fungicides that will also reduce the dependency on chemical fungicides (Yusuf *et al*., 2011).

Plant extracts have been used by humans to treat human and animal diseases for many centuries. This is due to the high concentration of phytochemicals in plant extracts exhibitingantimicrobial properties. These extracts are environmentally friendlier and have minimal to no toxicity to consumers (Gifoni *et al.*, 2012; Batista *et al.*,2014; Datta *et al.*, 2014; El–Mohamedy and Abdalla, 2014). In the 1990's the use of plant extracts in integrated pest management began (Freire *et al*., 2015); leaf, seed and bark extracts are most commonly tested plant organ extract used against phyto-fungi. Leaf and bark extracts are highly effectiveagainst *Pythium infestans* and *Alternaria* sp. (Yusuf *et al*., 2011). *Moringa oleifera* Lam (moringa) is a plant that has gained popularity due to its unique phytochemical composition that has been proven, by various researchers, to encompass antifungal compounds (Kanyinda *et al*., 2018). Moringa is rich in compounds containing the simple sugar rhamnose and certain unique groups of compounds (glucosinolates, isothiocyanates and chitin-binding protein) (Freire *et al*. 2015). These phytonutrients are responsible for the antifungal action of moringa leaf extracts (MLEs) (El-Mohamedy and Abdalla, 2014).

Previous studies have shown that solvents used in moringa extraction affect the phytochemicals extracted, as the extraction efficiency for a certain compound largely depends on the polarity of the solvent. There are numerous organic solvents used in moringa extraction, such as acetone, ethanol, and methanol, while water is also commonly employedas an extraction solvent.

The aim of this study was to investigate the antifungal efficacy of MLEs. The objectives were to investigate the effect of different extraction solvents (acetone, ethyl acetate, methanol, and water) on the antifungal efficacy of MLEs; further, it was attempted to determine the minimum inhibitory concentration (MIC) of MLEs.

3.2Materials and methods

3.2.1 Culturing *Fusarium oxysporum*

Fusarium oxysporum culture (Accession number 32931) was obtained from the Agricultural Research Council - Plant Health and Protection (ARC-PHP), National Collection of Fungi, in Roodeplaat, Pretoria. The fungus was cultured on potato dextrose agar (PDA). As per manufacturer's instruction, an amount of 39 g PDA (OXOID®) was mixed with 1 L of distilled water. The mixture was autoclaved for 15 minutes at 121°C. After cooling, the molten PDA agar was poured into petri dishes (90 mm diameter) and was left to solidify overnight under sterile conditions. A 4 x 4 mm^2 PDA plugs containing *F. oxysporum* mycelium were cut and placed at the centre of the freshly prepared PDA plates; and these plates were then incubatedat 28°C for 14 days.

3.2.2 Cell count

Conidia were collected 14 days post-incubation. This was achieved by flooding the surface ofthe PDA plates with 5 ml of sterile distilled water. The surface of the media was gently scrapedwith an L-shaped glass rod to dislodge the conidia as described by Ahmed (2017). Mycelial fragments were removed by straining the conidial suspension through a sterile cheese cloth.The conidia suspension was adjusted to a concentration of $1x10^6$ conidia ml^{-1} using a haemocytometer (Soleimani and Kirk, 2012). The conidial suspension was stored in 30% glycerol at -80 °C for subsequent use.

3.2.3 Morphological characterisation

The identification of *F. oxysporum* was performed based on the characteristics described by Leslie and Summerell (2008).

Macroscopic observation was performed on the previously cultured *F. oxysporum*. The colourof the colony and pigmentation were observed on potato dextrose agar (PDA) as per colour chart by Kornerup and Wancher (1978).

For microscopic characterization, a wet mount was prepared by transferring a loopful of the suspension onto a glass slide and viewed under a light microscope using 40X magnification, to confirm the presence of conidia.

3.2.4 Pathogenicity test

The fungus*, F. oxysporum,* was cultured on PDA for seven days at 28°C. A wet mount was prepared to observe macroconidia formation under the microscope at 40X magnification. The solution

containing *F. oxysporum* was prepared by washing cultured *F. oxysporum* with 5 ml of distilled water and gently scraping the mycelium off, using an L-shaped glass rod. The solution was filtered through a sterile cheese cloth, and the resultant suspension of conidia was used to inoculate fresh, locally obtained 'Mondial' and 'Valor' potato tubers. Tubers were slightly wounded using sandpaper, before 1 ml of the *F. oxysporum* solution was pipetted onto this wounded area. Tubers were then incubated at ambient temperature for three days. Presence of fusarium dry rot symptoms were evaluated daily.

3.2.5 Preparation of MLE dilutions

The MLE solution was prepared as described in Chapter 2. The experiment was laid out in a complete randomized design with five treatments and three replications. Five treatments were: 1) *F. oxysporum* cultured in methanol-MLE, 2) *F. oxysporum* cultured in acetone-MLE,
3) *F. oxysporum* cultured in ethyl acetate-MLE, 4) *F. oxysporum* cultured in aqueous-MLE and
5) *F. oxysporum* cultured in distilled water (control).

From each extraction solvent, three concentrations (30%, 50% and 80%) were used to prepare eleven stock solutions. From each of the ten MLE stock solutions, 2% (v/v) MLE dilutions were prepared by diluting 2 ml of the stock solution with 8 ml distilled water, making a total amountof 10 ml MLE dilution.

Similarly, the 4% MLE dilution was prepared by diluting 4 ml stock solution with 6 ml distilledwater, making a total volume of 10 ml. Data were recorded after four days of incubation at 28^{o}C.

3.2.6 Determination of fungal inhibition activity

Discs to be used were sterilized by autoclaving for 15 minutes at 121°C.

The confirmed *F. oxysporum* plugs (see Section 2.2) were cut from the edge of the actively growing colonies of the pathogen culture (see Section 2.1), before placing them at the centre of newly prepared PDA plates. The disc diffusion method was used (Beur *et al*., 1966), whereby each PDA plate was divided into four quadrants. Four discs, each absorbing 2% dilution of (30%, 50% and 70%) either acetone, ethyl acetate, methanol and water-MLE, wereplaced at the centre of each quadrant. There were three replicates for each plate. The same procedure was repeated for 4% MLE dilutions and used to investigate fungal inhibition activityof MLEs.

The inoculated plates were incubated at 28^{o}C for four days. A transparent ruler was used to measure the diameter of the inhibition zones (in mm) around the discs after four days of incubation.

Control treatment, discs were impregnated with distilled water. They were then placed at the centre of each quadrant, with *F. oxysporum* at the centre of the PDA plate.

The diameters of inhibition zones were compared with that of the control. To calculate the inhibition of fungal growth by MLEs, the following formula was used: Inhibition percentage (IP) = (C-T/C) × 100, where IP referred to the inhibition of mycelium growth by MLE, T = diameter of treated fungal growth, C= diameter of non-treated fungal growth (Hlokwe, 2018).

The minimum inhibitory concentration (MIC) was defined as the lowest concentration able toinhibit mycelial growth according to Latifa *et al*. (2016). Mycelial growth was visually monitored daily, and the MICs were determined 4 days after the mycelia of positive controlshad completely covered the PDA plate.

3.2.7. Data analysis

Data obtained were subjected to analysis of variance (ANOVA) using Genstat (VSN International, Hemel Hempstead, England, UK, version 18th edition) to determine any significant difference between fungal inhibition activity by MLE treatments. Differences wereconsidered significant at $P<0.05$.

3.3 RESULTS

3.3.1 Pathogenicity test

Three days after inoculation with *F. oxysporum*, potato tubers from both cultivars ('Valor' and 'Mondial') developed dark depressions, one of the symptoms of fusarium dry rot (Fig. 3.1b). Tubers alsohad visible, white mycelium growth on the tuber skin (Fig. 3.1a).

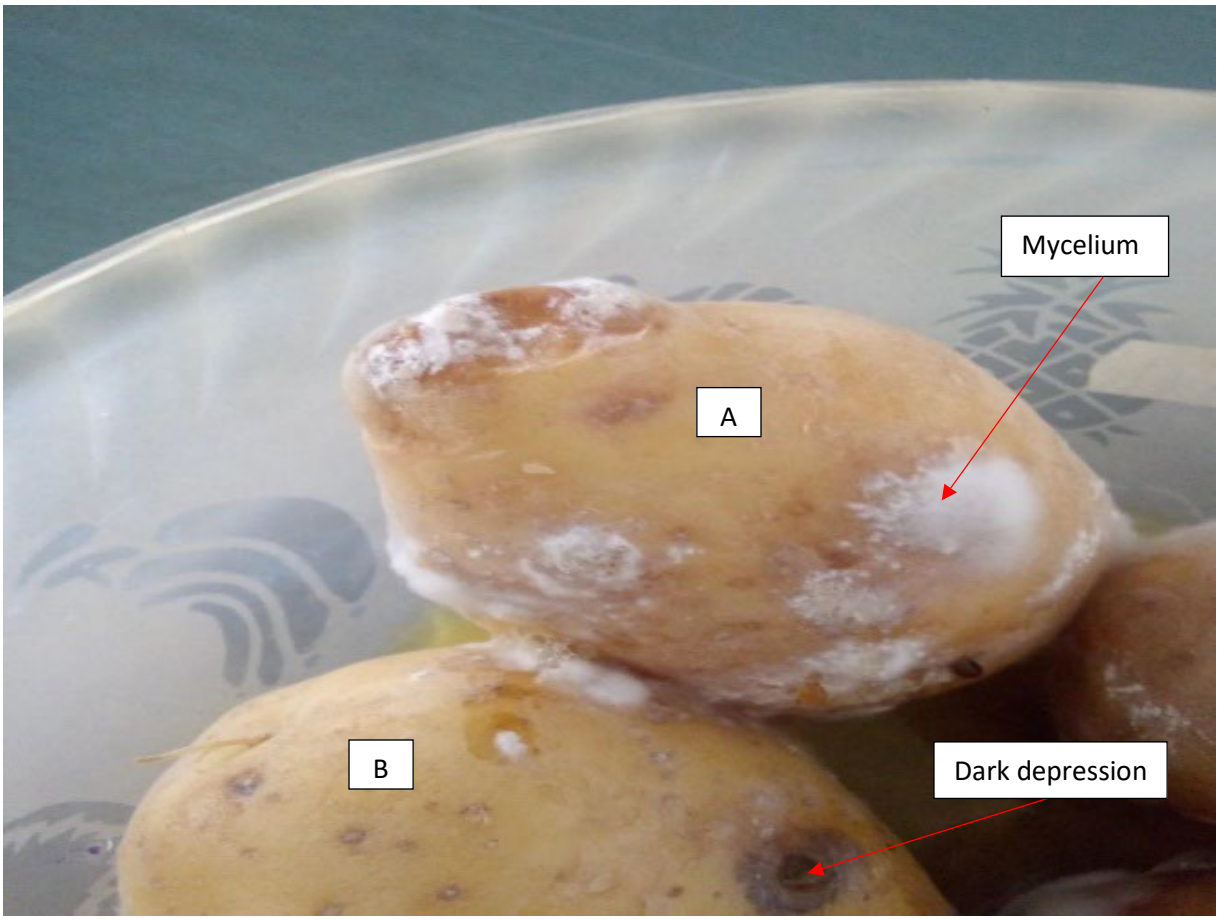

Figure 3.1: Lesions and mycelium of pathogenic *F. oxysporum* on 'Mondial' (A) and 'Valor' (B) tubers

3.3.2 Morphological characterisation of *F. oxysporum*

Fusarium oxysporum was viewed under the microscope to observe the morphological characteristics. The conidiogenous cell was long, branched and had septations. The *F. oxysporum* had a pinkish mycelium when viewed from the top of the PDA plate; at the bottomof the plate the media appeared to be violet.

Table 3.1: Macroscopic characteristics of *F. oxysporum* culture

	Macroscopic characteristics
[a]Colony colour	Pinkish
[b]Pigmentation	Dark violet
Growth rate	4.4±0.7
Conidiogenous cell	Long and branched
Macroconidia septation	3-5

a = Colony colour was determined by observing the upper surface of the colony; b = Pigmentation was determined by observing the surface of the colony on the PDA plate

3.3.3 Inhibition activity of moringa leaf extracts

All treatments were found to be effective in inhibiting fungal growth; however, as the solvent concentration increased from 30% to 70%, inhibition decreased (Table 3.2). The two lower solvent concentrations (30%, 50%) had 100% inhibitory activity, while the 70% solution did not result in complete inhibition of *F. oxysporum* growth. Water extracts (MWLE) resulted in 87.5% fungal inhibition. There was no significant difference between the 2% (v/v) and the 4% (v/v) dilution ($P > 0.05$).

