APPLICATION OF MICRONUTRIENT, PLANT EXTRACT AND DEFENSE INDUCER AS MANAGEMENT STRATEGY AGAINST *ALTERNARIA* LEAF SPOT OF TOMATO

BY

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Table of contents

Content Page

DEDICATION iv
ACKNOWLEDGEMENTS v
LIST OF TABLES vi
LIST OF FIGURES vii
LIST OF APPENDICES viii
MINI-DISSERTATION STRUCTURE ix
ABSTRACT x
CHAPTER 1 1
GENERAL INTRODUCTION 1
1.1. Background of the study 1
1.2. Problem statement 3
1.3. Motivation of the study 4
1.4. Purpose of the study 5
1.4.1. Aim 5
1.4.2. Objectives 5
1.4.3. Hypotheses 6
1.5. Reliability, validity, and objectivity 6
1.6. Bias 6
CHAPTER 2 7
LITERATURE REVIEW 7
2.1. Introduction 7
2.2. *A*lternaria leaf spot diseases 7
2.2.1 Causal organism: Taxonomy and Biology 7
2.2.2 Alternaria solani 8
2.2.3 Disease development and symptoms 8
2.2.4 Distribution and economic importance 9
2.3. Work done on the problem 10
2.3.1 Management strategies against Alternaria leaf spot 10
2.3.2 Application of fungicides 10
2.3.3 Cultural control 11
2.4. Work not done on the problem 25
CHAPTER 3 27

MATERIALS AND METHODS 27
3.1. Achieving objective one: 27
3.2.1 Study site and experimental design 27
3.2.2 Collection of diseased Tomato plant materials and pathogen isolation 27
3.2.3 *Monsonia burkeana* collection, processing and extract preparation 28
3.2.4 Combined nano zinc and copper micronutrient treatments preparation 28
3.2.5 *A. solani* mycelial growth suppression with *Monsonia burkeana* and combined nano zinc and copper micronutrient 28
3.2.6 Data collection and analysis 29
3.2 Achieving objective two and three 29
3.2.1 Study site and experimental design 29
3.2.2. Preparation of pathogen inoculum 31
3.2.3. Acibenzolar-S-Methyl (ASM) treatments preparations 31
3.2.4. Inoculation and treatment application 31
3.2.6 Data collection and analysis 32
CHAPTER 4 33
RESULTS 33
4.1.The efficacy of *M. burkeana* and combined nano zinc and copper micronutrienton pathogen growth (*Alternaria solani*) under laboratory conditions. 33
4.1.1 Antifungal activity assay 33
4.2 Evaluation of combined nano zinc and copper micronutrient, Acibenzolar-s-methyl, and *M. burkearna* under greenhouse and micro-plot. 36
4.2.1 Efficacy of combined nano zinc and copper micronutrient, Acibenzolar-s-methyl, and *M. burkeana* on *Alternaria* leaf spot development and severity. 36
4.2.2 Micro-plot experiments 37
4.2.3 The effect of *M. burkeana*, Nano-zinc micronutrient, and ASM defense inducer on yield of tomato. 38
CHAPTER 5 40
DISCUSSION 40
5.1 Antifungal activity assay 40
5.2 Efficacy of combined nano zinc and copper micronutrient, Acibenzolar-s-methyl, and *M. burkeana* on *Alternaria* leaf spot development and severity. 43
CHAPTER 6 46
SUMMARY, CONCLUSION, AND RECOMMENDATIONS 46
REFERENCES 48
APPENDICIES 67

DECLARATION

I declare that the mini-dissertation submitted to the University of Limpopo, for the degree of Master of Science in Agriculture (Plant Protection) has not been previously submitted by me for a degree at this or any other university; that it is my work in design and execution, and that all material contained herein has been duly acknowledged.

Candidate: R SIBUYI Signature:…… Date:……

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Co-Supervisor: PROF K.K AYISI Signature:…… Date:……

DEDICATION

This work is dedicated to my mother Alsina Marry Sibuyi. For endless support and guidance.

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First and foremost, I would like to thank the Lord God Almighty for his grace and mercy and the perfect gift of life. Every good gift and every perfect gift is from above and cometh down from the Father of lights, with whom is no variableness, neither shadow of turning (James 1:7).

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LIST OF TABLES

		Page
Table 2.1	Antifungal potential of nano-micronutrients and nano-formulated micronutrients	23
Table 3.1	List of treatments for laboratory experiments	27
Table 3.2	List of treatments for greenhouse and micro-plot experiments	30
Table 3.3	Disease severity rating scale	32
Table 4.1	Assessment of crop physiological responses and yield of tomato.	38

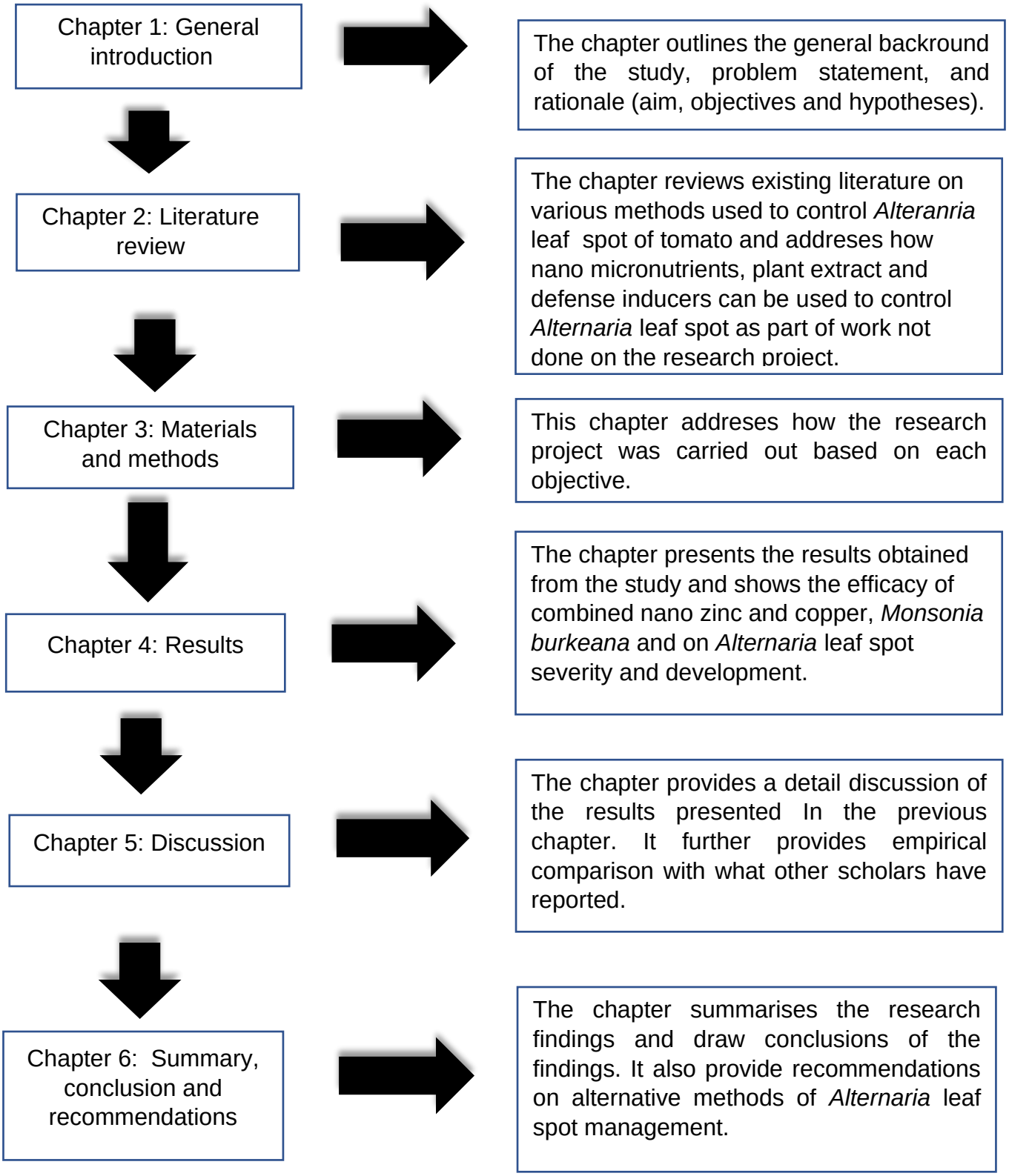
LIST OF FIGURES

		Page
Figure 2.1	Infection process, development, and symptoms of diseases caused by *Alternaria solani*	9
Figure 3.1	Mountain-view research and training farm	29
Figure 4.1	Effects of combined nano zinc and copper micronutrient concentrations on mycelial growth of *A. solani* after inoculation	34
Figure 4.2	Effects of *M. burkeana* concentrations on mycelial growth of *A. solani* after inoculation	35
Figure 4.3	*Alternaria* leaf spot severity under greenhouse conditions after 21 days. Bars followed by the same letter do not differ significantly (P ≤ 0.05) according to Duncan's multiple range test.	36
Figure 4.4	*Alternaria* leaf spot severity under micro-plot condition after 21 days. Bars followed by the same letter do not differ significantly (P ≤ 0.05) according to Tukey`s HSD	37

LIST OF APPENDICES

		Page
Appendix 4.1.1	Analysis of variance for mycelial growth of *A. solani* at different concentrations of combined nano zinc and copper micronutrient at 4 DAI	67
Appendix 4.1.2	Analysis of variance for mycelial growth of *A. solani* at different concentrations of combined nano zinc and copper micronutrient at 7 DAI	67
Appendix 4.2.1	Analysis of variance for mycelial growth of *A. solani* at different concentrations for *Monsonia burkeana* at 4 DAI	67
Appendix 4.2.2	Analysis of variance for mycelial growth of *A. solani* at different concentrations for *Monsonia burkeana* at 7 DAI	67
Appendix 4.3.1	Analysis of variance for disease severity under greenhouse	68
Appendix 4.3.2	Analysis of variance for disease severity under micro-plot	68
Appendix 4.4	Analysis of variance for yield parameters	68

MINI-DISSERTATION STRUCTURE



ABSTRACT

Alternaria leaf spot, caused by *Alternaria solani* is one of the most economically important foliar diseases in tomato production. Its management is mainly based on the application of synthetic fungicides. However, the negative side effects of these fungicides on the environment, human health, and increased pathogen resistance have prompted the identification of alternative control measures. This study was established to develop a management strategy against *Alternaria* leaf spot based on plant nutrition, application of protective plant extract, and induction of a defense mechanism. The antifungal activities of *Monsonia burkeana* and nano-zinc and copper micronutrient were first tested under laboratory conditions using a food poisoning assay. Potato dextrose agar was amended with different concentrations of *M. burkeana* (1, 2, 3, 4, 5 g 100 ml^{-1}), Combined nano zinc and copper micronutrient (0.09, 0.12, 0.18, 0.24, 0.30 ml ℓ^{-1}) and un-amended PDA served as control. Treatments were arranged in a Completely Randomized Design (CRD) replicated four times. Both *M. burkeana* and Nano-zinc micronutrient significantly ($P \leq 0.05$) inhibited the mycelium growth of *A. solani* when compared to the control. A significant reduction was recorded at 5 g 100 ml^{-1} and 0.30 ml ℓ^{-1} for both *M. burkeana* (32.81 %) and combined nano zinc and copper micronutrient (39.69 %) respectively. Both concentrations were further tested under greenhouse and field micro-plot experiments, in a randomized complete block design (RCBD) replicated four times along with *Acibenzolar-S-methyl* (ASM) defense inducer to determine their effect on *Alternaria* leaf spot development and severity. All tested treatments significantly reduced *Alternaria* leaf spot development and severity under both greenhouse and micro-plot when compared to untreated control. The results of the study demonstrated that *M. burkeana*, nano-zinc and copper micronutrient, and ASM defense inducer significantly reduce *Alternaria* leaf spot.