Table 3.2: Antifungal activity of MLE extracted with acetone, ethyl acetate, methanol, and water at three solvent concentrations (30, 50, 70%) tested *in vitro* against *F. oxysporum*

	Percentage inhibition			
Solvent percentage	Acetone (MALE)[1]	Ethyl acetate (MELE)[2]	Methanol (MMLE)[3]	Water (MWLE)[4]
30%	100%[a]	100%[a]	100%[a]	87.5%[d]
50%	100%[a]	100%[a]	100%[a]	
70%	95.0%[b]	86.3%[d]	90.3%[c]	

[1]Moringa acetone leaf extract, [2]Moringa ethyl acetate leaf extract; [3]Moringa methanol leafextract; [4]Moringa water leaf extract. Different letters within a column denote significant differences at $P < 0.05$.

3.4 DISCUSSION

Moringa is the only genus in the Moringaceae family, containing 13 tree or shrub species thatare widely distributed in the tropics and subtropics (Freire *et al*., 2015). All moringa species are known to be rich in phytochemicals that are highly effective in inhibiting both animal and phyto-pathogens. Moringa leaves are rich in secondary metabolites that exert antifungalactivities, containing a rich and rare combination of zeatin, quercetin, β-sitosterol, caffeoylquinic acid and kaempferol (El-Mohamedy and Abdalla, 2014).

All MLEs used in this study were found to be effective inhibitors of *F. oxysporum* growth. Theresults

obtained (Table 2) are in accordance with those reported by Sánchez-Maldonado *et al*. (2016), who reported application of MLE to inhibit mycelial growth of *F. oxysporum f. sp. lycopersici*. Furthermore, high concentrations of moringa had higher fungal inhibition efficacy;these extracts were found to be exceptional in hindering spore germination, growth and in reducing dry mycelia weight (El-Mohamedy and Abdalla 2014).

The fungal inhibition percentage differed with the type of extraction solvent used, forinstance, methanolic MLE was found to be the most efficient solvent for extraction of phytochemicals from moringa leaf powder (Chapter 2). This is in accordance with results obtained by Truong *et al.* (2019) who found methanol to be the best moringa leaf extractionsolvent, as it seemingly extracted more phytochemicals than acetone, ethyl acetate, or water.This high extraction yield was evident by the fungal inhibitory activity of these methanolic MLEs. The level of inhibition was probably due to the antifungal phytochemicals that may have been tightly bound to the leaf tissue; such phytochemicals include phenolics, saponins and flavonoids (Truong *et al*., 2019).

From this study, it is evident that the extraction method plays a vital role in the inhibition activity of MLEs; this is likely due to the extraction method used to release phytochemicals from leaf tissues. The means of extraction affects the concentration and type of phytochemical compounds available in the extracts, as solubility of phytochemicals differs with solvent (Truong *et al.,* 2019). The aim of using a certain extraction method is to extract as many phytochemical compounds as possible. In this study, methanol, acetone, ethylacetate, and distilled water were used to extract moringa leaf powder; the resulting MLEs showed high antifungal efficacy against *F. oxysporum*. Results obtained from this study are inaccordance with those of other researchers (Das *et al*., 2010; Gurjar *et al*., 2012; Emad El Din*et al*., 2016 and Truong *et al*., 2020), who reported that different extraction methods result in specific extract-solvent systems that have different characteristics. These variations are caused by different polarities amongst solvents which affect solubility of active phytochemicals and, thus, influencing the antifungal activity of an extract. According to Ahmadu *et al.* (2020), organic solvent extracts tend to have higher antifungal efficacy compared with aqueous extracts, regardless of the plant tissue used. Amongst organic solvents, however, methanolic leaf extracts have better antifungal activity, as demonstrated in this study. The MLEs extracted with 30% and 50% organic solvents were more efficient in *F. oxysporum* inhibition than the 70% organic solvents. The antifungal capability of water MLEresults from the ability of water to extract water-soluble phytocompounds, because of hydrolases and phenolases that regulate active principles in

extracts.

Solvent concentration also seemed to affect fungal inhibition activity of MLEs. Lower solvent concentrations (30% and 50%) from all MLEs had 100% inhibition activity; this may be due tosolvents extracting certain phytochemicals from the leaf tissue. It is speculated that higher solvent concentrations might have destroyed or partially broken-down active phytochemicals. There were no significant differences in *F. oxysporum* inhibition activity between MLEs diluted to 2% (v/v) and 4% (v/v) ($P>0.05$). Chiejina and Onaebi (2016), however, found that MLE concentrations have a directly proportional relationship with inhibition activity. There might have not been enough difference between 2% (v/v) and 4% (v/v) concentrations to cause a noticeable difference in the *F. oxysporum* inhibition activity inthis study. The minimum inhibition concentration of MLE was found to be 2%, as there was no significant difference between the inhibition activity of 2% MLE and 4% MLE ($P>0.05$).

The 100% inhibition activity of the 30 and 50% acetone-MLE, ethyl acetate-MLE, and methanolic-MLE extracts (Table 2) is in support of the results obtained by Chiejina and Onaebi(2016). This fungal inhibition activity of MLEs can be attributed to its antifungal constituents that confer fungal inhibition activity through different mechanisms. Amongst these is the moringa chitin-binding protein (Mo-CBP3), a protein resistant to high temperatures and pH changes (Freire *et al*., 2015).

The cell wall of *F. oxysporum* is composed of several polysaccharides that aim to protect the fungus from external stressors. The main component of the fungal cell wall is the carbohydrate polymer chitin, together with 1,3-glucan; these carbohydrate polymers maintain the rigidity of the cell wall (Gulia and Yadav, 2015). To attack the fungus, Mo-CBP3 acts by binding to the chitin of the cell wall and disrupts its functioning, which results in the complete degradation of the cell wall (Saini *et al*., 2016). This protein has the ability to preventfungal growth, ultimately destroying the fungus due to hindering sporulation and mycelial growth. This depends, however, on the protein concentration and the developmental stage of the fungus (Gifoni *et al.*, 2012; Batista *et al.*, 2014; Freire *et al.*, 2015). In a previous study by Gifoni *et al*. (2012), Mo-CBP3 was found to destroy the structural symmetry of the fungal cells and made them distorted and wrinkled. This protein is advantageous for utilization in agriculture as a fungicide, as it has a high efficacy of inhibiting sporulation, thereby disturbing mycelial growth (Gifoni *et al*., 2012). Apart from Mo-CBP3, moringa has other, small peptidesthat are vital in fungal growth inhibition. Moringa leaf extracts contain small peptides which could play an important role in the plant's antimicrobial defence system, enlarging the membrane

or cell wall of the fungal hyphae.

It, thus, cannot be ruled out that Mo-CBP3 may eventually pass through the cell wall barrier,interact with the cell membrane receptors, and induce secondary effects internally in *F. solani*to promote cell death. It can, therefore, be concluded that Mo-CBP3 can have similar effecton *F. oxysporum* (Batista *et al*. 2014). Furthermore, there various isomers of Mo-CBP3 with different possible modes of actions (Fig. 3.3), it has not yet been investigated if these isomers has similar antifungal efficacy or not.

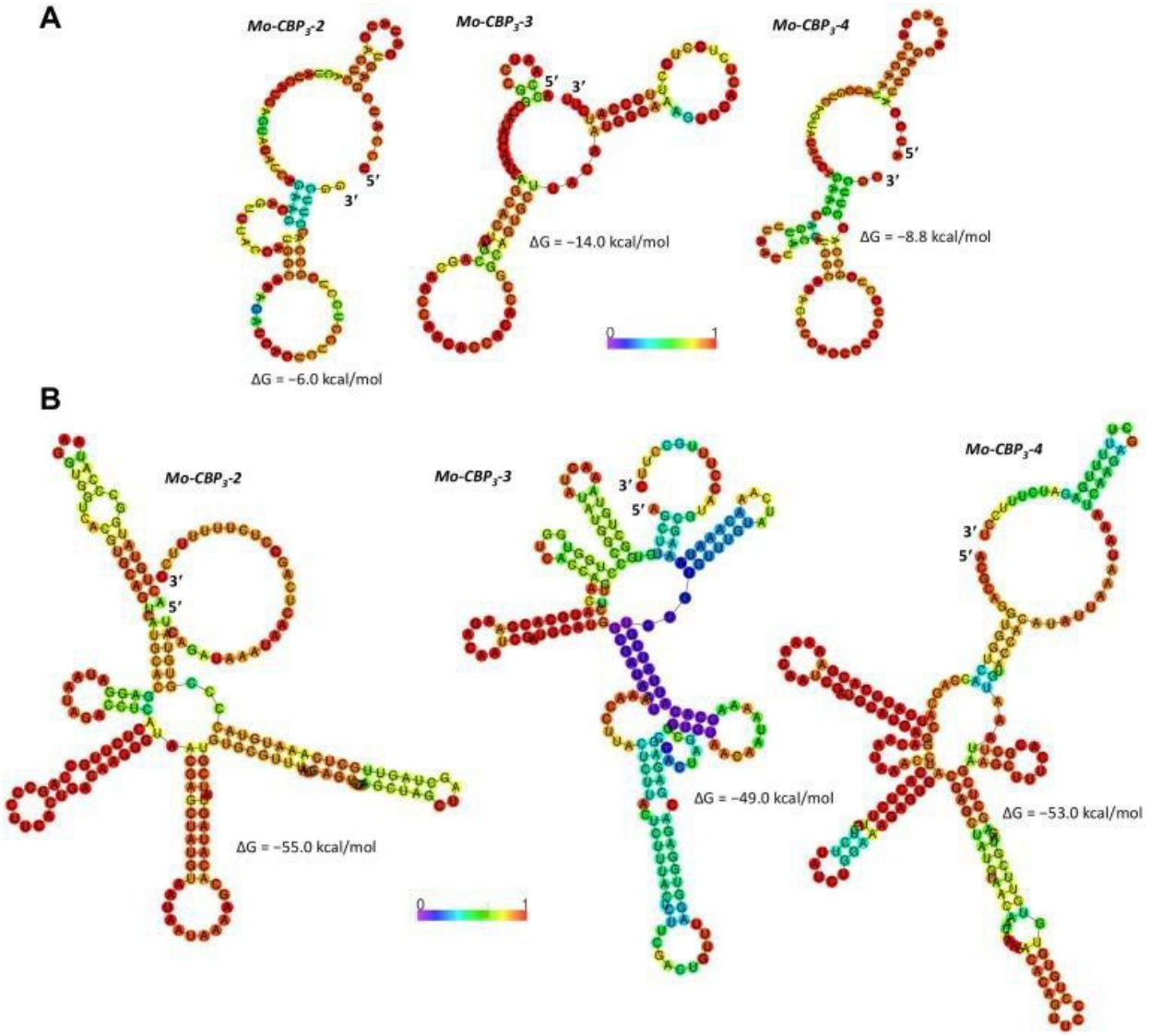

Figure 3.2: Potential MFE 5’ (A) and 3’ UTRs (B) secondary structures of Mo-CBP 3 mRNAs (Freire et al., 2015)

Moringa phenolics deactivate fungal enzymes and, subsequently, hinder amino acid production, essential in fungal sporulation (Ahmadu *et al*., 2020). The other method of phenolic action is to disrupt fungal growth, when present at higher concentrations. This is achieved, when a non-polar

part of the phenolic compound passes through a fungal membrane and a hydroxyl group combined with delocalized electrons, conferring an acidic nature to the molecules and therefore destroy fungus cell membrane (Pizzolitto *et al.*, 2015).Phenolics directly destroy the lipoprotein membranes of the fungi.

Tannins act by increasing the strength of the epidermal layer of the potato, creating rigid complexes that hinder fungal penetration. As large-sized glycosides, saponins are equipped with a glycone structure that destroys the cells of fungi. Terpenoids directly destroy the fungal membrane tissue by lysis of the fungal cell wall. Flavonoids (Fig. 4) form complexes with the cell wall by attaching to extracellular proteins and, thus, destroying the fungus.

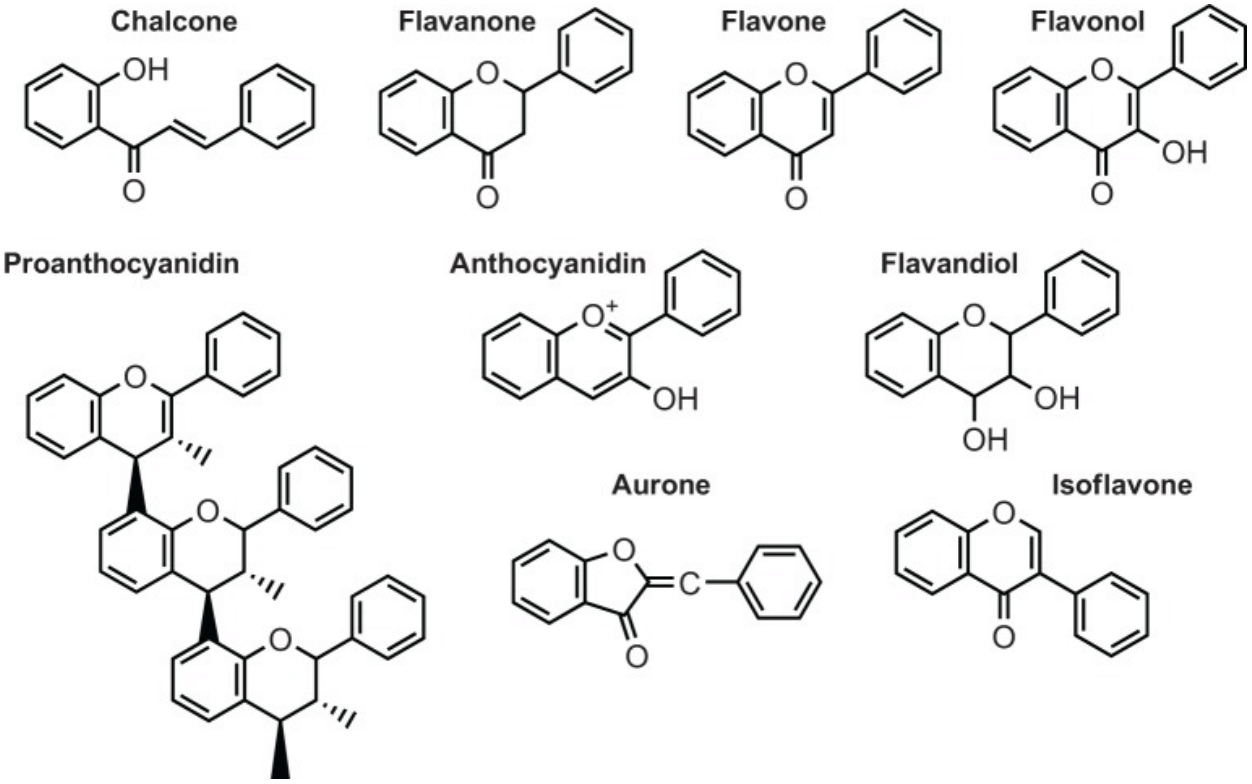

Figure 3.3: Main group of flavonoids found in higher plants exerting antifungal properties (Falcone *et al*., 2012)

Glucosinolates are a diverse class of S- and N-containing secondary metabolites that play a variety of roles in plant defence (Falcone *et al*., 2012). They exert antibacterial, antifungal, and anti-herbivoral properties. Glucosinolate derivatives, such as 4-(4'O-acetyl-R-L- rhamnosyloxy)-isothiocyanate and 4-(4'O-acetyl-R-L-rhamnosyloxy)-benzaldehyde have beenproven to have several antimicrobial activities (Bennett *et al*., 2003; Handiseni *et al.* 2016; Mvumi *et al.* 2018). Glucosinolate

derivatives are both electrophilic and lipophilic, which makes it easier for these compounds to penetrate fungal cellular membranes. Once they have penetrated fungal cellular membranes, glucosinolate derivatives then react with specific intracellular compounds (Chen *et al*., 2020).

3.5 Conclusion

The handling and chemical control of phytopathogenic fungi results in large expenses throughout the world. Nevertheless, about one third of the world's agricultural production islost every year because of pests and diseases. In conclusion, our results show that the methanol extract of moringa leaves has a strong antifungal efficacy on mycelial growth and spore germination, and, thus, reduces the dependence on synthetic fungicides.

From this study, it can be concluded that moringa antifungal activity is both solvent-type andsolvent-concentration-dependent. The results of this study pave the way for further studies on chemical constituents found in MLE and their mechanisms of exhibiting certain biological activities. Further investigations are needed to understand the complex pathological effects of this plant species.

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Chapter 4
Investigating the potential of moringa leaf extracts (MLEs) as potential antifungal agents against fusarium dry rot of potatoes

Potato is an economically important crop in South Africa; it is also one of the most-consumed starch crops worldwide. Potato production is, however, limited by fusarium dry rot, a potato disease caused by different *Fusarium* spp. Controlling these fungi has been problematic for the past decades, due to the resistance developed by *Fusarium* spp. to the available fungicides. This study was carried out to investigate the antifungal efficacy of moringa leaf extracts (MLEs), prepared using either acetone, ethyl acetate, methanol, at three concentrations (30%, 50%, 80%) as well as a watery extract against fusarium dry rot of potato. Moringa leaf extracts were used curatively by pre-infecting tubers of two potato cultivars ('Mondial' and 'Valor') with the pathogen, *Fusarium oxysporum* and, thereafter, treating tubers with the various MLEs. Further, a preventive study was carried out by pre-treating potato tubers with MLEs, before inoculation with *F. oxysporum*. The MLEs were found to be more effective as a preventive measure, as acetone-MLE (A-MLE) and methanolic-MLE (M-MLE) were able to prevent disease development, while water-MLE was able to delay disease development by at least one week in comparison with the control, using the preventive method. The MLEs were found to be less effective when used as a curative method. Tubers treated with A-MLE had the smallest average lesion diameter (3.67 mm and 3.44 mm for 'Valor' and 'Mondial', respectively). Ethyl acetate-MLE (EA-MLE) was found ineffective in controlling fusarium dry rot in both cultivars. Further, tubers of both cultivars treated with EA-MLE were prone to secondary infections by bacterial soft rot. The ability of MLE to delay and slow disease development is an indication of its antifungal potential. MLEs can be used in organic farming, and reduce reliance on synthetic fungicides, chemical pollution, and subsequently lead to a greener environment.

Keywords: Antifungal Compounds, Phytochemicals, Phenolics, Potato Defence Mechanism

4.1 Introduction

Amongst the non-grain starch crops, potatoes (*Solanum tuberosum* L.) are South Africa's most important crop with an estimated annual production value of R2.4 billion (FAOSTAT, 2018). The potato industry provides jobs to hundreds of thousands of South Africans, from farm labourers to logistic, processing, retail, and packaging personnel. Potato, furthermore, contributes to the SA economy through export markets and empowerment of traders in the informal sector, bringing revenue to the country, villages, and small towns. Potato productionalso plays a critical role in the eradication of food insecurity and poverty, as it is widely cultivated, throughout the country, by subsistence and commercial farmers. Potato cultivation, thereby, generates revenue in villages, where food insecurity is a common problem. The South African potato industry also contributes to food security in neighbouring countries through exportation (NAMC, 2017).

The production of potatoes is limited by fusarium dry rot caused by *Fusarium oxysporum* (*F. oxysporum*). This disease is one of the most important potato diseases in South Africa, causing estimated annual production losses between 10-15%. There is, however, no sufficient data on total product losses, as fusarium disease symptoms can develop postharvest or anywherefrom the field to the retailer to consumers' houses (www.agric.wa.gov. za). Even pre-harvest losses dueto fusarium can occur, which may be due to infection of seed tubers prior to planting; furthermore, the infection of seed tubers can happen in storage, in transit, orafter planting in contaminated soils. Severe losses, however, occur after planting due to the fungus exiting dormancy. Infection by *F. oxysporum* often leads to secondary infections by *Erwinia* species, bacteria that cause soft rot in potatoes (Wharton et al., 2007; Chiejina and Onaebi, 2016).

Potato dry rot is caused by *Fusarium* spp., soil-inhabiting fungi that can overwinter in soils andon plant debris. These saprophytic species target the root system and invade the vascular system of potatoes, causing symptoms, such as dark depressions, wrinkled skin, and lesions on tubers. The skin of the tuber may have pinkish to dark-red spores. Dark, rotten cavities inside the tuber may also develop; such cavities may be filled with fungal threads (wwww.agric.wa.gov.a). Field infections by fusarium dry rot, particularly of seed tubers, can lead to failure of crop establishment due to damage of the sprouted tubers (Wharton et al., 2007). Michielse et al. (2009) further state that fusarium field infections delay potato plant emergence and reduce crop vigour.

Postharvest infections of tubers usually occur through open wounds that tubers might have acquired during handling and mechanical harvesting. These wounds provide entry points to *Fusarium* spp.

that colonize the tubers, resulting in necrotic tissue. Infections usually occur in spring and winter, with *Fusarium sambucinum* and *Fusarium solani* dominating spring infections. To minimize tuber infections and disease development, cooler storage temperatures must be maintained (Sinha et al., 2018).

Potatoes have several defence mechanisms against fusarium, activated in response to fusarium infection; these mechanisms include the manufacture of physical barriers, such as periderm tissue and cell walls, as well as the synthesis of chemical compounds, such as polyunsaturated fatty acids (PUFAs) (Barel and Ginzberg, 2008; Gosset et al., 2009; Michielse and Rep, 2009).There are currently 101 registered potato cultivars in South Africa, amongst these, 'Sifra' and'Mondial' are the most common-traded cultivars in South Africa (NAMC, 2017)]. Thesecultivars differ in susceptibility to fusarium dry rot; nevertheless, there is no single cultivar with complete resistance to dry rot. In attempts of improving the resistance of crops,medicinal plant extracts have been used (Goss et al., 2017; Khanyinda et al., 2018; Kalsoom et al., 2019; Liao et al., 2021). These extracts contain various phytochemical constituents thatpossess antifungal properties.

Moringa oleifera (moringa) is a medicinal plant with a vast profile of phytochemicals exhibiting known antifungal properties. Moringa phytochemicals include phenolics, chitin- binding proteins, flavonoids, zeatin, glucosinolates and isothiocyanates (Datta et al., 2014; Kafi et al., 2015). These phytochemicals exert antifungal activity through various mechanisms(Chiejina and Onaebi 2016). The moringa extracts have been found to inhibit spore germination and to hinder fungal growth of several plant pathogenic fungal species (Batista etal., 2014; Mvumi et al., 2018).

This study aimed to investigate if compounds extracted from moringa can be used to controlfusarium dry rot incidents, either as a preventive measure or as a curative method. Further, the antifungal activity of MLEs, prepared using various extracting solvents (acetone, ethyl acetate, methanol, and water) and extract concentrations, were compared. It was further attempted to determine, if the antifungal activity of MLE differs between potato cultivars.

4.2 Materials and methods

4.2.1 Surface sterilization of potatoes

Two potato cultivars, namely 'Mondial' and 'Valor' were obtained from local supermarkets based on availability (Woolworths Food, Pietermaritzburg, Pick n' Pay, Pietermaritzburg). Potato pockets that were well before their 'best before' date were selected. The cultivar 'Mondial' was chosen as it

is the most commonly produced cultivar in South Africa and is known to have a high resistance to several diseases and to environmental stresses (de Gouveia, 2020). 'Valor' was selected as it was widely available in the experimental season.

Upon arrival in the laboratory, all tubers were surface-sterilized by soaking in 0.2% (v/v) sodium hypochlorite solution for five minutes; thereafter, potatoes were rinsed twice with distilled water and left to dry at ambient temperature for three hours. The experiment was conducted between 29 October and 30 November 2020.

4.2.2 *Fusarium oxysporum* inoculum preparation

Potato dextrose agar (PDA) plates, containing *F. oxysporum* culture (preparation as outlined in Chapter 3) were washed with 5 ml distilled water. The *F. oxysporum* mycelia were lightly scrapped off using an L-bent glass rod. The suspensions were then filtered through cheesecloth into a 100 ml beaker. A wet mount was prepared by transferring a loopful of thesuspension onto a glass slide which was viewed under a light microscope using 40X magnification, to confirm the presence of conidia.

4.2.3 Pathogenicity test

Surface sterilised tubers from both cultivars were inoculated with *F. oxysporum* by woundingthem with sandpaper and inoculating 1.0 mL ($10x^6$) of *F. oxysporum* conidia into the wounds.Thereafter, tubers were incubated at 27°C and observed daily for symptom development. Uninoculated tubers were used as control. A completely randomized block design was used for this experiment. The two treatments were i) inoculation with *F. oxysporum* solution ii) inoculation with distilled water. Cultivar type ('Mondial' and 'Valor') was used as blocks.