Key words; *Alternaria* leaf spot, *Alternaria solani*, *M. burkeana*, combined nano zinc and copper micronutrient, Acibenzolar-S-methyl (ASM), Alternative management

CHAPTER 1

GENERAL INTRODUCTION

1.1. Background of the study

Tomato (*Solanum lycopersicum*) is regarded as one of the most important vegetables worldwide ranking second after potato (Mujtaba and Masud, 2014). It is consumed in different forms which makes it common in different countries, it can be consumed raw as salsa, sandwiches, and fruit salads or processed into different preferences which include soups, juices, pastes, and preserves, (Mujtaba and Masud, 2014; Pinheiro *et al.,* 2014). In South Africa, tomatoes are cultivated commercially by large-scale farmers, subsistence, resource-poor farmers, and home gardeners. It is produced in all Provinces, however, Limpopo Province with its warm climate is best suited for the production of tomatoes, and it accounts for more than 75% of the total planted area of South Africa (DAFF, 2018; TPO, 2017). According to NDA (2015), the South African tomato industry contributes significantly to the national Gross Domestic Product (GDP) and it is considered one of the most important components in the agricultural sector. It contributed 24% towards GDP in 2014 and has shown great growth in terms of production for the past decade.

Nonetheless, numerous factors are threatening tomato production in South Africa, resulting in large losses in fresh and processed tomato production. These include biotic factors, such as bacteria, viruses, nematodes, fungi, and abiotic factors resulting in serious yield reduction leading to economic losses in the agriculture sector (Gimenez *et al.,* 2018; Pereg and Tolessa, 2019). Several foliar diseases, including *Alternaria* leaf spots and bacterial spots, are favoured by warm temperatures or prolonged periods of wetness (Sallam and Kamal, 2012). *Alternaria* leaf spot which is caused by *Alternaria solani* and other *Alternaria* spp complex such as *A. alternate*, is one of the most prevalent foliar diseases in tomato production that results in poor crop quality and yield (Faheed *et al.,* 2005). The disease infects the plant leaves at all stages of growth and causes destructive necrotic symptoms that lead to yield losses (Reddy *et al.,* 2002). The incidence of *Alternaria* leaf spot is gradually increasing in tomato production areas as a result of its sophisticated nature to overwinter during unfavourable conditions or in the absence of a suitable host plant and increase in growth of susceptible cultivars (Khan *et al.,* 2003). Synthetic fungicides remain an

integral component of managing *Alternaria* leaf spots in agricultural systems (Abdalla *et al.,* 2014). However, many synthetic fungicides have negative side effects, including environmental pollution, damage to human health, and loss of efficacy due to increased pathogen resistance to fungicides. In addition, *Alternaria* leaf spot is difficult to control, regardless of attempts of eradication, disease outbreaks continue to occur across several growing seasons, often originating from spores that have to remain dormant (McConnell *et al.,* 2003). Furthermore, frequent application of synthetic fungicides accounts for the development of resistant strains of *Alternaria spp*, reducing the efficacy of synthetic fungicides (Ma and Michailides, 2005). Therefore, alternatives to synthetic fungicides are being considered as a key constituent of *Alternaria* leaf spot management.

According to Toor *et al.* (2021), micronutrients, play a paramount role in plant disease management. They contribute to the reduction of disease incidence and severity through active participation in the biochemistry and physiology of the plant since most of the micronutrients are involved in processes that affect the plant's response to pathogens (Van Bockhaven *et al.,* 2013). The rate of disease development can be reduced significantly under optimum nutrient supply. Similarly, plant nutrients also influence host resistance or susceptibility to the disease and the pathogen virulence. Saharan *et al.* (2015), reported that infection risk is minimal when an ideal nutrient supply is sufficient and this has been attributed to the activation of biochemical processes including those responsible for defense activities, Moreover, plant nutrition has been shown to play a critical role in *Alternaria* leaf spot management, for instance, a low rate of nitrogen fertilization in potatoes leads to a greater lesion area for potato early blight while minimal under normal fertilization (Dong *et al.,* 2018; Ninh *et al.,* 2015). Furthermore, MacDonald *et al. (*2007), reported that when cotton (*Gossypium hirsutum*) plants are deficient in potassium (K) they become more susceptible to *Alternaria alternata* than those that are not deficient.

Micronutrients including zinc (Zn), play a major role in improving plant defenses through oxidative enzyme activation and reduction in pathogen activities (Datnoff and Heckman, 2014). One of its roles is to maintain the structural integrity of cell walls. For instance, when plants are deficient in Zn, they become highly susceptible to root diseases (Dordas, 2008; Singh *et al.,* 2021). Datnoff *et al.* (2007), reported that the addition of Zn fertilizers reduces the incidence and severity of *Fusarium* root-rot in

wheat. Furthermore, a study of *Rhizoctonia solani* infection levels in wheat indicated that the application of Zn can dramatically reduce the degree of infection (Schurt *et al.,* 2014). Besides its ability to reduce root disease severity, Zn may also play a role in airborne pathogen tolerance. When deficient in Zn, the plant's biosynthesis of proteins is inhibited, and an extensive accumulation of amino acids occurs. Similarly, an increase in susceptibility to airborne disease is found when Mg or K is deficient and leaves accumulated sugar and amino acids (Datnoff and Heckman, 2014; Heckman, 2013).

Other micronutrients such as boron (B), manganese (Mn), and calcium (Ca) can significantly reduce disease invasion in plants by improving and maintaining the structural integrity and rigidity of the cell wall (Kablan *et al.,* 2012). Nutrient deficiencies in plants reduce their ability to defend themselves against attack by pathogens thus resulting in high disease susceptibility. For example, plants deficient in potassium (K) and magnesium (Mg), are more susceptible to infection from airborne pathogens due to an increase in soluble sugars and amino acids (Dallagnol *et al.,* 2011; Datnoff *et al.,* 2007). Adequate plant nutrition can also improve drought and head-stress tolerance and further tolerance to attack by various fungal pathogens. Balanced crop nutrition plays a key role in assisting to prevent disease infections and is another valuable tool in your toolbox to manage the disease. Therefore, alternative environment-friendly methods for the management remain important for sustainable production.

1.2. Problem statement

Alternaria leaf spot is a fungal disease caused by *Alternaria spp* which infects both leaves and fruits. *Alternaria* leaf spot remains a major foliar disease in tomato and potato production, for both commercial and small-scale farmers in all areas where tomato is produced. The disease is highly destructive and can result in 100% yield reductions under favourable conditions (El-Khallal, 2007). Fungicides and resistant cultivars are the major control measures used against this disease (Van Bockhaven *et al.,* 2013). However, resistant cultivars are often overcome by the pathogen`s genetic variability, environmental interactions, and genotype (Bayles *et al.,* 2000). Furthermore, the continuous application of synthetic fungicides has led to the development of resistance by *Alternaria* isolates resulting in a loss of control for *Alternaria* leaf spot (Wharton *et al.*, 2012). Resistance to fungicides by *Alternaria* has been attributed to several factors including the presence of gene mutations which are

due to the positioning of phenylalanine and leucine in amino acid 129 resulting in the development of new populations (Thomas *et al.*, 2012). *Alternaria* has a species diversity that can maintain high morphological and pathogenic variability thus, high incidences of fungicides resistance have been reported (Patil *et al.,* 2002). The abovementioned led to reduced efficacy of commonly used fungicides to control *Alternaria* leaf spot worldwide. In addition, fungicides pose risks to the environment, since they have a long residual effect and drift to water reservoirs and other non-target areas (Kibria *et al.,* 2010). Agrochemicals have a long degradation period that leads to environmental pollution, to promote an effective and sustainable agricultural system, a balance between management of fungal pathogens, threats to crops, and the conservation of marine and terrestrial habitats should be established. Thus, the main aim of the study was to develop management strategies that are environmentally sound, effective, and efficient in the control of *Alternaria* leaf spot.

1.3. Motivation of the study

Alternaria leaf spot is controlled by the application of various measures including implementation of cultural practices such as modification of irrigation systems to reduce prolonged periods of leaf wetness, planting of resistant cultivars, and application of synthetic fungicides. The most commonly used fungicides include Copper oxychloride, Mancozeb, carbendazim, and benomyl (Visvas *et al.*, 2017). Despite their effectiveness in managing *Alternaria* leaf spot, recent reports have shown an increase in the loss of pathogen sensitivity in crops, particularly in potato, thus suggesting the development of resistant genes (Van der Waals *et al.,* 2004). Moreover, synthetic fungicides also have a negative impact on the environment, health, and ecosystems. Plant nutrition plays an important role in plant disease management. In plants deficient in Zn, protein biosynthesis is inhibited resulting in an extensive amino acid accumulation. This is comparable to the increase in airborne disease sensitivity observed when Mg or K is deficient and sugar and amino acids accumulated in the leaves. According to Printz *et al.* (2016), copper (Cu) compounds has been widely used in agriculture as inorganic agrochemicals as fertilizers and as well as for crop protection. However, concern has been raised over the excessive application which negative effect on crop and environmental pollution (Cruz *et al.,* 2021). Cu nanoparticles (NPs) emhnance antimicrobial properties which reduces the application frequency and application rate. Shende *et al.* (2021), reported that Cu-

based NPs are effective against different plant pathogens and have advantages over conventional agrochemicals.

Natural plant products are considered an essential alternative for controlling plant diseases as they contain secondary metabolites which have antifungal activities. According to Barman *et al.* (2016), plant extracts can significantly decrease pathogen populations and suppress the development of plant diseases. Numerous natural plant species have been reported to have natural properties that are toxic to plant pathogenic fungi. Kagale *et al.* (2004), reported that under *in vivo* and *in vitro* conditions different natural extracts demonstrated antifungal properties toward fungal pathogens. Furthermore, systemic acquired resistance (SAR), an inducible resistance system has been identified as a promising environmentally-sound technique in combating plant diseases, especially those caused by viruses, fungi, and bacteria. Besides being induced biologically, SAR can be stimulated by the application of chemicals that contains a salicylic acid in its synthetic analogs, such as aminobutyric acid (BABA) and potassium salts, 2,6-dichloroisonicotinic acid (INA) and salicylic acid (SA) induce SAR in plants (Hartmann *et al.,* 2019). Thus, the study aimed to provide an alternative management strategy against *Alternaria* leaf spot using plant nutrition, plant extract, and a defense inducer.

1.4. Purpose of the study

1.4.1. Aim

The study aimed to develop a management strategy against *Alternaria* leaf spot of tomato, by using *Monsonia burkeana,* combined nano zinc and copper micronutrient, and ASM defense inducer.

1.4.2. Objectives

The objectives of this study were:

i) To evaluate the efficacy *of M. burkeana* and combined nano zinc and copper micronutrient on pathogen growth (*Alternaria solani)* under laboratory conditions.

ii) To determine the effects of *M. burkeana*, combined nano zinc and copper micronutrient, and ASM defense inducer on *Alternaria* leaf spot development and severity under greenhouse and micro-plot conditions.

iii) To determine the effects of *M. burkeana*, ASM defense inducer, and combined nano zinc and copper micronutrient on crop yield.

1.4.3. Hypotheses

i) *Monsonia burkeana* and combined nano zinc and copper micronutrient will suppress pathogen growth under *in vitro* conditions.

ii) *Monsonia burkeana*, combined nano zinc and copper micronutrient, and plant defense inducers will reduce the incidence of *Alternaria* leaf spot development and severity under greenhouse and micro-plot conditions.

iii) Plant defense inducer, *M. burkeana*, and combined nano zinc and copper micronutrient will improve crop yield.

1.5. Reliability, validity, and objectivity

Data reliability was determined through statistical analysis at a probability level of ($P \leq 0.05$) and was achieved by ensuring that the findings are discussed based on empirical values, thereby eliminating all types of subjectivity, and the validity was obtained by repeating the experiments twice.

1.6. Bias

Bias was minimized by ensuring that the experiment error was reduced through adequate replications and randomization of treatments within the selected experimental designs (Leedy and Ormrod, 2005).