4.2.4 The activity of MLEs in controlling fusarium dry rot

4.2.4.1 Effectivity of MLEs in delaying fusarium dry rot disease progression

Potato tubers ('Mondial' and 'Valor') were surface-sterilised and wounded as previously described under Section 4.2.3. The wounded spot served as entry point for F. oxysporum. Tubers were then inoculated with F. oxysporum by pipetting 1.0 ml of the F. oxysporum conidia solution into the wound site of each tuber, which was circled with a marker pen. Tubers were placed on trays and then left at ambient temperature to develop F. oxysporum symptoms (dry lesions and skin wrinkling), before they were treated with MLEs by soaking.

When tubers began to wrinkle and develop dry lesions, they were soaked in either acetone- MLE,

ethyl acetate-MLE, water-MLE, or methanol-MLE. Three drops of TWEEN 20 were added to each solution to aid coating. Each tuber was soaked for five minutes and, thereafter, incubated at ambient temperature. Disease progression was evaluated daily for 21days. Lesion diameters were measured in millimetres using a transparent ruler.

Tubers treated with distilled water were used as controls. .

4.2.4.2 MLE as a preventive measure against fusarium dry rot

Surface-sterilized tubers were soaked in either acetone-MLE, ethyl acetate-MLE, water-MLE, or methanol-MLE (see Chapter 2). To each solution, three drops of TWEEN 20 were added, before tubers were soaked in the solution for five minutes. Such coated tubers were then left to air-dry in trays at room temperature overnight.

Coated tubers were wounded by rubbing with sandpaper, before each tuber was inoculated with F. oxysporum as described above (Section 4.2.3). The wounded parts were marked with a marker pen and tubers were incubated at ambient temperature. Symptom development was evaluated daily. Lesion diameter and depth were measured and used as parameters of disease progression.

Tubers treated with distilled water were used as control.

4.2.5 Statistical analysis

Data obtained were subjected to analysis of variance (ANOVA) using Genstat (VSN International, Hemel Hempstead, England, UK, version 18th edition) to determine any significant difference between treatments. Graphs were plotted using Microsoft Excel (Microsoft Office 2010). Differences were considered significant at $P \leq 0.05$.

4.3 Results

Treatment with all MLE solutions (acetone-MLE, water-MLE, methanol-MLE and ethyl acetate-MLE) slowed disease development in tubers; some tubers did not develop anydisease symptom at all.

4.3.1 Preventive treatment of fusarium dry rot

Solvent concentration and solvent type were found to affect the level of disease development. Higher solvent concentrations (50% and 80%), particularly of ethyl acetate- MLE, had a negative impact on disease development, as fusarium dry rot lesions increased insize (Fig. 4.4); furthermore, tubers treated with ethyl acetate-MLE were more prone to secondary infection by bacterial soft rot than the other treatments (Fig. 4.4).

Response to MLE treatment also depended on cultivar used. 'Mondial' seemed to have a better performance in terms of lesion sizes than 'Valor' compared with the respective control (untreated tubers) as the average lesion diameter in 'Mondial' tubers ranged from approximately 6 mm to 11 mm; on the other hand, the average lesion diameters in 'Valor'tubers ranged from 4.7 mm to 14 mm.

Fusarium lesions started to develop three days after inoculation with F. oxysporum in control tubers and in tubers treated with ethyl acetate-MLE. After four days, lesions and wrinkles started to develop in other treatments. The lesions in control tubers developed rapidly and grew deeper into the tuber, while in MLE-treated tubers no further disease development occurred. All lesions in treated tubers were shallow (depth < 2 mm).

4.3.2 Fusarium dry rot disease development in tubers treated with MLEs: 'Valor'

The 50% acetone-MLE and 30% methanol-MLE treatments (average lesion diameter of 3.7 mm and 6.3 mm, respectively (Figs 4.2 and 4.3) were more effective against fusarium dry rot than the control. Ethyl acetate-MLE was found to be least effective in controlling fusarium dry rot. Lesions were wider in tubers treated with 80% ethyl acetate-MLE; with 30% ethyl acetate-MLE providing the best control than other treated tubers, resulting in an average lesion diameter of 7.7 mm and an average 6.0 mm lesion depth. In contrast, control tubers had an average 16.0 mm lesion diameter and an average lesion depth of 8.3 mm. Furthermore, 'Valor' tubers treated with 80% ethyl acetate-MLE and control tubers were prone to secondary infection by bacterial soft rot

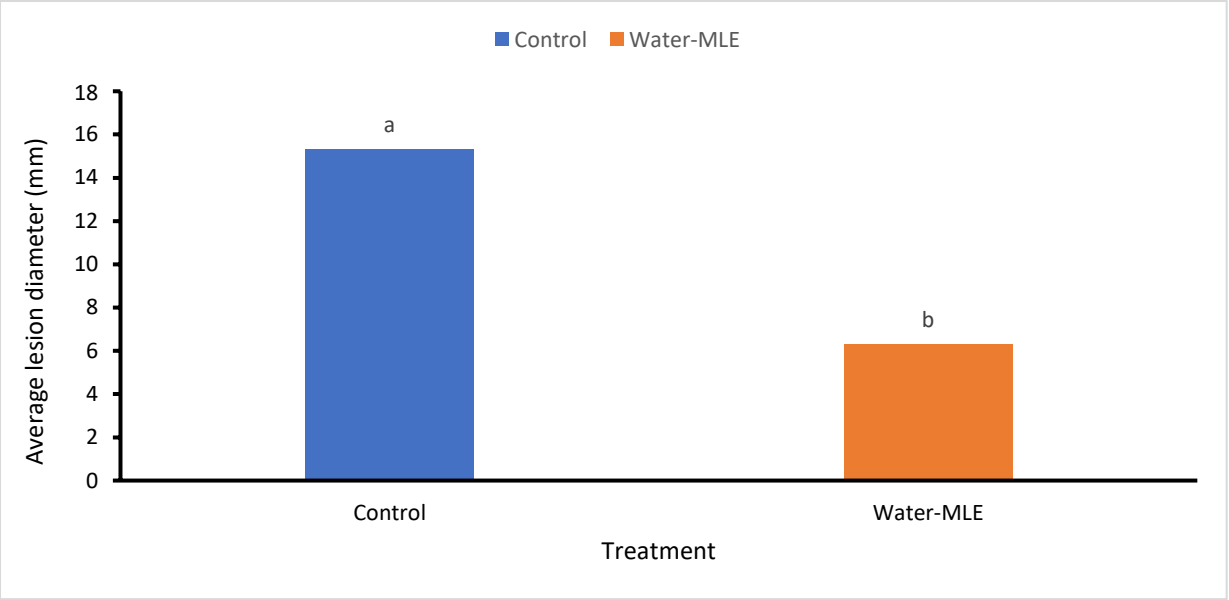


Figure 4.1: Average lesion diameter in tubers treated with water-MLE after an incubation period of two weeks. Different letters above the columns denote significant difference at P< 0.05.

Acetone-MLE provided satisfactory control of fusarium dry rot in potato tubers, as it was able to slow down disease development; the average lesion diameters in tubers treated with 50% Acetone-MLE was 4.7 mm. The lesions in these tubers were shallow (less than 3 mm deep); there were no skin wrinkles on tubers treated with acetone-MLE. There was no significant difference between tubers treated with 30% and 80% acetone-MLE (P>0.05), but there was a significant difference between tubers treated with 50% and 80% acetone-MLE, with 50% acetone-MLE reducing disease development more so than the 30% and 80% acetone MLEs (Fig. 4.2).

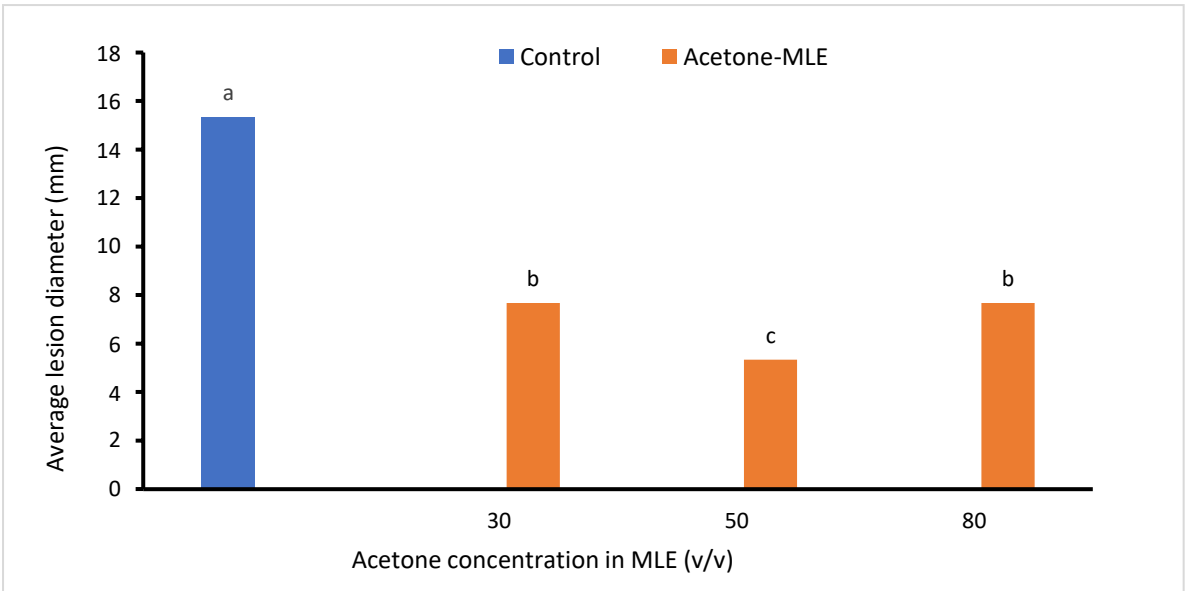


Figure 4.2: Average lesion diameter in 'Valor' tubers treated with acetone-MLE after two weeks

of storage. Different letters above the columns denote significant difference at P< 0.05

Methanol-MLE was found to be an effective treatment against fusarium dry rot, as such treated tubers had an average lesion diameter of only 6.0 mm and a lesion depth of less than 2.0 mm.There was no significant difference between tubers treated with 30% and 50% methanol-MLE(P≥ 0.05) (Fig. 4.3), but there was a significance difference between control tubers and all treated tubers (P<0.05). Tubers treated with 80% methanol-MLE were characterised by visible skin shrinkage.

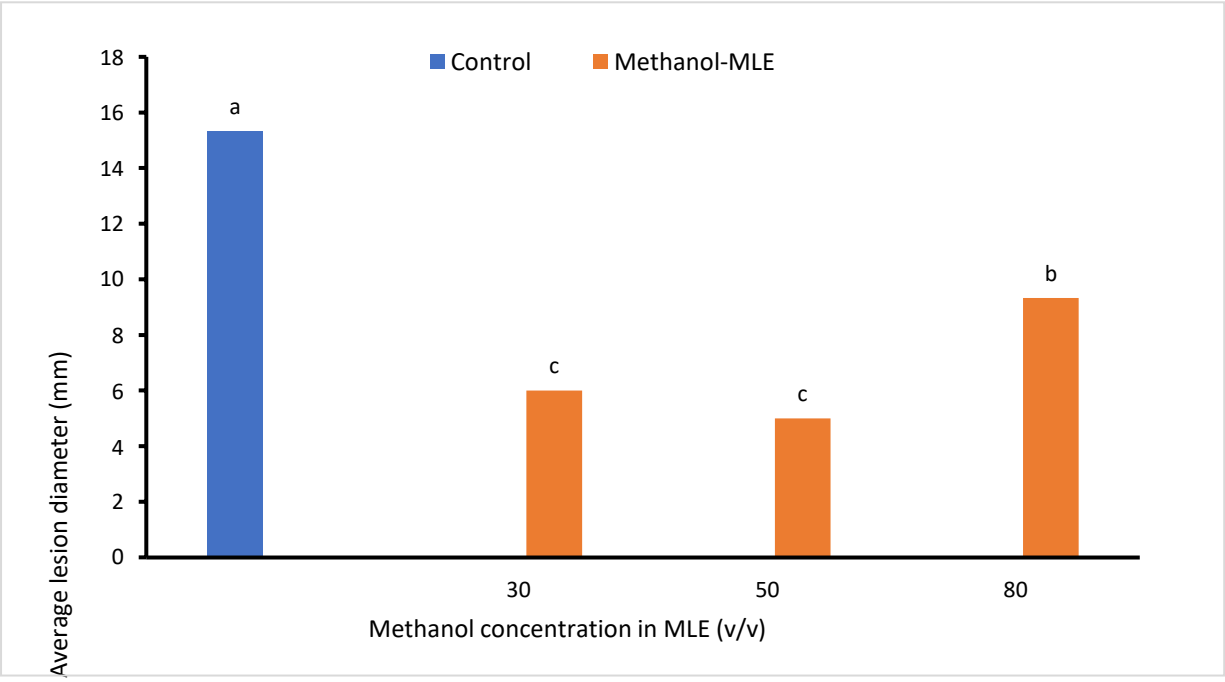


Figure 4.3: Average lesion diameter in 'Valor' tubers treated with methanol-MLE after two weeksof storage. Different letters above the columns denote significant difference at P< 0.05.