CHAPTER 2

LITERATURE REVIEW

2.1. Introduction

Tomato (*Solanum lycopersicum*) is amongst the most important and valuable vegetables worldwide. Tomato ranks second among the *solanaceous* vegetable crops after potatoes (FAOSTAT, 2009). However, tomato ranks first among the processing crops in the world. Tomato is commonly consumed in our daily life and it is a good source of antioxidants (Sgherri *et al.,* 2008). Tomato contains 95.3% of water, 0.07% calcium, and niacin, all of which have great importance in the metabolic activities of humans (Olaniyi *et al.,* 2010). Tomato, like any other crop, is constantly under threat from fungi, bacteria, viruses, and nematodes. Among the fungal diseases, *Alternaria* leaf spot caused by *Alternaria solani* is one of the most destructive and most common foliar diseases of tomato (Foolad *et al.,* 2002; Raza *et al.,* 2016). This pathogen infects the plant leaves at all stages of growth and causes destructive necrotic symptoms that lead to yield losses and possibly up to 100% yield reduction if control measures are not put in place. The pathogen causes infection on leaves, stems, petiole, twigs, and fruits as well as leads to the defoliation, drying of twigs, and premature fruit drop which ultimately reduces yield (El-Khallal, 2007). Moreover, disease development is favoured by relative humidity, rainfall, high temperatures as well as prolonged periods of leaf wetness. However, the plants are more susceptible during the fruiting period (Khan *et al.,* 2003).

2.2. *A*lternaria leaf spot diseases

2.2.1 Causal organism: Taxonomy and Biology

The genus Alternaria

Alternaria genus was first identified in the late 1700 and since then about 1200 *Alternaria* species have been identified (Simmons, 2007). From the 1200 species identified, only 300 are currently accepted as members of this group (Simmons, 2007). Morphological similarities between *Alternaria* and some related fungal genera have resulted in more confusion in their identification and classification (Woundenberg *et al.*, 2013*)*. Currently, new 32 combinations and 10 new species names based on molecular characterization and morphology have been proposed for this genus (Woundenberg *et al.*, 2013). The Genus *Alternaria* includes both pathogenic and

saprophytic as well as borderline species, capable of becoming one or other with varying environmental conditions (Agrios, 2005). *Alternaria* is the ascomycete fungi that contribute significantly to plant pathogens (Laurence *et al.,* 2012). They are omnipresent in the ecosystem and the natural part of the fungal flora is present almost everywhere. The absence of *Alternaria spp* in any given location has been attributed to the lack of suitable host plants and unfavourable environmental conditions (Rotem, 1994). The spores are air-borne and found in both soil and water. The formation of dark-coloured conidia with longitudinal and transverse septa remains a major key to the taxonomic characterization of this genus (Dube, 2014; Pryor and Gilberson, 2002).

2.2.2 Alternaria solani

Alternaria solani is an imperfect fungus that reproduces asexually via multi-cellular conidia with an unknown sexual cycle (Agrios, 2005; Rogers, 2007; Van der Waals *et al.,* 2001). The fungi *A. solani* are classified under the *Eukaryota* Domain, Kingdom Fungi, phylum *Deuteromycota* (formerly) or *Ascomycota* (present), class *Hyphomycetes* and order *Hyphales* (Simmons, 2007; Van der Waals *et al.,* 2001). Like other members of the genus *Alternaria, A. solani* has transverse and longitudinal septate conidia, multinucleated cells, and dark-coloured (melanized) cells (Rotem, 1994). Melanin gives protection against adverse environmental conditions including resistance to antagonistic microbes and their hydrolytic enzymes (Rotem, 1994).

2.2.3 Disease development and symptoms

The asexual spores, mainly conidia or chlamydospores, which normally survive on dead plant materials germinate under moist and near-saturated humidity conditions and produce few or more germ tubes. The germ tubes subsequently penetrate the host epidermal cells through direct means of penetrating hyphae or through natural openings (stomata) or mechanical damage (wounds). According to Schultz, and. French (2009), it takes about 40 minutes for conidia to germinate. Due to the ability of desiccated germ tubes to re-grow when re-wetted, the infection can occur when wet and dry periods alternate. *Alternaria* leaf spot symptoms begin with tiny dark brown dots on the lower and older leaves. The tissue surrounding the main lesions turns into a bright yellow colour, and the leaves become necrotic and chlorotic. The spots increase in size, and form a concentric rings, giving them the appearance of a bull's eye. Diseases thrive in warm temperatures, causing outbreaks to multiply and plants to defoliate, reducing the quantity and quality of tomato fruits (Kouyoumjian, 2007).

Alternaria solani can infect any part of the plant and can disrupt the plant's growth at any stage (Abada *et al.,* 2008). Generally, a long period of wetness is essential for spore production however, spores are also produced during changing dry and wet conditions. Conidiophores are the first to develop during a wet night and are followed by conidia or spores in the following wet night after daylight and dryness. The pathogen has a relatively short life cycle hence, it has the potential of causing polycyclic infection (Kemmitt, 2002; van der Waals, 2001).

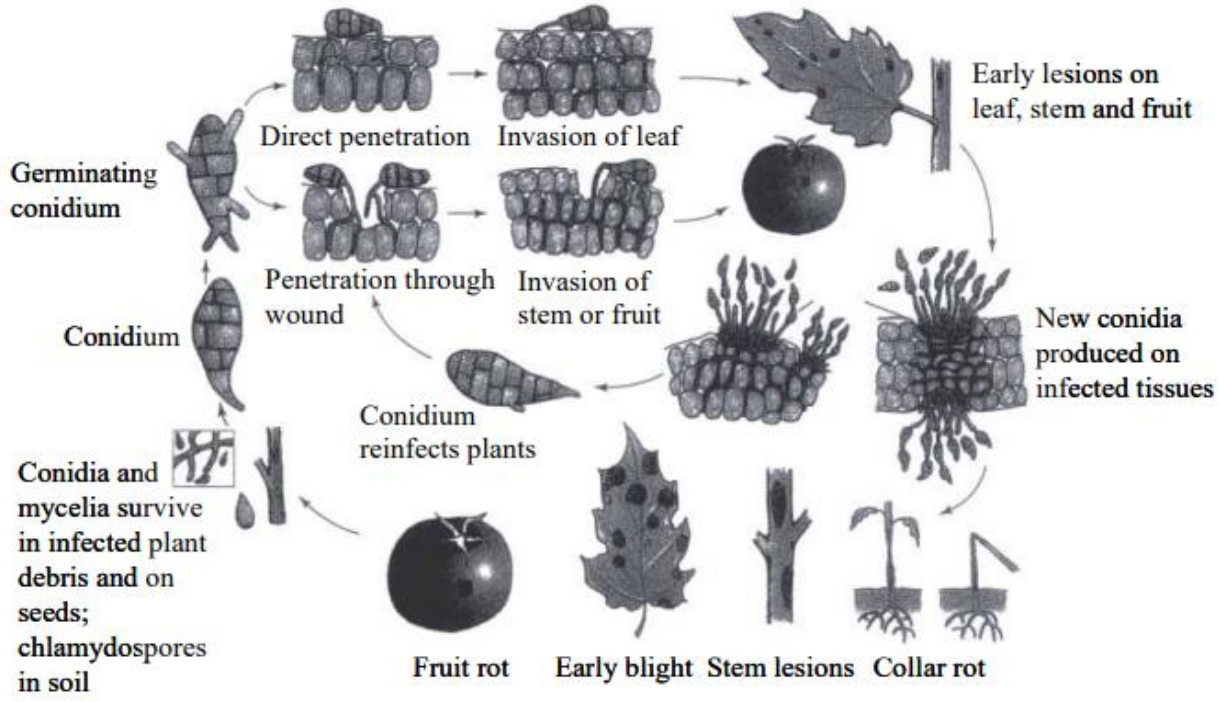


Figure 2.1: Infection process, development, and symptoms of diseases caused by Alternaria solani (adapted from Agrios, 2005)

2.2.4 Distribution and economic importance

Alternaria leaf spot is a common disease in the temperate and tropical zones, however, it is predominately found in most areas where potatoes and tomatoes are produced. *Alternaria* leaf spot is caused by *A. solani* and is regarded as one of the most destructive tomato diseases in most tomato-growing areas (Kamble *et al.,* 2016; Kumar *et al.,* 2017). The pathogen attacks the crop at all developmental stages and can significantly reduce the economic value of the crop. However, plants become more susceptible to chronological stages of development, with epidemics development coinciding with physiological maturity (Grigolli *et al.,* 2010**;** Nikam *et al.,* 2015).

Affected plants result in premature defoliation leading to increased photosynthetic rates. Van der Waals (2002), suggested that infected fruits tend to be more susceptible to other secondary pathogens resulting in a drastic reduction of marketable produce.

2.3. Work done on the problem

2.3.1 Management strategies against Alternaria leaf spot

Various methods or strategies have been used to control *Alternaria* leaf spot. They include fungicides application, cultural practices, the use of resistant cultivars, and disease-free planting materials (Sarfraz *et al.,* 2018). The use of synthetic fungicides is considered to be the most effective method for controlling *Alternaria* leaf spot of tomatoes (Shamurailatpam and Kumar, 2020). There are numerous registered synthetic fungicides to control *Alternaria* leaf spot which include but not limited to mancozeb, pyraclostrobin, ziram, and azoxystrobin. The use of resistant varieties is also an important method for the management of *Alternaria* leaf spot (Baibakova *et al.,* 2018; Nxumalo *et al.,* 2021). However, the worldwide trend towards safe environment methods for controlling plant diseases in sustainable agriculture practice needs reduced usage of synthetic chemicals (Bais *et al.,* 2019). Despite all strategies incidence of *Alternaria* leaf spot continue to occur in areas where tomatoes and potatoes are produced. Thus, it has exacerbated pressure for alternative methods of controlling *Alternaria* leaf spot.

2.3.2 Application of fungicides

Synthetic fungicides remain the most commonly used method to control *Alternaria* leaf spot. Fungicides application at a regular interval has shown a significant effect on inhibiting spore germination, penetration, and pathogen growth (Gondal *et al.*, 2012; Leiminger *et al.*, 2014). Commonly used fungicides in the management of *Alternaria* leaf spot include, mancozeb, chlorothalonil, and copper oxychloride (Horsfield *et al.*, 2010; Pasche and Gudmestad, 2008). However, Mphahlele *et al.* (2018), reported that some *A. solani* isolates have lost their sensitivity to fungicides such as chlorothalonil, thiram, and mancozeb and this further suggests pathogen resistance against these fungicides. The development of fungicides resistance in *A. solani* and other fungal pathogens has been attributed to the high genetic diversity that enables these pathogens to adapt to fungicides and shift the population towards more resistant isolates (Horsfield *et al.*, 2010). Frequent application of synthetic fungicides and induced population genetic variations in *Alternaria spp* are the main reasons behind

the development of new resistant strains (Rosenzweig *et al.*, 2008). The limitations of synthetic fungicides have prompted the identification of alternative control measures. Thus, the current study aimed to develop a management strategy against *Alternaria* leaf spot based on plant nutrition supplement, application of protective plant extract, and induction of defense mechanism.

2.3.3 Cultural control

Sanitation

Plant diseases have been managed successfully using cultural methods for several years. Cultural control includes manipulating farming practices to make the environment unfavourable for pathogen growth. Crop rotation is regarded as one of the most effective techniques in reducing levels of *Alternaria* leaf spot on tomatoes. Alternating crops from different families are effective in reducing the initial inoculum of *Alternaria* leaf spot (Soni *et al.,* 2015). According to Labrada (2008), crop rotation assists by reducing the pathogen population in soil and effectively controlling soil-borne pathogens.

A broader definition of sanitation, according to Tumwine *et al.* (2002), encompasses the elimination of inoculum sources both within and outside the crop. This can be accomplished through a variety of methods, ranging from simple crop hygiene to the removal of alternate hosts (Kunjwal and Srivastava, 2018). The practice has been recommended for controlling fungal diseases of perennial crops and other diseases such as dumping-off. For controlling *Alternaria* leaf spot removing crop residues and eliminating weeds is very critical since infection can persist in the residues from season to season.

Disease-free plants

A strategy that involves the use of pest-resistant and pest-tolerant cultivars developed through traditional breeding or genetic engineering (Douglas, 2018; Nelson *et al.,* 2018). These cultivars possess physical, morphological, or biochemical characteristics that reduce the plant's attractiveness or suitability for the pest to feed, develop, or reproduce successfully. However, there are currently no known resistant cultivars to *Alternaria* leaf spot available on the market for farmers.

2.3.4 Plant extracts

With the recent focus to develop environmentally safe, long-lasting and effective alternative management of plant disease, natural plant products are considered an essential source of non-synthetic agrochemicals for controlling plant diseases (da Cruz Cabral *et al.,* 2016; Kagale *et al.,* 2004). Reports show that plants with medicinal properties are also effective against different groups of plant pathogenic organisms (Dissanayake, 2014). They employ various modes of action including suppression of pathogen growth and improved plant defense mechanisms (Rahman *et al.,* 2018). According to Muthomi *et al.* (2017), plant extracts are regarded as environmentally safe alternatives and an important component in integrated disease management**.** Goussous *et al.* (2010), conducted a study on antifungal activity for several medicinal plants against the early blight fungus (*Alternaria solani*) and concluded that many natural plant species possess natural substances that are toxic to plant pathogenic fungi**.** Iran Nejad *et al.*, 2012; Muthomi *et al.,* 2017, studied the effect of 11 different plant extracts on the mycelial growth of *Alternaria spp* and found that leaf extracts of some plants, for example, *Tamarix aphylla* and *Salsola baryosma*, totally inhibited the growth of the pathogen *in vivo*. Wszelaki and Miller (2005), suggested that garlic extracts can reduce *Alternaria* leaf spot on tomatoes.

2.3.5 Application of defense inducers

In most agricultural systems, fungicide resistance has been reported and in other cases, environmental concerns revolving around the usage of synthetic chemicals have been raised (Ons *et al.,* 2020). The aforementioned have exacerbated pressure for an alternative approach for disease management that is sustainable, economic, and environmentally sound as well as effective and efficient (Hartmann *et al.,* 2019). The inducible resistance system, known as systemic acquired resistance (SAR), has been identified as the most prominent environment-friendly technique for combating plant diseases, especially those caused by viruses, fungi, and bacteria. Besides being induced biologically, SAR can be stimulated by the application of chemicals that contains a salicylic acid in its synthetic analogs, such as salicylic acid (SA) and 2,6-dichloroisonicotinic acid (INA), potassium salts, and aminobutyric acid (BABA), were reported to induce SAR in plants (Hartmann *et al.,* 2019; Oostendorp *et al.*, 2001; Tripathi *et al.,* 2019). According to Lopez and Lucas (2002), benzothiadiazole derivative benzo (1, 2, 3) thiadiazole-7-carbothioic acid-S-methyl ester (*acibenzolar-*

S-methyl, ASM) is an effective SAR inducer. ASM does not have antimicrobial properties, however, it induces crop resistance to diseases by activating the SAR signal transduction pathway in several plant species. Induction of SAR by ASM was reported in many plants against a broad spectrum of fungal, viral, and bacterial pathogens (Anfoka, 2000; Brisset *et al.*, 2000; Cole, 1999; Godard *et al.*, 1999; Klessig *et al.*, 2018**;** Lopez and Lucas, 2002). The compound has been commercially released in some countries as a plant health promoter of annual crops under the names Bion or Actigard.

2.3.6 Application of nanoparticles and Nano-formulated micronutrients

Table 2.1 Antifungal potential of Nano-micronutrients and Nano-formulated micronutrients.

Nano-micronutrient	Disease	Causal pathogen	Reference
ZnO-nCuSi	Citrus canker	*Xanthomonas citri* subsp. *citri*	Young *et al.,* 2018
ZnO NPs	Grey and black mold	*Botrytis cinerea* and *Alternaria alternate*	Hassan *et al.,* 2019
ZnO NPs	Postharvest diseases	*Botrytis cinerea* and *Penicillium expansum*	Singh *et al.,* 2013
Zn NPs	Tomato root rot	*Fusarium oxysporum* f. sp. *radicis-lycopersici*	Kashyap *et al.,* 2015
ZnO NPs	Citrus canker	*Xanthomonas citri* subsp*. Citri*	Graham *et al.,* 2016
$CuSO_4$ NPs	Rust disease	*Uromyces viciae–fabae*	Singh *et al.,* 2013
Cu NPs		*Peronospora tabacina*	Wagner *et al.,* 2016
Cu-NPs	Vascular wilt	*Fusarium oxysporum* f. sp. *lycopersici*	Lopez-Lima *et al.,* 2021
Cu-NPs	Anthracnose and fruit rot	*Colletotrichum capsici*	Lliger *et al.,* 2021
Ag NPs		Tomato mosaic virus, Potato virus	Elbeshehy *et al.,* 2015
Nano-sized silica-silver	Powdery mildew	*Podosphaera xanthii*	Park *et al .,* 2006
Chitosan	Bunch rot	*Botrytis cinerea*	Kashyap *et al.,* 2015
Chitosan	*Fusarium* wilt	*Fusarium oxysporum*	Maluin *et al.,* 2019

The application of nanotechnology in the management of plant diseases is relatively new, however, several reports have already shown the positive impacts of this technology in the suppression of different plant pathogens (Table 2.1). According to Saravanan *et al.* (2018), nanoparticle (NP), refers to a particle with a size that ranges from 1 to 100 nanometres, it is an emerging field of research, with various applications in science and technology. Reports further show that nanoparticles can significantly suppress the development and activities of different fungal organisms, including plant pathogenic groups (Mujeebur and Tanveer, 2014). For example, the application of micronutrients $CuSO_4$ and $Na_2B_4O_7$ significantly reduced rust severity in peas under field conditions (Singh *et al.,* 2013). In other studies, zinc oxide nanoparticles were found to be highly effective against postharvest rot of fruits and vegetables caused by *Botrytis cinerea* and *Penicillium expansum* (Mujeebur and Tanveer, 2014). The suppressiveness of zinc nanoparticles has been reported under both laboratory and field conditions (Nandhini *et al.,* 2019; Malandrakis *et al.,* 2019). Silver is another nanoparticle with high suppressiveness against several fungal pathogens including *Rhizoctonia solani, Alternaria alternata*, *B. cinerea,* and *Macrophomina phaseolina* (Jayaseelan *et al.,* 2012).

The application of nanoparticles is an attractive tool to enhance plant protection against *Alternaria* leaf spot. Copper, silica, and silver are the candidates for this method, which increases the antioxidant enzymes in plant tissues (Derbalah *et al.,* 2019; Quiterio-Gutierrez *et al.,* 2019). Other work conducted by Zakharova *et al.* (2017), has demonstrated the impact of biosynthesized silver nanoparticles in reducing early blight and increasing plant growth as well as photosynthesis. Copper (Cu) is an essential micronutrient that is incorporated into many proteins and enzymes and it is also important in the health and nutrition of plants (Kasana *et al.,* 2017) and photosynthetic reactions (Ouda, 2014). According to Ponmurugan *et al.* (2016), Cu in a nanoparticle form has a strong antifungal activity. Moreover, Ouda (2014), reported that Cu NPs and Ag NPs assist in the inhibition of pathogenic fungi such as *A. alternata* and *Botrytis cinerea*.

Nanoparticles are directly involved in plant defense mechanisms as structural components and metabolic regulators, they act as the first line of defense. Moreover, plant nutrient status can influence the pathogen-host interaction by increasing the plant`s susceptibility to pests and pathogens (Brokbartold *et al.,* 2012; Walters and

Bingham, 2007). Datnoff *et al.* (2007), suggested that plant nutrient status directly affects plant health by stimulating enzymes that produce defense secondary metabolites that have antifungal properties such as phenols, and phytoalexins. This also indirectly affects plant health by altering root exudates, rhizosphere pH, and microbial activity.

Zinc nanoparticles and their mode of action against plant pathogenic fungi

Zinc has been widely used for the development of several commercially available agricultural pesticides and also in combination with copper (Prasad *et al.,* 2014; Rajasekaran *et al.,* 2016). It is directly involved in plant protection as a structural component and metabolic regulator. Nano-zinc materials have potential targeted antimicrobial properties and low to minimal phytotoxicity activities, making them well-qualified to be used for the management of plant pathogenic fungi (Kalia *et al.,* 2020). According to Marschner and Marschner (2012), crop nutritional status act as a primary line of defense against pathogen attacks and can determine the plant`s susceptibility to pathogens and pests. Moreover, He *et al.* (2011), reported that when nano-zinc is applied, it is adsorbed by the hyphal cell surface of the plant leading to morphological alterations in the cell wall and cell membrane.

These changes in the cell wall and cell membrane result in thickening of the cell wall, dislocation of cytoplasmic organelles, and detachment of the cell wall from cytoplasmic contents (Arciniegas-Grijalba *et al.,* 2017), which then improves the ability of the plant to withstand adverse conditions thus, playing an important role against pathogen attack (Datnoff and Heckman, 2014). In addition, nano-zinc influences plant-pathogen interactions by activating metalloenzymes, which play a critical role in the plant's response to pathogen attack or stress when Zn levels are low (Fones and Preston, 2012). Application of nano-zinc leads to a delay in spore germination and complete spore inhibition showing that Zn materials have sporistatic to sporicidal properties.

2.4. Work not done on the problem

The application of fungicides is the most effective method to control *Alternaria* leaf spot. A variety of synthetic fungicides are registered to control *Alternaria* leaf spot of tomato are suggested by (Abu-El Samen *et al.,* 2016; Song *et al.,* 2011). However, the recent occurrence of fungicide-resistant isolates of *A. alternata* and *A. solani* in tomato fields has warned of frequent uses of the synthetic fungicides (Dulta *et al.,* 2022). The

growing public admiration and the challenges encountered in the treatment of fungal infections have stimulated researchers to find alternative synthetic fungicides. Towards the direction of finding, suitable alternatives to synthetic fungicides plant defense inducers (ASM), Nano-micronutrients, and natural plant products (extracts) have been utilized in combating plant disease. For example, ASM has been used on various crops to control diseases such as downy mildew (*Plasmopara helianthi*) and *Orobanche cumana* attacking sunflower, fire blight of pear, *Sclerotinia* white mould of soybean, and brown spot and narrow brown leaf spot in rice.

Furthermore, nanoparticles or their composites have been well identified against phytopathogenic fungi belonging to diverse taxonomic groups such as *Fusarium oxysporum, Pythium ultimum. Aspergillus niger, Botrytis cinerea,* and many other fungal pathogens. Natural plant products are considered the best alternative to synthetic chemicals due to being less hazardous to the environment as well as human life. However, most studies have reported on the effectiveness of plant extracts under *in vitro* and *in vivo* conditions with fewer reports on their application under greenhouse or field conditions (Kagale, 2004). Despite the effort in finding the best suitable alternatives to date, there is limited or no documentation on the use of ASM defense inducer, nano-micronutrients, and plant extracts in the management of *Alternaria* leaf spot and early blight of tomato. Thus, the current study was initiated to test the antifungal activity of *M. burkeana,* combined nano zinc and copper micronutrient, and ASM defense inducer on *Alternaria* leaf spot management.

CHAPTER 3

MATERIALS AND METHODS

3.1. Achieving objective one:

3.2.1 Study site and experimental design

The experiment was conducted at the Plant Pathology Laboratory, the University of Limpopo, South Africa (23°53”12’S, 29°44”19’E). Both *M. burkeana* and combined nano zinc and copper micronutrient experiments were arranged in Completely Randomized Design (CRD) with six treatments each and replicated four times.