Ethyl acetate was found to be ineffective in extracting moringa components that can control fusarium dry rot, particularly the 80% ethyl acetate-MLE extract, as these tubers had an average lesion diameter of 11 mm, while potatoes soaked in 30% and 50% ethyl acetate -MLE had an average lesion diameter of 7.7 mm and 8.7 mm, respectively. Tubers treated with 80% ethyl acetate-MLE had an average lesion depth of 10 mm (Fig. 4.10). 'Valor' tubers were very susceptible to secondary infection by bacterial soft rot (Fig. 4.13). Disease progressed faster in ethyl acetate-MLE treated tubers than in control tubers. There was a significance difference in lesion diameter between treated tubers and control tubers (P<0.05) (Fig 4.4). There also was a significant difference in disease progression between tubers treated with 30%, 50% and 80% ethyl acetate as disease progressed slower in tubers treated with 30% ethyl acetate-MLE, and progressed much faster in tubers treated

treated with 80% ethyl acetate-MLE.

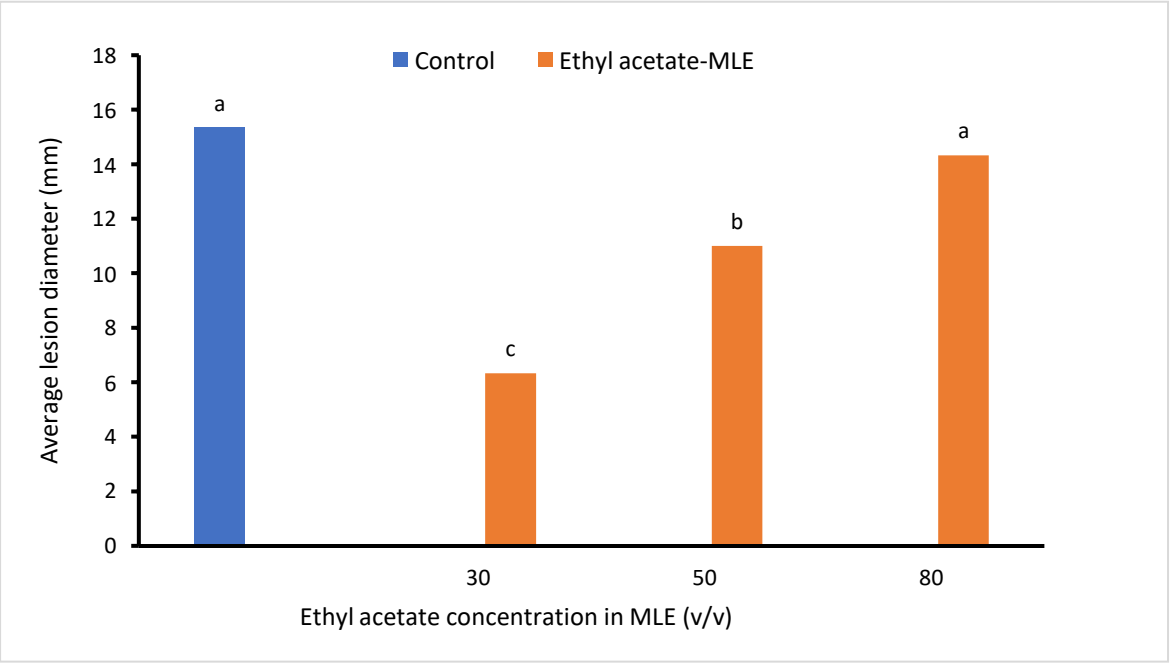


Figure 4.4: Average lesion diameter in 'Valor' tubers treated with ethyl acetate-MLE after two weeks of storage. Different letters above the columns denote significant difference at P< 0.05.

4.3.4 Fusarium dry rot disease development in tubers treated with MLEs: 'Mondial'

The most effective treatment in controlling fusarium dry rot was the 50% methanol- MLE treatment, as such-coaed tubers had an average lesion diameter of 1.6 mm with no skin wrinkles and no bacterial soft rot infection. There was a significant difference between tubers treated with 30%, 50% and 80% methanol-MLE (P< 0.05). The 50% acetone-MLE treatment was an effective control against fusarium dry rot, as tubers had an average lesion diameter of 3.4 mm and were significantly different from tubers treated with 30% and 80% acetone-MLE (P≤ 0.05) (Fig. 4.6.). Control tubers had an average lesion diameter of 15.3 mm and lesion depth of 8.3 mm. There was satisfactory dry rot control in tubers treated with water MLE, as these tubers had an average lesion diameter of 3.0 mm; it is noteworthy, that these tubers had undamaged skin and no secondary infection by bacterial soft rot. Treatments with 50% and 80% ethyl acetate-MLE were ineffective against fusarium dry rot with average lesion diameters of 11.0 mm and 14.0 mm, respectively. There was no significant difference between tubers treated with 50% and 80% ethyl acetate (P≥ 0.05); furthermore, both treatments were prone to secondary infections by bacterial soft rot (Fig. 4.12.).

Water-MLE also proved to be effective in inhibiting fusarium dry rot development, as these tubers had an average lesion diameter of 6.7 mm with 1.0 mm lesion depth (Fig. 4.5). There was a significant difference (P≤0.05) between control tubers and water MLE-treated tubers. Controltubers had an average lesion diameter of 15.3 mm and showed secondary infections by bacterial softrot, resulting in the complete damage to tubers (Fig. 4.13).

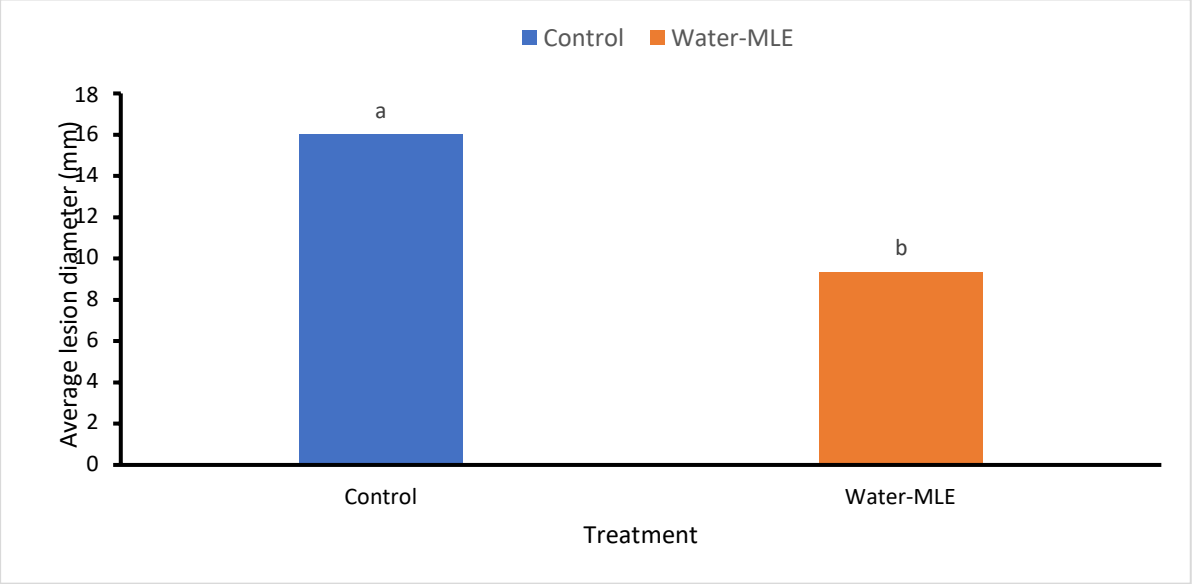


Figure 4.5: Average lesion diameter, after two weeks of storage in 'Mondial' tubers treated with water-MLE and inoculated with *F. oxysporum*. Different letters above the columns denote significant difference at P< 0.05.

The 50% acetone-MLE treatment was found to be effective in slowing down the rate of disease development in 'Mondial' tubers, as tubers treated with acetone-MLE had an averagelesion diameter of 2.3 mm with no wrinkles and no secondary infection (Fig. 4.9). On the otherhand, tubers treated with 30% and 80% acetone-MLE had an average lesion diameter of 6.3 mm and 8.3 mm, respectively, wider than the 50% acetone-MLE treatment (Fig. 4.6).

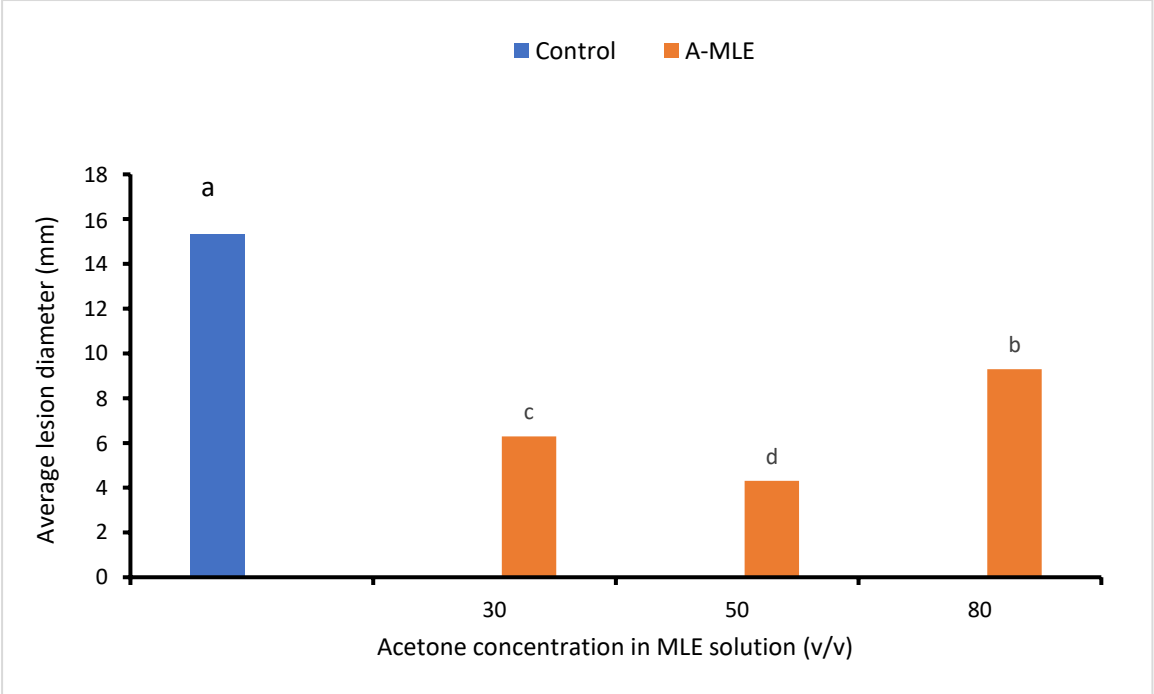


Figure 4.6: Average lesion diameter after two weeks of storage in 'Mondial' tubers treated with acetone-MLE (A-MLE) and inoculated with *F. oxysporum*. Different letters above the columnsdenote significant difference at P< 0.05.

The 30% ethyl acetate-MLE treatment also positively impacted on disease control, as tubers treated with ethyl acetate-MLE had an average lesion diameter of 6.3 mm. There was no significant difference between tubers treated with 30% ethyl acetate and tubers treated with 50% ethyl acetate. On the other hand, 80% ethyl acetate-MLE was ineffective in controlling fusarium dry rot, as the average lesion diameters in tubers treatedwith 80% ethyl acetate had an average lesion diameter of 14.3 mm (Fig. 4.7).