Table 3.1: List of treatments for laboratory experiments

	M. burkeana	Nano zinc and copper micronutrient
1.	Control = 0g 100 ml^{-1}	Control = 0 ml ℓ^{-1}
2.	MB_1 = 1g 100 ml^{-1}	NZC_1 = 0.09 ml ℓ^{-1}
3.	MB_2 = 2g 100 ml^{-1}	NZC_2 = 0.12 ml ℓ^{-1}
4.	MB_3 = 3g 100 ml^{-1}	NZC_3 = 0.18 ml ℓ^{-1}
5.	MB_4 = 4g 100 ml^{-1}	NZC_4 = 0.24 ml ℓ^{-1}
6.	MB_5 = 5g 100 ml^{-1}	NZC_5 = 0.30 ml ℓ^{-1}

MB: *Monsonia burkeana,* CNZC: Combined nano zinc and copper micronutrient

3.2.2 Collection of diseased Tomato plant materials and pathogen isolation

Diseased tomato leaves showing symptoms of *Alternaria* leaf spot were collected from the field in February 2020 at the Mountain view research and training farm (MVRTF). Infected leaves were placed in labelled brown paper bags for further use. The labelling included information on the area of collection, the date of collection, and the name of the cultivar. Collected tomato leaves were cut into small pieces using a sterile knife, and the cut pieces were surface sterilized with 1% sodium hypochlorite (jik) solution for 2 minutes and rinsed three times with sterile distilled water. Following rinsing, the cut pieces were placed in a petri dish with a Potato Dextrose Agar (PDA) amended with antibiotics to suppress bacterial contamination. Petri dishes were incubated at 25 ± 2 °C for 48 hours. Growing mycelium was picked with a flame sterilized needle and transferred to Petri plates containing PDA and incubated at 25 ± 2 °C for seven days. A light microscope (10×20 Boeco microscope) at 60x magnification was used to confirm the culture through morphological characteristics such as the number of septa, conidial length, and presence or absence of horizontal and transverse septa

(Alhussaen, 2012; Nikam *et al.,* 2015). The isolated pathogen was further confirmed using its morphological similarity and confirmed cultures were stored at 4°C and subculturing was done every 14 days to maintain viability.

3.2.3 *Monsonia burkeana* collection, processing and extract preparation

Whole plants of *M. burkeana* were collected in February 2020 at the University of Limpopo experimental farm, Limpopo Province, South Africa (23°53'10" S, 29°44'15" E). The leaves were gently washed with tap water, placed in a paper towel to remove excess water, and shade-dried for approximately 21 days. The leaves were ground into powder using a laboratory grinder (Model FZ-102). The powder was added to 700 ml of methanol and placed on a rotary shaker for 24 h and evaporated on a rotary evaporator under reduced pressure at 64°C. The powder was stored at room temperature until further use. The extract was prepared in five concentrations by adding 1, 2, 3, 4, and 5g of the extract powder in 100ml sterilized distilled water then autoclaved at 121 °C. Following the concentration used by Hlokwe *et al.* (2018), of 2, 4. 6, 8, and 10 g/ml. Extract treatments were prepared by adding different concentrations in separate 100 ml bottles of sterilized PDA medium and transferred to Petri dishes. The extract amended PDA was then allowed to solidify overnight.

3.2.4 Combined nano zinc and copper micronutrient treatments preparation

Aqua nano micronutrient (Aqua salveo CC, 58 Norite Rd, Helderkruin, Roodepoort, 1724) was used as the source of nano zinc and copper micronutrient. The stock solution was prepared by adding 0.9ml of Aqua salveo micronutrient in 1 ℓ sterile distilled water. The stock solution was used to prepared five concentration levels (0.09 (standard rate), 0.12, 0.18, 0.24 and 0.30 ml ℓ^{-1}). The concentrations were amended with PDA and the media was transferred into Petri dishes, allowed to solidify overnight.

3.2.5 *A. solani* mycelial growth suppression with *Monsonia burkeana* and combined nano zinc and copper micronutrient

The antifungal activity of *M. burkeana* and combine nano zinc and copper micronutrient were tested at different concentrations as described above. Extract amended Malt Extract Agar (MEA) was poured into 90 cm Petri plates and left to solidify overnight. A seven-day-old pure culture of *A. solani* was used to inoculate both *M. burkeana* and combined nano zinc and copper amended Petri dishes and inoculated Petri dishes were incubated at 25 ± 2°C for fourteen days. Non-amended

PDA medium plates served as control treatments. Both *M. burkeana* and combined nano zinc and copper micronutrient were arranged in Complete Randomized Design (CRD) with six treatments replicated four times. The experiment was repeated twice.

3.2.6 Data collection and analysis

Mycelial growth: The diameter of *A. solani* mycelial growth inhibition was collected at four, seven, and fourteen days after inoculation (DAI) by measuring the mycelium growth (mm) using a transparent ruler as modified by (Sallam and Kamal, 2012). Mycelium growth inhibition was calculated using the formula of (Tegegne *et al.,* 2008).

Inhibition (I) = $\frac{dc-dt}{dc}$, where, dc= Average diameter of mycelium growth of the control (PDA only) and dt= Average diameter of mycelium growth in amended (extract and Nano-zinc and copper micronutrient plates). Finally, the data were subjected to analysis of variance (ANOVA) using Statistix 10.0 software. Mean separation was achieved through Tukey's HSD at P ≤ 0.05.

3.2 Achieving objective two and three

The effects of *M. burkeana*, combined nano zinc and copper micronutrient, and ASM defense inducer on *Alternaria* leaf spot development and severity under greenhouse and micro-plot conditions and the effects of *M. burkeana*, ASM defense inducer, and combined nano zinc and copper micronutrient on crop yield.

3.2.1 Study site and experimental design

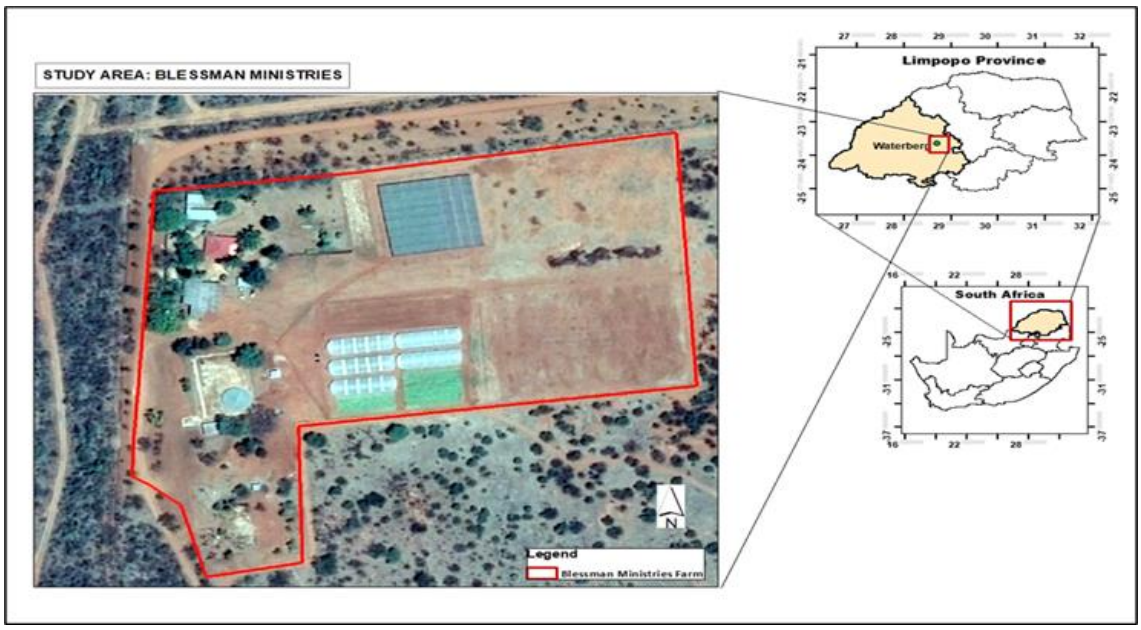


Figure 3.1: Mountain-view research and training farm

The study was conducted under greenhouse and micro-plot at Mountain View Research and Training Farm, (24º 11' 2" S, 29º 0' 46" E), South Africa with an ambient average day and night temperature of 28˚C and 18°C respectively. The experiment was arranged in a Randomized Completely Block Design (RCBD) with eight treatments replicated four times and was conducted twice. First season planting was carried in March to July 2021 and the average temperatures for the season were 14°C to 28°C and the second season planting was August to December 2021 with average temperatures of 18°C to 34°C.

Table 3.2: List of treatments for greenhouse and micro-plot experiments

	Treatments	Description
1.	Healthy (negative) control	Non-inoculated and non-treated
2.	Unhealthy (positive) control	Inoculated but not treated
3.	ASM_1	0.0750 g ℓ^{-1}
4.	ASM_2	0.0563 g ℓ^{-1}
5.	ASM_3	0.0375 g ℓ^{-1}
6.	ASM_4	0.0190 g ℓ^{-1}
7.	CNZC	0.30 ml g ℓ^{-1}
8.	MB	5 g 100 ml^{-1}

ASM= Acibenzolar-S-Methyl, CNZC= Combined nano zinc and copper, MB= *Monsonia burkeana*

3.2.2. Preparation of pathogen inoculum

A 7-day-old *A. solani* pure culture plate (PDA) was flooded with 10 ml of sterile distilled water. Following gentle scrapping of the mycelium with a sterilized needle to dislodge the spores. The concentration of the spore suspension (10^6 ml^{-1}) was adjusted using a haemocytometer (Hosseini *et al.,* 2020). The conidial suspension (10^6 ml^{-1}) was added to a sterilized hand sprayer and 45-day-old tomato plants were inoculated with the *A. solani* pathogen. To enhance natural infection and to obtain uniform disease pressure, leaves were injured before inoculation, by rubbing the thumb and forefingers which were sterilized with 70% ethanol. Ten healthy leaves from each plant were selected, tagged, and inoculated with *A. solani* conidial suspension. Control plants were sprayed with sterilized distilled water. After inoculation, plants were covered with polyethylene bags for 48 hours to increase humidity and accelerate the infection.

3.2.3. Acibenzolar-S-Methyl (ASM) treatments preparations

Five liters (5 ℓ) beaker was filled with tap water, and ASM granules (purchased from Syngenta (Pty) Ltd, Halfway house, Johannesburg, RSA) were weighed out according to the assigned five treatments (0, 25, 50, 75, and 100%) and dissolved in 1 ℓ of tap water. For each treatment, the ASM solution was transferred into a clean 1 ℓ polystyrene spray bottle and then labelled. According to the ASM application specifications, the recommended dosage on tomato plants is 0.075 g $ℓ^{-1}$. The treatments in this study was derived from this recommended dosage resulting in: 0.075 g $ℓ^{-1}$ (100%, full strength), 0.0563 g $ℓ^{-1}$ (75%, three-quarter strength), 0.0375 g $ℓ^{-1}$ (50%, half strength) and 0.019 g $ℓ^{-1}$ (25%, quarter strength).

3.2.4. Inoculation and treatment application

Four weeks old healthy tomato seedlings (cultivar Rodade) were transplanted into 25 cm diameter pods filled with steam pasteurized sand and Hygromix at a ratio of 3:1. Exactly forty-five days after planting inoculation was done on actively growing plants by spraying the leaves and branches of every plant with 100 ml of the inoculum using a hand sprayer. Inoculated plants were then treated with: 1) *M. burkeana* (5g 100 ml^{-1}) and 2) combined nano zinc and copper micronutrient (0.30 ml $ℓ^{-1}$). These concentrations were the most effective in pathogen growth suppression in chapter 3. ASM was applied as previously described. Check treatments were maintained to serve as a positive and negative control, where positive control was inoculated but not treated and negative control was non-inoculated and non-treated. Treatments were

applied 7-days prior to inoculation and at an interval of 7 days post-inoculation for three weeks through foliar sprays conducted using a hand sprayer.