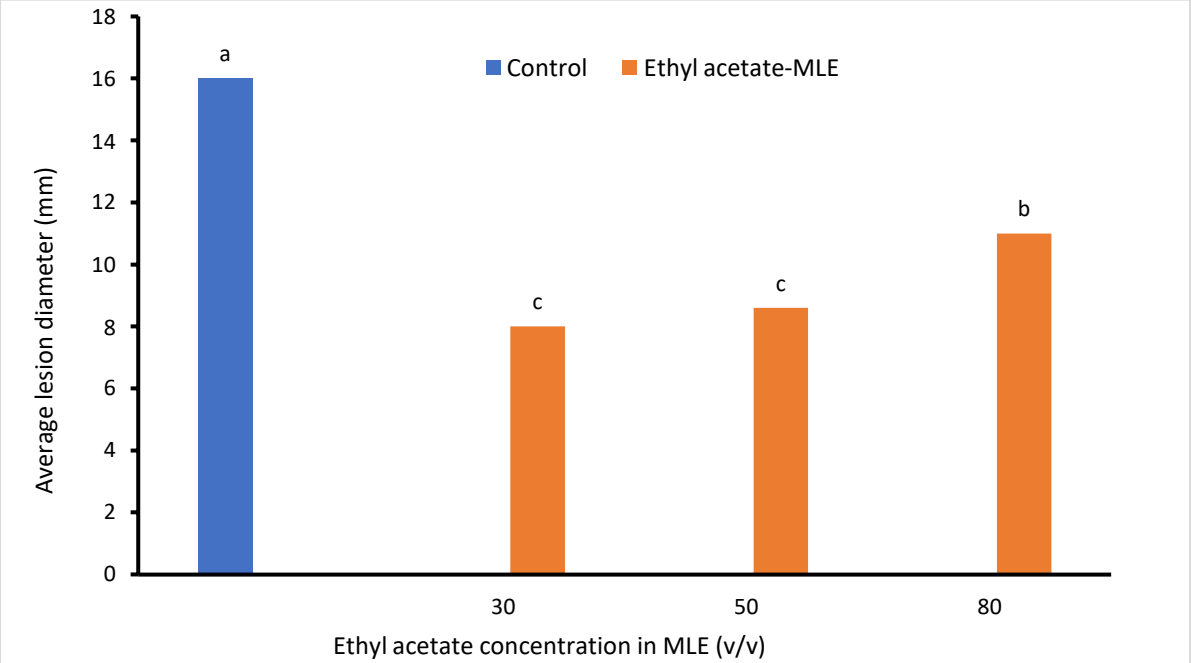


Figure 4.7: Average lesion diameter after two weeks of storage in 'Mondial' tubers treated withethyl acetate-MLE and inoculated with *F. oxysporum*. Different letters above the columns denote significant difference at P< 0.05.

Methanol-MLE provided better disease control than ethyl acetate-MLE treatments. There was no significance difference between treatments with 50% and 80% methanol; however, 30% methanol-MLW treatement significantly differed from the 50% and 80% methanol-MLE (Fig. 4.8). This indicates the ability of methanol- MLE to provide good control of fusarium dry rot.

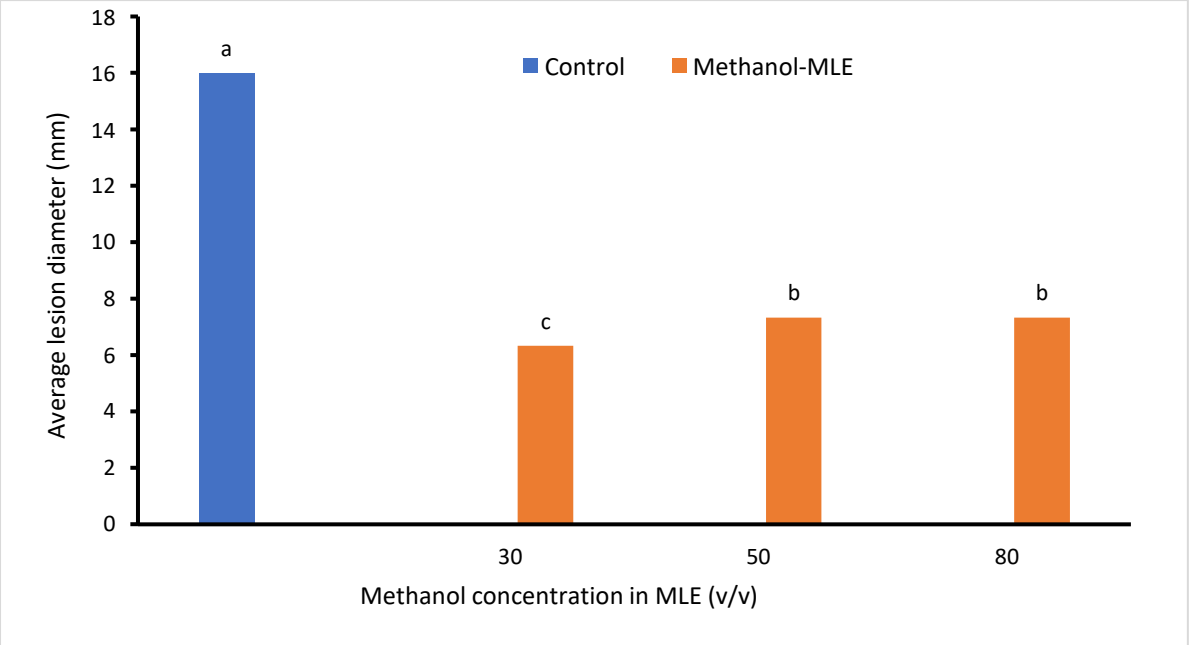


Figure 4.8: Average lesion diameter in tubers treated with methanol-MLE and no treated tubers after inoculation with *F. oxysporum* and stored for two weeks. Different letters above the columns denote significant difference at P< 0.05

4.2.1 Efficacy of MLEs in slowing down fusarium dry rot progression

After 7 days of treatment with MLEs, all 'Valor' tubers had an average lesion diameter greater than 6 mm (tab. 4.1). Wrinkles were, however, only observed in tubers treated with all ethyl acetate- MLEs, 80% methanolic-MLE and in control tubers. Disease progressed slowed in tubers treatedwith 30% and 50% acetone-MLEs, 30% methanolic-MLE and water MLE, where an average lesion diameter less than 6 mm was recorded 14 days post treatment (tab. 4.1); while disease progressed faster in other treated tubers. These tubers also had no wrinkles, and had lesion diameters less than 6 mm; while the lesion diameter of tubers treated with other MLEs had increased to an average of 8 mm diameter. 'Valor' tubers treated with all ethyl acetate-MLEs, and control tubers (non-treated 'Valor' tubers) developed wrinkles within 7 days post treatment. In these 'Valor' tubers, disease symptoms, characterised by wrinkles and lesions, developed faster compared with other tubers. Ethyl acetate-treated

tubers further developed bacterial soft rot. The 30% methanolic-MLE treatment maintained a healthy tuber appearance, as there were no wrinkles observed; furthermore, disease progression slowed 14 days post treatment as compared with ealier disease development; however, bacterial soft rot symptoms were observed 14 days post treatment with MLEs; these symptoms were severe in ethyl acetate-MLE treated tubers (tab 4.1).

Table 4.1. Effectivity of moringa leaf extracts in slowing down disease development in 'Valor' tubers

Treatments	Day 7		Day 14		
	Lesion ≥ 5 mm diameter	Wrinkles	Lesion diameter		Wrinkles
			≤ 7.9 mm	≥ 8mm	
Water-MLE	X		X		
Acetone-MLE					
30%	X		X		
50%	X		X		
80%	X			BSR	X
Ethyl acetate-MLE					
30%	X	X		X	X
50%	X	X		BSR	
80%	X	X		BSR	
Methanol MLE					
30%	X		X		
50%	X			X	X
80%		X		BSR	
Control	X	X		X	X

BSR = Bacterial soft rot

Seven days post treatment with MLEs, 'Mondial' tubers treated with 30% acetone-MLE and water

MLE had no wrinkles and had lesion diameters less than 5 mm (tab. 4.2). The other ‘Mondial’ tubers (treated with 50% and 80% acetone-MLE, ethyl acetate-MLEs, methanol-MLEs and water-MLE) had an average lesion diameter of ≥5 mm within 7 days post-treatment with MLEs (tab. 4.2). ‘Mondial’ tubers treated with all ethyl acetate-MLEs, and control tubers (non-treated ‘Mondial’ tubers), developed wrinkles within 7 days post treatment (tab. 4.2). In these ‘Mondial’ tubers, disease symptoms (wrinkles and lesions) developed faster compared with methanol- and acetone-MLE treated tubers. These tubers further developed bacterial soft rot symptoms. The 30% methanol-MLE treatment maintained the disease-free appearance of tubers, as there were no wrinkles observed 14 days after treatment; furthermore, general disease progression slowed 14 days post treatment. At this time, ‘Mondial’ tubers treated with water-MLE still had lesion diameters less than 6 mm, while lesion diameters of tubers treated with 30% acetone-MLE had increased to an average of 8 mm (tab 4.2).

Table 4.2: Lesion development in 'Mondial' tubers to moringa leaf extracts treatment and inoculation with *F. oxysporum,* a causal agent of fusarium dry rot.

Treatments	Day 7		Day 14		
	Lesion diameter ≥ 5mm	Wrinkles	Lesion Diameter		Wrinkles
			≤7.9 mm	≥ 8mm	
Water-MLE			X		
Acetone-MLE					
30%				X	
50%	X				
80%	X				
Ethyl acetate-MLE					
30%	X	X		X	X
50%	X	X		X	X
80%	X	X		BSR	X
Methanol MLE					
30%	X		X		
50%	X		X	BSR	
80%				BSR	
Control	X	X		BSR	

BSR = Bacterial soft rot

All 'Mondial' tubers had small lesions after 7 days of inoculation with *F. oxysporum*. Tubers were then treated with MLEs (acetone-MLE, ethyl acetate-MLE, methanolic-MLE and watery-MLE). Seven days post treatment with MLEs, 'Mondial' tubers treated with 30% acetone-MLEand water-MLE had no wrinkles and had lesion diameters less than 5 mm. The other 'Mondial'tubers had an average lesion diameter of ≥5 mm within 7 days post treatment with MLEs. 'Mondial' tubers treated with all ethyl acetate-MLEs, and control tubers (non-treated 'Mondial' tubers), developed wrinkles within 7 days post treatment. In these 'Mondial' tubers, disease symptoms were characterised by wrinkles and lesions, and developed faster compared with other tubers. These tubers further developed

bacterial soft rot symptoms (fig. 4.12 and 4.12). The 30% methanolic-MLE treatment remained disease-free, there were no wrinkles observed; furthermore, disease progression slowed 14 days post treatment (fig. 4.9). 'Mondial' tubers treated with water-MLE still had lesion diameters less than 6 mm, while lesion diameters of tubers treated with 30% acetone-MLE had increased to an average of 8 mm. Secondary infection by bacterial soft rot was more severe in 'Valor' than in 'Mondial' tubersand quickly destroyed the tubers, as 'Valor' internal structures were completely destroyed within three days.

Figure 4.9: 'Mondial' tubers two weeks after treatment with 50% methanol-MLE and inoculated with *F. oxysporum*; small lesions developed (3 mm) but did not expand into the tuber

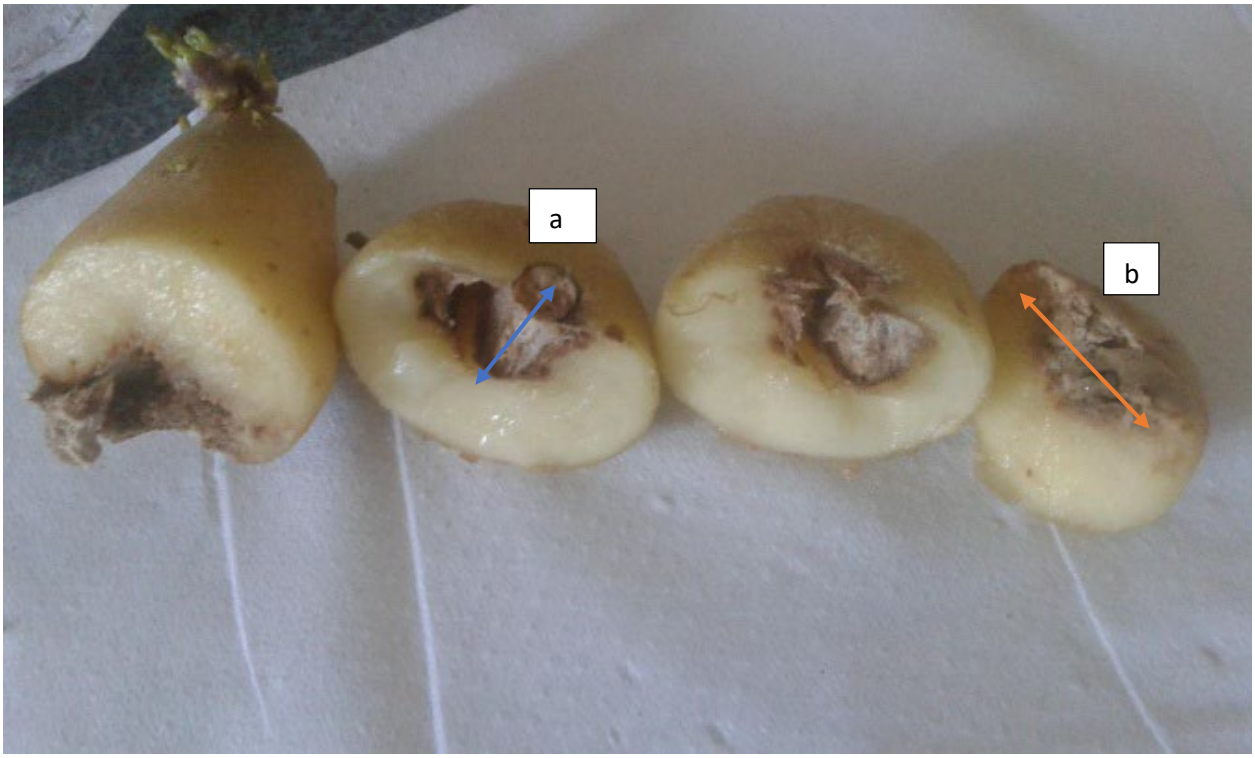
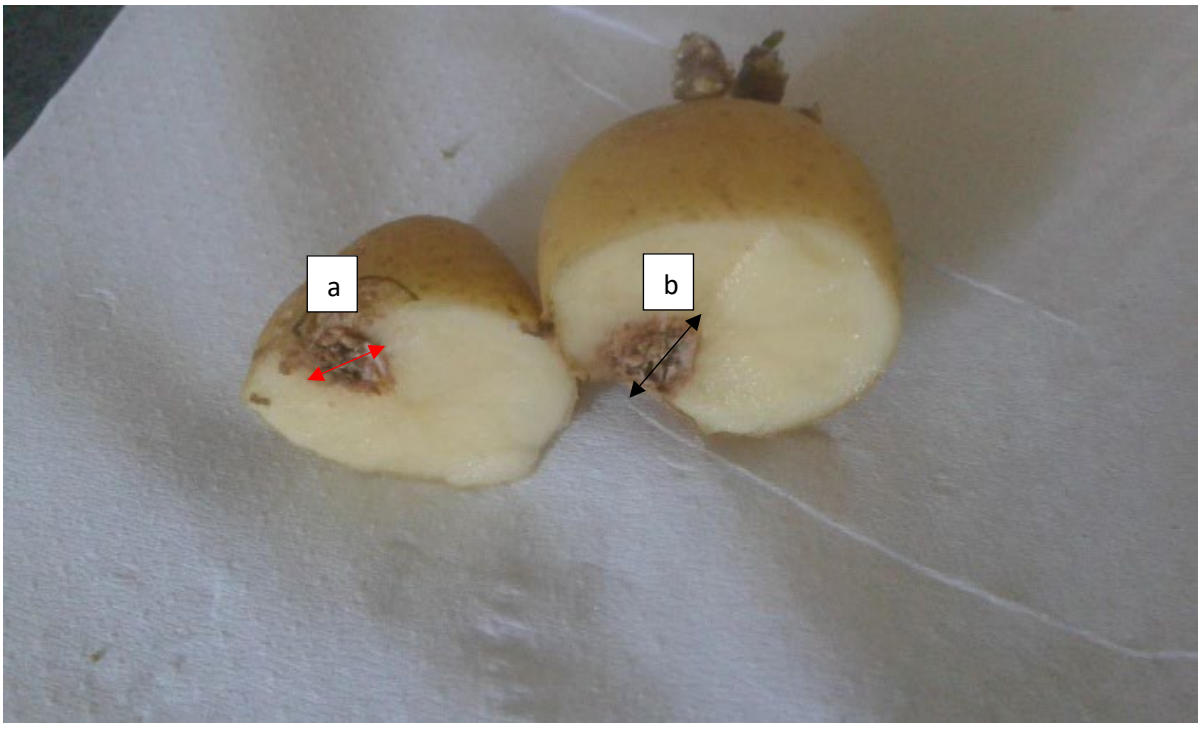


Figure 4.10: 'Valor' tubers treated with 50% ethyl acetate-MLE showing fusarium dry rot, characterized by dry lesions, progressing in both diameter and depth. (a - lesion depth; b -lesion diameter)

Figure 4.11: 'Mondial' tubers treated with 30% ethyl acetate-MLE and infected with fusarium dry



rot; lesions did not grow in diameter, but progressed deeper into the tissue (a - lesion diameter; b - lesion depth).

Fig. 4.12: 'Mondial' tuber treated with ethyl acetate-MLE, infected with fusarium dry rot and developed bacterial soft rot characterized by water-soaked areas of soft tissue

Figure 4.13: 'Valor' tubers treated with ethyl acetate-MLE with symptoms of bacterial soft rotcharacterized by soft tissue

4.4 Discussion

Pathogens continue to develop resistance to fungicides, creating an urgent need to develop new control strategies to minimize losses in potato production. Yanar *et al*. (2011) pointed out that as synthetic fungicides are detrimental to the environment, the public demands reduction of fungicide residues on marketed products. Antifungal agents derived from medicinal plants are “the hope” of effective biological control strategies of fusarium dry rot. These measures potentially inhibit the growth of *Fusarium* spp. and, thus, improve yield andquality of potatoes (Yanar *et al*., 2011; El-Mohamedy and Abdalla, 2014).

In this study, MLEs showed high efficacy as antifungal control agents against fusarium dry rot of potato. Potatoes coated with ethyl acetate-MLE developed severe fusarium dry rot symptoms in the curative and preventative experiments; these MLs were also not effective in slowing down disease progression. This indicates failure of ethyl acetate-MLE as an antifungal agent, as ethyl acetate seemed to have damaged the tuber cell structure (membranes and amyloplasts) thereby opening access for F. oxysporum to develop and colonize the entire tuber. Another possibility for the failure of the ethyl acetate treatment could be the low polarity of ethyl acetate (4.4), so that the solvent was not able to extract sufficient antifungal phytochemicals from the moringa leaf tissue, thereby not sufficiently inhibiting disease development in tubers or not sufficiently triggering the potato’s defence system. These results further rule out ethyl acetate-MLE as a potential biofungicide solvent.

Tubers coated with ethyl acetate-MLE that had already developed fusarium dry rot symptoms were prone to secondary infection by *Erwinia* spp., the bacterium that causes bacterial soft rot (van der Waals and Krüger, 2020). Under high humidity conditions *Erwinia* spp. takes advantage of the already developed dry rot lesions and invades the potato tissue (Czajkowski*et al*., 2015). As opposed to fusarium dry rot, bacterial soft rot is characterized by water-soaked tissues and slimy rots that progress rapidly and surpass fusarium dry rot development, resulting in the characteristic bad soft rot odour (Wharton *et al*., 2007).

All extracts showed a certain level of antifungal activity; however, MLE extracted with highersolvent concentrations had a rather detrimental effect on tubers, particularly the high ethyl acetate concentrations. Extraction of MLE with solvent concentrations of 50% and 30% resulted in more effective MLE treatments, preventing disease development more so than the 80% ethyl acetate, methanol, and acetone extracts (Figs 4.9-4.11). Thus, extraction with these lower solvent

concentrations improved disease control of fusarium dry rot (Fig. 4.9). The results obtained from this study are in accordance with reports by El-Mohamedy and Abdalla (2014), who found MLEs to be an exceptional inhibitor of *F. oxysporum*. According to these authors, MLEs act by inhibiting mycelium growth, hence, preventing the mycelium from further infecting adjacent tissues. This leads to localized disease symptom, as observed in tubers treated with MLEs (Fig. 4.9). All MLEs were found to be rather fungistatic than fungicidal, as they failed to stop disease development. When used as a curative method, acetone-MLE and water-MLE were able to delay disease progression by at least one week. Our results confirm reports by Datta *et al*. (2014) and Mvumi *et al*. (2018) that moringa leaf extracts can be used as natural bio-fungicides.

The improved disease control to fusarium dry rot in tubers treated with acetone-MLE, methanol-MLE and water- MLE could be attributed to certain secondary metabolites (SMs) produced by the tubers, following MLE application. Such SMs are plant chemical compounds that do not seem to play a role in plant growth and development, but rather are synthesized by plants as a defence mechanism against environmental stressors and pathogens (Ibrahim et al., 2013). Phenolic compounds present in potato (Chapter 2, Table 2.1) are comprising flavanols, isoflavones, coumarins and pterocarpans; these metabolites all contribute to the antifungal and antimicrobial activity of MLEs (Bennett et al., 2003; Yaseen and Hájos, 2020).

Results from this study imply that the efficacy of moringa extracts also depends on the specific potato cultivar (Figs 4.9 - 4.13). Disease symptoms were not as severe in 'Valor' as in 'Mondial'. 'Mondial', the most-cultivated potato cultivar in South Africa (de Gouveia, 2020), is an inbred cultivar that has high resistance towards several potato diseases and environmental stressors. This may be the reason why 'Mondial' had lower disease severity than 'Valor'. Nevertheless, treatment with MLEs significantly improved disease control in 'Mondial', as there was a significant difference between control 'Mondial' tubers and treated tubers ($P<0.05$) (Figs 5-8). Treatment with MLEs also significantly improved disease control in 'Valor', as there was a significant difference between treated tubers and untreated tubers ($P>0.05$) (Fig. 10). The improved control of both cultivars may be attributed to the action of certain SMs present in MLEs. In Chapter 2 (Fig. 2.1 and Fig. 2.2), tuber treatment with MLEs significantly increased the concentration of certain SMs, such as phenolics, in both cultivars.

According to Boddy (2016), wall-bound phenolics accumulate in cell walls to strengthen the wall structure, so that the fungus cannot spread further after penetrating the potato tissue. Latif and

Mohamed (2016) found that MLE enhanced the mesophyll cell strength by increasing the concentration of plastoglobuli. Matern et al. (1995) revealed that the concentration of wall-bound phenolics increased under pathogen attack; hence, this indicates that one of the antimicrobial mechanisms of phenolics is via enhancing the strength of cellular structures thereby preventing microbial growth and development development.It is also important to note that not all solvents were of similar efficacy in extracting moringa leaf powder (Chapter 2, Table 2.1). The use of particular moringa extraction solvents is important, as certain solvents can/ should not be used on food products, as these solvents may have negative consequences on human health and the ecosystem at large (Joshi and Adhikari, 2019). Although methanolic-MLE seemed to be the most effective treatment, it cannot be used in the potato industry due to its toxicity (Joshi and Adhikari 2019; Chemicalsafetyfacts.org, 2021). On the other hand, acetone-MLE can be potentially used in extracting moringa leaves, as the U.S. Food and Drug Administration (FDA) and World Health Organization (WHO) have determined acetone as safe for use as an indirect food additive in adhesives and food-contact coatings, rendering acetone as a Generally Recognized as Safe (GRAS) substance at certain concentrations (Chemicalsafetyfacts.org, 2021).

4.5 Conclusion

Results obtained point to MLEs as promising bio-fungicides controlling fusarium dry rot; this could be highly advantageous, because moringa is rather widely cultivated in Africa and is drought-resistant; hence, even with increasing droughts due to climate change, moringa will be locally available. Moreover, moringa is adapted to different soil types of different pH, making it suitable for most South African soils. It is, however, important to note that an environmentally friendly and non-toxic extraction solvent with similar strength to methanol is needed, as methanol is regarded as toxic and should not be part of the MLE preparation. Acetone-MLE and aqueous MLE were also found to be effective in controlling fusarium dry rot; therefore, water, as the simplest and most-widely available solvent, should be used by subsistence farmers in townships and villages; further, water-MLE has no toxic effects on the ecosystem. Water-MLE should, therefore, be used in controlling fusarium dry rot postharvest. Furthermore, antifungal properties of aqueous MLEs indicate an abundance of phytochemicals, crucial in preventing diseases. It is important to efficiently dry tubers after treatment with MLEs, as excessive moisture may favour fungal and bacterial pathogen development.