3.2.6 Data collection and analysis

Alternaria leaf spot severity was evaluated for 21 days after inoculation using a visual scale rating of 0-5 (Table 4.2) as modified by Mphahlele, (2017)**.** Final disease severity was calculated using the formula:

Disease severity (%) = [(Number of leaves scored for each rating x the rating value) / Total plants scored] x 100.

Table 3.3 Disease severity rating scale

Scale	Description
0	No symptoms on the leaf area
1	≤ 2 mm-3 mm on the main lesion with one to few spots on the leaf area
2	3 mm- 5 mm on the main lesion with few spots on the leaf area
3	5 mm-7 mm on the main lesion with few to many spots on the leaf area
4	5 mm-7 mm on the main lesion with few to many spots on the leaf area
5	≥ 10 mm on the main lesion on leaf area being fully necrotic

Treatment effects on yield, plant height (cm), stem diameter (mm), chlorophyll content (µmol m^{-2}), and normalized differential vegetation index (NDVI) were measured at 43, 64, and 85 days after planting (DAP). Leaf chlorophyll content was measured on fully expanded leaves using SPAD 502 Plus Chlorophyll Meter. The stem diameter was measured using a Vernier caliper and NDVI for leaf greenness of vegetation photosynthetic activity was measured using a Green Seeker handheld crop sensor (Trimble, USA). After harvesting, the yield was expressed as the number of fruits per plant. The data were subjected to Analysis of Variance (ANOVA) using Statistix 10.0 software. Mean separation was achieved through Tukey's HSD at $P \leq 0.05$.

CHAPTER 4

RESULTS

4.1.The efficacy of *M. burkeana* and combined nano zinc and copper micronutrienton pathogen growth (*Alternaria solani*) under laboratory conditions.

4.1.1 Antifungal activity assay

Combined nano-zinc and copper micronutrient and *Monsonia burkeana* were assessed for their inhibitory effect on *A. solani* under laboratory conditions (Figures 4.1 and 4.2). All tested concentration of combined nano-zinc and copper and *M. burkeana* significantly ($P \leq 0.05$) inhibited *A. solani* growth at 4 and 7 DAI compared to control, however, there was no significant difference at 14 DAI. Notably, the inhibition was concentration specific with an increase in the concentration of combined nano zinc and copper and *M. burkeana* resulting in an increased inhibition percentage. For instance, combined nano zinc and copper inhibited *A. solani* growth at the highest concentration of 0.30 ml ℓ^{-1} by 41.3%, and 26.8% at 4 and 7 DIA respectively. Similarly, *M. burkeana* effectively inhibited the mycelium growth of *A. solani* at the highest concentration of 5 g 100 ml^{-1} by 43.4%, and 38.0% at 4 and 7 DIA respectively. Both combined nano zinc and *M. burkeana* were effective in suppressing mycelium growth of *A. solani* particularly at 4 and 7 DAI relative to the control, however, the pathogen (*A. solani*) reached peak growth at 14 DAI and there was no significant difference amongst the treatments at 14 DAI. This could indicate that for effective management of *Alternaria* leaf spot treatments must be applied in a seven-day interval.

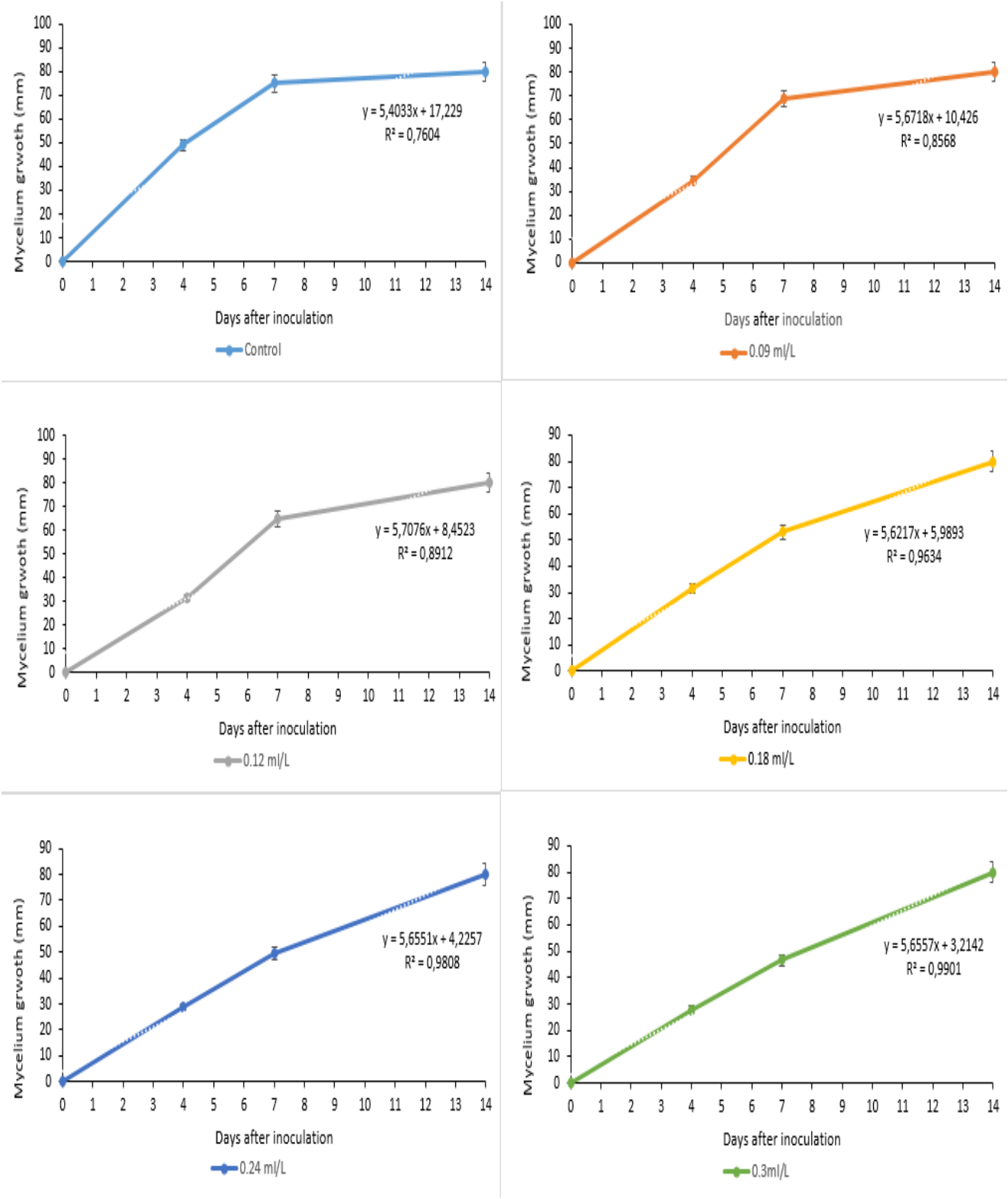


Figure 4.1: Effects of nano-zinc and copper micronutrient concentrations on mycelial growth of A. solani after inoculation.

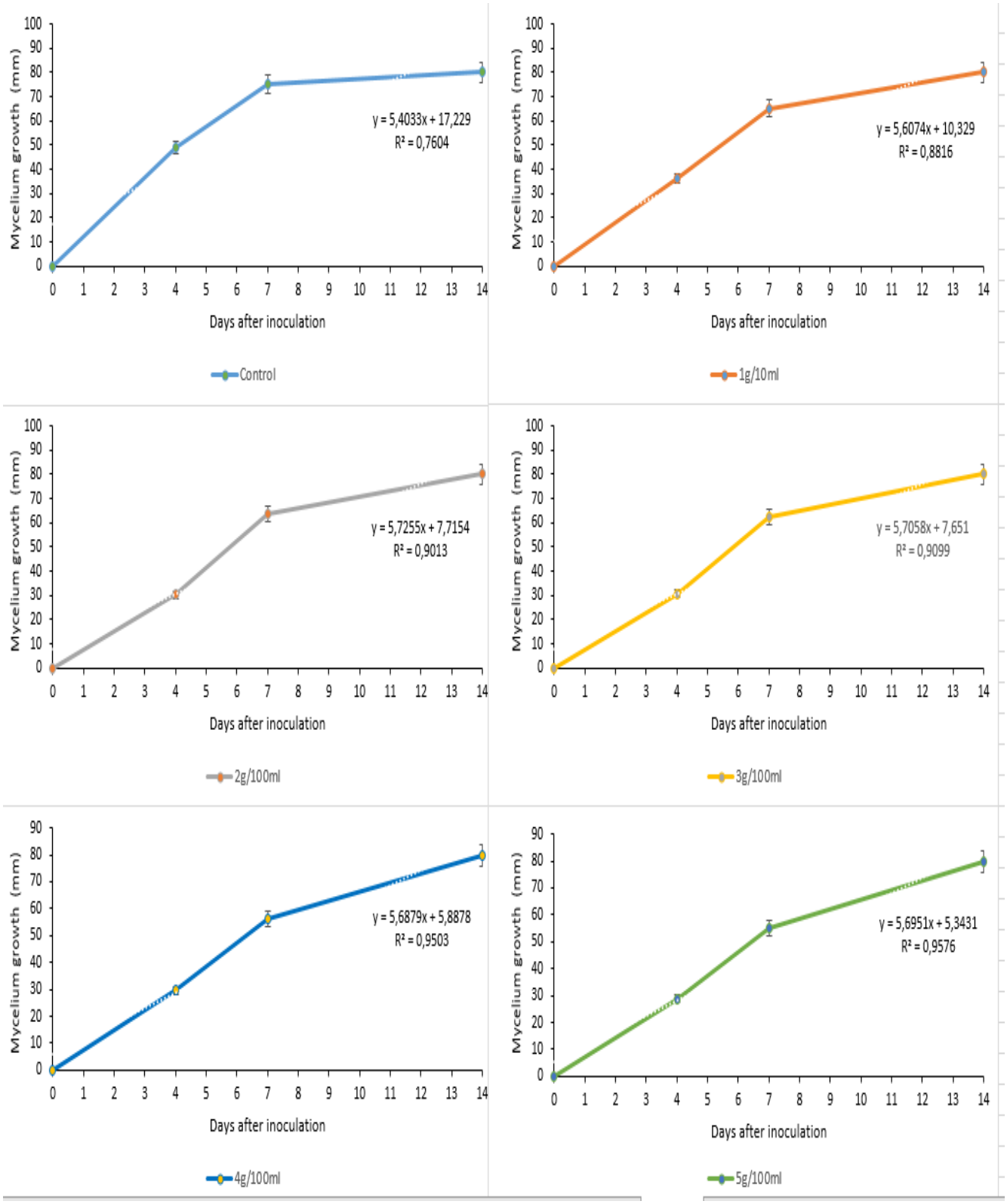


Figure 4.2: Antifungal activities of Monsonia burkeana against A. solani in vitro after inoculation.

4.2 Evaluation of combined nano zinc and copper micronutrient, Acibenzolar-s-methyl, and *M. burkearna* under greenhouse and micro-plot.

4.2.1 Efficacy of combined nano zinc and copper micronutrient, Acibenzolar-s-methyl, and *M. burkeana* on *Alternaria* leaf spot development and severity.

Combined nano zinc and copper micronutrient, ASM, and *M. burkeana* significantly ($P \leq 0.05$) reduced *Alternaria* leaf spot severity under both greenhouse and micro-plot conditions (Figures 4.3 and 4.4). Combined nano zinc and copper and *M. burkeana* concentrations at 0.3 ml ℓ^{-1} and 5 g 100 ml^{-1} respectively displayed a high level of suppression of *Alternaria* leaf spot, resulting in 28% and 30% disease severity compared to controls. On the other hand, ASM_3 [75% (0.056 g ℓ^{-1})] was the most effective when compared to the other levels of ASM. Nonetheless, there was a significant difference ($P \leq 0.05$) between all treatments compared to the positive control and negative control. The highest disease severity of 70% was observed in the positive control (inoculated and non-treated) treatment whilst the minimum, 10% was recorded in the negative control (non-inoculated and non-treated).

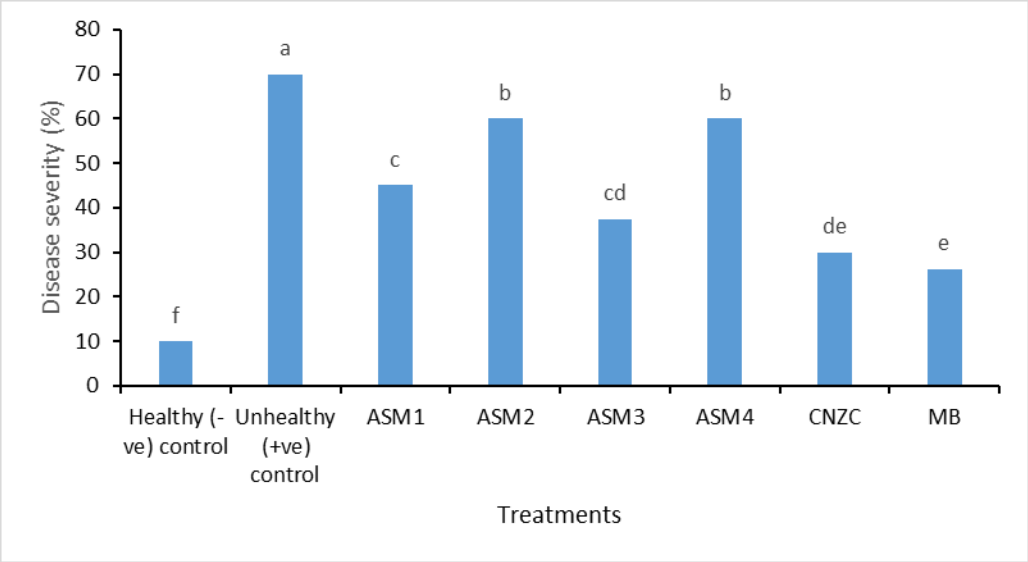


Figure 4.3: Alternaria leaf spot severity under greenhouse condition after 21 days. Bars followed by the same letter do not differ significantly ($P \leq 0.05$) according to Duncan's multiple range test. ASM: Acibenzolar-S-Methyl, CNZC: Combined nano zinc and copper, MB: Monsonia burkeana.

4.2.2 Micro-plot experiments

A similar trend was observed under micro-plot conditions, where all tested treatments varied significantly when compared to the positive and negative control. Notably, there was a significant difference ($P \leq 0.05$) among treatments. All the tested treatments reduced *Alternaria* leaf spot development and severity relative to control. Combined nano zinc and copper and *M. burkeana* were the most effective treatments resulting in minimal disease severity of 10% and 15% respectively. Both positive control and ASM_1 [25% (0.018 gL^{-1})], recorded the highest disease severity of 30% and there was no incidence of disease in the negative control (Figure 4.4).

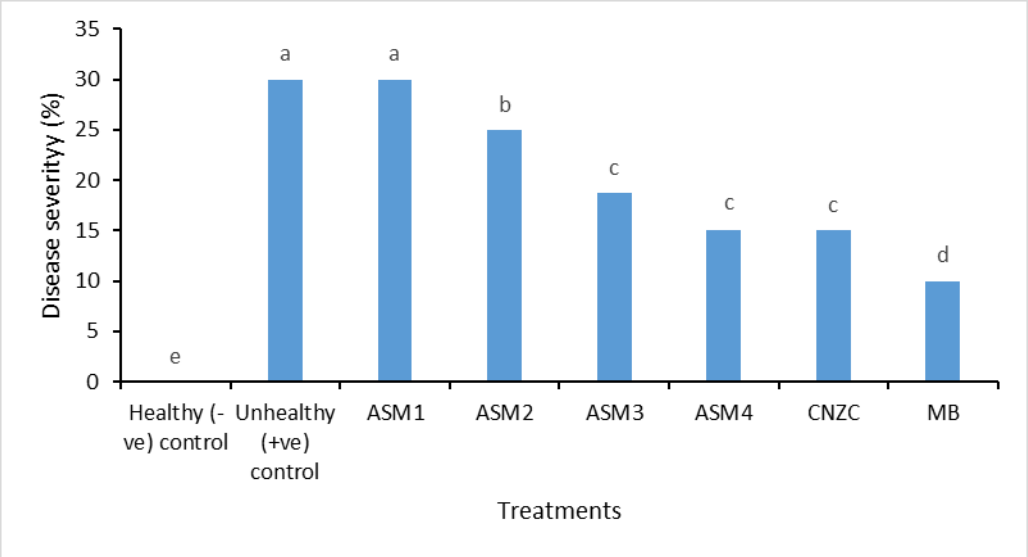


Figure 4.4: Alternaria leaf spot severity under micro-plot condition after 21 days. Bars followed by the same letter do not differ significantly ($P \leq 0.05$) according to Tukey`s HSD. ASM: Acibenzolar-S-Methyl, CNZC: Combined nano zinc and copper, MB: Monsonia burkeana.

4.2.3 The effect of *M. burkeana*, Nano-zinc micronutrient, and ASM defense inducer on yield of tomato.

Table 4.1: Assessment of crop physiological responses and yield of tomato.

	Plant height (cm)	Stem diameter (mm)	NDVI			Chlorophyll content (µmol m^{-2})			Yield (number of fruits $plant^{-1}$)
			DAP						
			44	65	90	44	65	90	
Positive control	58.00^{b}	8.25	0.57	0.57	0.44^{b}	76.65	89.85^{ab}	87.55^{a}	12.25
Negative control	49.00^{b}	9.50	0.65	0.67	0.67^{a}	73.63	84.43^{ab}	64.03^{ab}	12.50
MB	59.50^{ab}	9.00	0.66	0.66	0.63^{a}	63.20	86.23^{ab}	50.43^{b}	11.25
Nano-Zinc	55,00^{b}	10.50	0.65	0.69	0.63^{a}	65.86	90.60^{a}	55.05^{ab}	12.50
ASM_1	49.00^{b}	8.50	0.63	0.61	0.58^{ab}	51.54	63.00^{ab}	88.60^{a}	13.25
ASM_2	37.75^{c}	9.00	0.50	0.56	0.57^{ab}	71.45	70.15^{ab}	73.93^{ab}	10.75
ASM_3	54.00^{b}	9.25	0.57	0.61	0.51^{ab}	59.00	51.60^{a}	76.20^{ab}	6.50
ASM_4	73.00^{a}	9.25	0.60	0.62	0.56^{ab}	47.93	89.43^{ab}	88.65^{ab}	11.75
	**	Ns	ns	Ns	**	ns	**	**	ns

**

Numbers within the same column followed by the same letter are not significantly different according to the LSD All-Pairwise Comparisons Test (P < 0.05).

MB: Monsonia burkeana, ASM: Acibenzolar-S-Methyl: NDVI: Normalized differential vegetation index, DAP: Days after planting

Treatment effect on physiological responses and yield plant high, stem diameter, NDVI, SPAD, and yield were measured (Table 4.3). Plant height was significantly different across all treatments, while stem diameter was not significantly different from each other. NDVI was not significantly different at 44 and 65 DAP while significantly different at 90 DAP. A similar, trend was observed in chlorophyll content which was not significantly different from each other at 44 and 65 DAP but significantly different from each other at 90 DAP. The yield was not significantly different across all the treatments tested

CHAPTER 5
DISCUSSION

5.1 Antifungal activity assay

Exclusive dependency on synthetic fungicides for the management of plant disease has caused residue and environmental disturbances (Guo *et al.,* 2021). As a result, efforts are being directed toward utilizing botanicals and plant nutrients as a tool for disease management system integration since they do not produce bio-accumulation in consumables or contamination of the environment. In modern times, disease management through botanicals and plant nutrients is becoming a significant component (Campos *et al.,* 2019; Pavela, 2016). The results obtained from the current study demonstrated that combiend nano zinc and copper micronutrient and *M. burkeana* (plant extract) significantly inhibited mycelium growth of the test pathogen (*A. solani)* under laboratory conditions (Figures 4.1 and 4.2).

The development of resistant variants of microorganisms to antimicrobial agents increased pressure to find novel ways to inhibit such resistant variants (Abdel-Shafi *et al.,* 2019; Enan *et al.,* 2018). According to El-Gazzar and Ishmail, (2020), nanoscience provides the potential for confirming the efficiency of metal nanoparticles. Nanoparticles (NPs) have been experimented with as antifungal agents against different pathogenic fungi (kashyap *et al.,* 2015). Zn, Cu nanoparticles, and chitosan exhibit a wide spectrum of antimicrobial activity against different bacterial (and fungal species (Ouda, 2014). The results of the present study showed that different concentrations of combined nano-zinc and copper micronutrient have antifungal activities and have significantly inhibited mycelium growth of *A. solani* in the laboratory at 4 and 7 DAI (Figure 4.1).

These results are in agreement with those reported by Malandrakis *et al.* (2019), where ZnO NPs significantly inhibited the mycelium growth of *Alternaria alternata.* He *et al.* (2011), reported that ZnPs significantly inhibited hyphal growth of *Fusarium oxysporum* and *Aspergillus niger* at a concentration of 3 mML^{-1}. Sharma *et al.* (2021), observed that zinc and copper showed the most significant antifungal activity at all tested concentrations and zinc sulphate displayed a maximum inhibition of mycelium growth (48.21%). Fahad *et al.* (2017), evaluated the antifungal properties of characterized zinc and copper nanoparticles and chitosan against *A. alternata*, *R.*

solani, and *B. cinerea* on tomato, cotton, and strawberry respectively, the results showed that the Zn NPs had highest antifungal activities against *R. solani* compared the control.

Rathi *et al.* (2015), evaluated the role of soil application of micronutrients in defense against white rust and *Alternaria* blight in Indian mustard and found minimum disease severity of both white rust (31.3%) and *Alternaria* blight (26.3%) when $ZnSO_4$ is applied as basal dose. The Cu-NPs showed inhibitory activity against *E. coli* and *S. aureus* with maximum inhibition against *E. coli* at the highest concentration of 150 mg/mL (Bakshi and Kumar, 2022). Furthermore, Cu-NPs synthesized using fruit juice of *Citrus medica* (citron) its antimicrobial activity was tested against five species of bacteria namely *Escherichia coli*, *Propionibacterium acne*, *Klebsiella pneumoniae*, *Salmonella typhi*, *and Pseudomonas aeruginosa* and three plant pathogenic fungi *Fusarium oxysporum*, *F. graminearum*, and *F. culmorum* (Shende *et al.,* 2015). In bacteria, *E. coli* was highly susceptible to the antimicrobial activity of Cu-NPs while in fungi *F. culmorum* was found to be most sensitive. Kumar *et al.* (2015), observed that K_2SO_4 showed maximum inhibition of mycelial growth (64.28%) in *Alternaria brassicae* followed by $ZnSO_4$ (63.88%) in their studies of the effect of eco-friendly chemicals on *Alternaria* blight disease of mustard. Most micronutrients influence disease development and the severity of most crops hence, diseases can be greatly decreased by proper nutrient management.

There are abundant reports of the control of phytopathogenic fungi and plant diseases by using plant extracts *in vitro* and *in vivo*. Mamphiswana *et al.* (2011), reported that *M. burkeana* has shown to have nematicidal and antimicrobial effects against plant-pathogenic bacteria and nematodes. The results obtained from the current study demonstrated that different concentrations of *M. burkeana* have antifungal activities against *A. solani* and significantly reduced the mycelium growth of the pathogen (Figure 4.2). However, the efficacy of the treatments was concentration-dependent with an increase in concentration correlating to mycelium growth inhibition.