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General discussion and conclusion

Potato is one of the world's most-popular and most-widely consumed agricultural commodities; it is estimated that more than one billion people consume potato worldwide (Potato facts and figures, 2020). Potato requires less water input than grains (rice, wheat and maize) and produces ten times the yield of grains per ha. Potato is, however, mainly composed of water, as it has an average moisture content of approximately 75-75%, while wheat has a moisture content of 18-20%, when harvested. Thus, wheat has a much higher dry matter content than potato.

Potato is not only a starch crop, but it contains high amounts of vitamin C and vitamin B6, amongst others. With the world's population concatntly increasing, potato demand is on the rise (Devaux et al., 2021). If yields would decline due to a lack of disease control strategies for potato, negative impacts on the food security status of South Africa would be realized, as many disadvantaged South African villages rely on cultivation of potato (Kumar et al., 2021). These rural communities consume the potatoes they grow and also use them as a trade object. One of the major challenges to potato production is fusarium dry rot, severely limiting potato production; this disease causes economic losses in the field and even more so in post-harvest storage (Kumar et al., 2021). Approximately 20% to 60% of potato production is lost annualy worldwide to fusarium dry rot (Tiwari et al., 2021).

Fusarium spp. are rapidly evolving pathogens with new strains continuously appearing. Whilethese species are adapting to climatic conditions, plants seem to be less able to cope with these changing conditions. Chemical fungicides have been used in controlling *Fusarium* spp.;however, these species develop resistance towards such fungicides, potentially threatening potato production. Therefore, these fungicides are not only becoming more ineffective, but are also generally expensive, making them unaffordable to rural farmers Fusarium spp. are not only problematic in the potato industry; these species have a broad host range and also attack many important crops, such as banana, tomato, cabbage, sweet potato, melons, and legumes(www.britannica.com, 2017).

Developing a bio-fungicide against fusarium dry rot that is effective, sustainable, and environmentally sound is of utmost importance. Research studies have shown that moringa

leaf extracts (MLEs) can be used as potential bio-fungicides. This is due to the vast numberof chemical compounds moringa leaves contain, which have antimicrobial activity.

The present study was carried out to investigate the potency of moringa leaf extract (MLE) as bio-fungicides, which will subsequently decrease fusarium dry rot prevalence and improve potato production.

This dissertation is categorized into sections, with each section focussing on a specific research goal.The categories are as follows:

- **Section I**: Review of the literature on the current knowledge of fusarium dry rot, and on the antifungal potency of MLEs, due to the presence of certain phytochemical compounds.
- **Section II:** The effectiveness of solvent type and solvent concentration in extracting moringa SM. The effect of MLE treatment on phytochemical composition of the tubers and their physical appearance.
- **Section III:** The antifungal potency of MLEs, both *in vitro* and *in vivo*.

In **Section I**, the efficacy of solvent type and solvent concentration in extracting different SMs was investigated (**Chapter 1**). From previous studies, MLEs seemed to enhance the phytochemical composition of field crops and, subsequently, increase yield and crop quality. Spectrophotometric analyses revealed the presence of flavonoids, tannins and glycosides in MLEs. Glycosides were, however, absent in the ethyl acetate MLE (Chapter 2; Fig. 2.2). In this study, the tuber treatment with various MLEs was found to enhance the bound and free phenolic concentrations. Treatment with 30% and 50% methanol-MLEs and 30% acetone-MLE resulted in higher free phenolic concentrations than other treatments in both 'Mondial' and 'Fandango' tubers (Chapter 2; Fig. 2.1). The 50% and 70% methanol-MLE treatment also significantly enhanced bound phenolic concentrations in both 'Mondial' and 'Fandango'. Phenolics play a crucial role in plant defence mechanisms; thus, the increased phenolic concentration could assist potato tubers to be more resistant to fungal diseases and to better withstand environmental stresses. Since potato is a staple crop and important in food security, enhancing the phytochemical concentration of potato could improve malnutrition prevalent in South Africa (Goyer, 2017; Zhang *et al*., 2017).

Treatment with MLEs also impacted on the percentage mass loss and firmness of tubers over the four- week storage period. The response to MLE treatments was found to be cultivar-dependent, as 'Mondial' tubers had a lower percentage mass loss compared with 'Fandango' tubers. The MLE

treatments were found to reduce percentage mass loss in treated tubers compared with control tubers. Tuber percentage mass loss is associated with rapid water loss, which has a negative impact on tuber quality and longevity. Tubers that had a higher percentage percentage mass loss were also found to be less firm. These are important traits, particularly in tuber marketing, as consumers are attracted to firmer and visually appealing tubers; thus, MLE treatments can potentially improve tuber marketability.

The water-MLE effectively enhanced both, free and bound phenolics concentrations in 'Fandango' and 'Mondial' tubers and was able to preserve tubers appearance and reduce average percentage percentage mass loss in both cultivars. These results are important for both, commercial farmers, and smallholder farmers, as these MLE solution are easily prepared and will improve the phytochemical concentration of tubers and thus, improve resistance to pathogens and environmental stresses as wellas marketability.

In **Section II**, MLEs were found to inhibit mycelial growth of *F. oxysporum*. Solvent concentration was found to influence fungal inhibition activity of MLEs, as MLEs extracted with higher solvent concentrations did not inhibit mycelial growth 100%. In contrast, MLEs extracted with lower solvent concentrations (30% and 50%) resulted in 100% mycelial growth inhibition. Watery-MLE was able to inhibit 87.95 mycelial growth. There was no significant difference between the mycelial growth inhibition activity of the two tested MLE dilutions (2% w/w and 4% w/w) ($P>0.05$). *Fusarium oxysporum* is known to causedevastating losses, not only in potato production. Thus, the results obtained from the *in vitro*assay could be a solution for many farmers whose production is suffering from *F. oxysporum*infections.

In **Section III**, the MLEs extracted with either 30% or 50% organic solvent were found to delay fusarium dry rot progression and severity. Treatments with MLEs extracted with 30% organicsolvent were the best treatments, where 30% acetone-MLE had the smallest average lesion diameter of 3.67 mm. These lesions were shallow and did not grow deep into the tuber. In both cultivars, 50% methanol-MLE was the best treatment as a preventive measure with an average lesion diameter of 1.67 mm in 'Mondial' tubers. Furthermore, water-MLE was found to significantly delay disease progression. Response to MLE treatment was also found to be cultivar-dependent, as 'Mondial' tubers had less severe disease symptoms and were not prone to secondary infection by bacterial soft rot. Treatment with 80% ethyl acetate had a rather detrimental effect on the tubers, as in these tubers disease was more severe than incontrol tubers; this was particularly true for 'Valor'. Ethyl

acetate might have destroyedtuber structure, which may have, in turn, created a pathway for easier colonisation by *F. oxysporum,* destroying the internal tuber structures. As opportunistic pathogens, *Pectobacterium* spp., exploited these tubers that were already invaded.

Although MLEs were not curative, they did, however, delay disease progression and reduce disease severity. After 28 days post-inoculation and -treatment, 'Valor' tubers treated with 30% and 50% acetone, 30% methanol-MLE and water-MLE had an average lesion diameter of less than 8 mm, had not wrinkled, and did not develop bacterial soft rot infection. 'Mondial' tubers, treated with 30% acetone, 30% and 50% methanol-MLE and water-MLEs, developed an average lesion diameter less than 8 mm, had no observable wrinkles, and did not developsecondary bacterial soft infection 28 days post inoculation and treatment. Therefore, these MLE seem to be potential antifungal agents that might claim a place in reducing postharvest diseases in potato.

These findings are important for the potato industry and for smallholder farmers who experience enormous production losses due to fusarium dry rot. These results also highlight moringa extract applications as important environmentally sustainable treatments, as waterMLE has a nor or minimal negative impact on the environment; thus, MLE treatments can potentially reduce reliance on synthetic fungicides and, subsequently, reduce environmental pollution. Agriculture is one of the leading contributors to global pollution, through chemical fertilizers. (https://www.iwmi.cgiar.org/Publications/Books/PDF/more_people_more_food_worse_wa ter-chapter-3.pdf).

Results from this research study have proven that MLE can be used to enhance the production of SMs, which are produced as part of a plant's defence system and play an important role in protecting plants against environmental stressors. Flavonoids were present in all MLEs. Flavonoids play a certain role in scavenging free radicals and in preventing oxidation, ultimately prolonging tuber longevity and, thereby, improving tuber marketability. Tuber treatment with MLEs was also found to prevent secondary infection by bacterial soft rot.

Development of MLEs as bio-fungicides will reduce synthetic fungicide dependency. This may reduce chemical pollution of the environment, evolution of new fungal strains and, hence, crop losses. In contrast with chemical fungicides, bio-fungicides are less harmful to mammals and are preferred by the majority of consumers (El-Mohamedy and Abdalla, 2014). Bio-fungicides derived from plant SMs, such as Mo-CBP3, could become important environmentally friendly and sustainable treatments (Freire et al., 2015). Given that MLE treatment enhanced the concentration of phytochemical

compounds in potatoes, consuming potatoes treated with MLEs can potentially decrease malnutrition, prevalent in many South African communities. Bio-fungicide application will also be beneficial, as these compounds are effective plant growth enhancers; furthermore, MLEs have been found to improve yield in crops, such as tomato, okra and sunflower (aEl-Mohamedy and Abdalla, 2014; Hanafy, 2017; Kanchani and Harris, 2019; Iqbal et al., 2020).

Proposed future research priorities

- To isolate the moringa chitin-binding protein (Mo-CBP3) and test its fungal efficacy on economically important fungal pathogens, such *Fusarium* spp., *Phytophthora infestans* and *Alternaria solani*
- To investigate the efficacy of Mo-CBP3 under greenhouse conditions and in field conditions
- To study the mode of action of Mo-CBP3 in a range of important fungal species
- To compare the efficacy of moringa seed, leaf, and bark extracts against *Fusarium* spp.
- To use different solvents to extract moringa leaves, solvents that are more environmentally sound and have lower toxicity
- To use different extraction techniques, such as Soxhlet extraction, microwave-assisted extraction, and ultrasound-assisted extraction in MLE preparation. These extraction methods may potentially optimize phytochemical compounds extracted from moringa leaves
- To further investigate the effect of MLE as a postharvest treatment on quality and longevity of potato tubers

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Appendix

Table 2.2: Free phenolic concentration standard deviation of various moringa leaf extract treatment

Variable	Observations	Obs. with missing data	Obs. without missing data	Minimum	Maximum	Mean	Std. deviation
30% Acetone	2	0	2	0,213	0,265	0,239	0,037
30% Methanol	2	0	2	0,281	0,316	0,298	0,025
30% Ethyl acetate	2	0	2	0,113	0,155	0,134	0,030
50% Acetone	2	0	2	0,278	0,287	0,283	0,006
50% Methanol	2	0	2	0,163	0,265	0,214	0,073
50% Ethyl acetate	2	0	2	0,148	0,234	0,191	0,061
70% Acetone	2	0	2	0,202	0,206	0,204	0,003
70% Methanol	2	0	2	0,161	0,235	0,198	0,052
70% Ethyl acetate	2	0	2	0,146	0,245	0,195	0,070
Water	2	0	2	0,164	0,238	0,201	0,052
Control	2	0	2	0,151	0,168	0,160	0,012

Table 2.3: Bound phenolic concentrations standard deviations after treatmenet with various moringa leaf extracts

Variable	Obser-vations	Obs. with missing data	Obs. with-out missing data	Minimum	Maximum	Mean	Std. deviation
30% Acetone	2	0	2	0,213	0,265	0,239	0,037
30% Methanol	2	0	2	0,281	0,316	0,298	0,025
30% Ethyl acetate	2	0	2	0,113	0,155	0,134	0,030
50% Acetone	2	0	2	0,278	0,287	0,283	0,006
50% Methanol	2	0	2	0,163	0,265	0,214	0,073
50% Ethyl acetate	2	0	2	0,148	0,234	0,191	0,061
70% Acetone	2	0	2	0,202	0,206	0,204	0,003
70% Methanol	2	0	2	0,161	0,235	0,198	0,052
70% Ethyl acetate	2	0	2	0,146	0,245	0,195	0,070
Water	2	0	2	0,164	0,238	0,201	0,052
Control	2	0	2	0,151	0,168	0,160	0,012