Similar to the results reported by Kena (2016), who studied the effect of *M. burkeana* and *E. ingens* against *P. digitatum* and citrus green mold development. The results showed that both plant extracts possess antifungal activities against *P. digitatum* and were able to suppress disease development and severity of citrus green mold and the

efficacy was concentration-specific. Similarly, Hlokwe *et al.* (2020), reported that methanolic extract of *M. burkeana* significantly suppressed pathogen growth at different concentrations. *M. burkeana* significantly reduced *Rhizoctonia solani* growth at 8 g/mL (71%) relative to control. The results could have been influenced by the presence of secondary metabolites, such as phenols, phenolic acids, quinones, flavones, flavonoids, and flavonols which are toxic to microorganisms and have antifungal properties. Concentration dependency appears to be a common phenomenon in pathogens as several plant extracts have been shown to suppress pathogen growth at different concentrations. According to Shazia *et al.* (2015), pathogen growth is significantly suppressed at high extract concentrations. This was reported where extracts of *A. absinthium, T. officinale, and M. sylvestris* caused significant inhibition in the mycelial growth of *A. alternata*. *A. absinthium* at the highest concentration, *A. alternata* and caused the highest inhibition in the mycelial growth (79.75%) followed by *T. officinale* (76.18%), *M. sylvestris* (72.61%), *R. obtusifolius* (71.43%) and *P. lanceolata* (63.11%).

Chohan *et al.* (2019), investigated 15 medicinal plant extracts as fresh and dry parts against tomato early blight disease (*Alternaria solani*) in *vitro* and in *vivo*. Their results showed that the fresh aqueous extracts of *Azadirachta indica*, *Allium sativum,* and *Ocimum sanctum* had a high antifungal activity when applied under both laboratory and field experiments. *Allium sativum* treatments showed the highest reduction (77.42%) in disease severity with an increase in yield parameters. Ravikumar and Garampalli (2013), studied the efficacy of 39 aqueous extracts, 13 of the extracts significantly reduced the mycelium growth of *A. solani.* Amongst the 13 *Citrus aurantifolia* (27.3%), *Azadirachta indica* (23.7%), *Polyalthia longifolia* (23.3%), *Muntingia calabura* (20.09%), and *Oxalis latifolia (*20.09%) were found to be highly effective in reducing disease severity and development of tomato early blight. Numerous natural plant extracts are found efficient in fungal disease management, particularly those that are caused by *Alternaria* spp without imposing risks on the environment. Therefore, *M. burkeana* can be used as an alternative to conventional practices of disease management.

5.2 Efficacy of combined nano zinc and copper micronutrient, Acibenzolar-s-methyl, and *M. burkeana* on *Alternaria* leaf spot development and severity.

The effectiveness of *M. burkeana,* combined nano zinc and copper, and ASM defense inducer was studied under greenhouse and micro-plot conditions. *Monsonia burkearna,* combined nano zinc and copper micronutrient, and ASM defense inducer separately reduced the incidence of *Alternaria* leaf spot under both conditions. The use of plant extracts in plant disease management has been widely studied since they tend to have minimal toxicity, minimal environmental effects, and wide public acceptance (El-Mougy *et al.,* 2004). Plant extracts and their secondary metabolites have been used in different areas of research like drug carriers, antioxidants, and for the synthesis of metal and non-metal nanoparticles (Ingale and Chaudhari, 2013; Momeni *et al.,* 2016; Sujitha and Kannan, 2013). To develop environment-friendly alternatives to synthetic fungicides for the control of fungal plant diseases, the interest in plant extracts has increased. Natural plant extracts have antifungal properties against different fungi by their antimycotic behavior on the mycelial growth of the fungi in media and disease control in plants (Akter *et al.,* 2019; Lu *et al.,* 2019).

In this investigation, *M. burkeana* plant extract was evaluated under greenhouse and micro-plot to determine its efficacy towards *Alternaria* leaf spot incidence. From the results, *M. burkeana* significantly reduced the incidence of *Alternaria* leaf spot and severity under greenhouse and micro-plot conditions. Similar results have been observed by Hlokwe, (2018), who reported that *M. burkeana* effectively significantly reduced the incidence of damping-off caused by *F. oxysporum* f. sp*. lycopersici* and *R. solani* under in greenhouse and, therefore, have the potential for use in the management of the fungal pathogens as bio-fungicides. Sharma *et al.* (2021), also studied the efficacy of natural plant extracts *in vivo* and reported that *Allium sativum* clove extract (garlic) showed the highest reduction followed by *Azadirachta indica* leaf extract against *A. alternata* causing early blight of tomato. *Helichrysum stoechas* extract had antifungal activity against plant pathogenic fungus under greenhouse and subsequently reduced disease severity (Bigović *et al.,* 2017).

Plant disease management through nanoparticles could be useful in protecting plants against pathogens and provide a lasting plant disease management alternative (Sathiyabama, 2019). The current study examined the effect of combined nano zinc and copper micronutrient on *Alternaria* leaf spot development and severity under

greenhouse and micro-plot. Results of the present study demonstrated that Nano-zinc micronutrient significantly reduced the incidence of *Alternaria* leaf spot under both greenhouse and micro-plot conditions. These findings are the same as those obtained by Sharma *et al.* (2021), who evaluated the effect of plant nutrients on *Alternaria alternata* and reported that copper sulphate was found to be most effective in reducing disease severity, followed by zinc sulphate compared to untreated control. Rathi *et al.* (2015), reported that $ZnSO_4$ applied as a basal dose showed a minimum disease severity against white rust and *Alternaria* blight in Indian mustard under greenhouse.

Ocsoy *et al.* (2013), reported that Ag NP effectively decrease *X. perforans* cell viability in culture and significantly reduced the severity of bacterial spot disease in a greenhouse experiment. Sharma *et al.* (2021), demonstrated that foliar applications of nutrients were proved effective in reducing disease under greenhouse and minimum disease severity (35.64%) was recorded by foliar application of copper sulphate (0.5%) with 46.94% decreased severity over control followed by zinc sulphate (37.78%) and ferrous sulphate (40.45%).

ASM has been reported to activate resistance in many crops against a broad spectrum of diseases which includes fungi, bacteria, and viruses through the release of systemic acquired resistance by which ASM confers resistance towards pathogen attacks (Burketova *et al.,* 2015). From the results obtained in this study, ASM significantly reduced *Alternaria* leaf spot development and severity under greenhouse and micro-plot conditions. Under greenhouse, all the ASM levels resulted in disease severity of less than 60%. The current results conquer with the findings of Havis *et al.* (2014), who reported that ASM induces SAR against fungal and bacterial pathogens. Similar results were reported by Mandal *et al.* (2008), that ASM reduces the lesions caused by Tomato spotted wilt virus (TWSV) in tobacco plants. Furthermore, Pute, (2016), reported that significant differences were observed among the treatments. Under micro-plot ASM_3 (75% (0.056 g ℓ^{-1}) and ASM_4 (100% (0.075 g ℓ^{-1}) were the most effective resulting in severity less than 20%. The results corroborate previous studies which demonstrated that 75 and 100% provide the most effective control (Pute, 2016; Madhusudhan *et al.*, 2008). According to Katiyar *et al.* (2015), chemical defense inducers affect various physiological responses such as plant immunity and defense mechanisms involving different enzymes such as polyphenol oxidase, tyrosine ammonia lyase, and antioxidant enzymes. Although these factors were not tested in

our study, the observed changes in plant growth parameters support this phenomenon. Recently, ASM is being used in agricultural operations as an alternative to fungicides, as a resistant catalyst, and as a growth promoter (Sharma, 2013). The effect of low concentrations of ASM in *Alternaria* leaf spot control provides a suitable alternative that can be used in integration with other disease management strategies to effectively manage the causal pathogen.

CHAPTER 6

SUMMARY, CONCLUSION, AND RECOMMENDATIONS

Tomato is one of the most widely grown and consumed vegetables worldwide. The crop is affected by a variety of diseases, among others *Alternaria* leaf spot caused by *Alternaria solani. Alternaria* leaf spot of tomato is an important and widespread disease of the cultivated tomato. The disease is the most devastating and economic foliar disease of tomato which cause a reduction in quantity and quality. High temperature and humidity (crowded plantations, high rainfall, and extended periods of leaf wetness from dew) favor disease development, and plants are more susceptible to infection during the flowering and fruiting periods (Deepti and Nidhi, 2015). *Alternaria* leaf spot is effectively controlled by synthetic fungicides. Recent efforts, on the other hand, have focused on developing environmentally safe, long-lasting effective biocontrol methods for the management of plant diseases. Kagale *et al.* (2004), reported that natural plant products can be used as a new source of agrochemical for plant disease management. Various natural products have antifungal properties which control plant diseases and the plant extracts have great potential as environmentally safe alternatives and effective components of integrated pest management programs (Nashwa and Sallam 2011; Raza *et al.,* 2016).

The findings of this study suggest that *M. burkeana* and combined nano zinc and copper micronutrient have antifungal properties which significantly inhibited mycelium growth of *Alternaria solani* under laboratory and significantly reduced *Alternaria* leaf spot severity under both greenhouse and micro-plot conditions. Furthermore, the results showed that the ASM defense inducer effectively reduced the incidence of *Alternaria* leaf spot under greenhouse and micro-plot conditions. In addition, *M. burkeana,* ASM defense inducer, and combined nano zinc and copper micronutrient have the potential to be used as alternatives to synthetic fungicides in the control of *Alternaria* leaf spot. Moreover, this suggests that *M. burkeana*, ASM defense inducer, and combined nano zinc and copper micronutrient can be used successfully as alternatives to synthetic fungicides that are more environmentally safe.

The present findings present an opportunity for *M. burkeana*, ASM defense inducer, and combined nano zinc and copper micronutrient to be used as a tool for integrated pest management (IPM), thus providing a wide scope for plant disease management that is effective, efficient and environmental sound. Moreover, these could limit the sole reliance to synthetic fungicides since they provide a similar level of efficacy and will reduce the incidence of pathogen resistance development to synthetic fungicides. In addition, *M. burkeana*, ASM defense inducer, and combined nano zinc and copper micronutrient are economically viable and providing a good advantage for smallholder farmers with limited production inputs.

It is recommended that more studies must be conducted to establish concentrations of extract and combined nano zinc and copper micronutrient that could provide high pathogen growth inhibition and prolong periods of disease suppression. More studies must be conducted on the biochemical changes of the plant after the application of the plant extract and combined nano zinc and copper micronutrient to investigate the potential chemical compounds which are responsible for pathogen growth inhibition which will assist in the development of alternative control of *Alternaria* leaf spot and possibly other plant diseases.

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APPENDICIES

Appendix 4.1.1: Analysis of variance for mycelial growth of *A. solani* at different concentrations for *Monsonia burkeana* at 4 DAI

Source	DF	SS	MS	F	P
REP	5	1209,83	241,98	32,5	0,00
Error	18	134,00	7,44		
Total	23	1343,83			

Appendix 4.1.2: Analysis of variance for mycelial growth of *A. solani* at different concentrations for *Monsonia burkeana* at 7 DAI

Source	DF	SS	MS	F	P
REP	5	1037,71	207,542	17,52	0
Error	18	213,25	11,847		
Total	23	1250,96			

Appendix 4.2.1: Analysis of variance for mycelial growth of *A. solani* at different concentration of combined nano zinc and copper micronutrient at 4 DAI

Source	DF	SS	MS	F	P
REP	5	1216,83	243,37	35,76	0,00
Error	18	122,50	6,81		
Total	23	1339,33			

Appendix 4.2.2: Analysis of variance for mycelial growth of *A. solani* at different concentration of combined nano zinc and copper micronutrient at 7 DAI

Source	DF	SS	MS	F	P
REP	5	2687,33	537,47	84,86	0,00
Error	18	114,00	6,333		
Total	23	2801,33			

Appendix 4.3.1: Analysis of variance for disease severity under greenhouse

Source	DF	SS	MS	F	P
REP	3	114,80	38,28		
TRT	7	11505,50	1643,64	59,62	0,00
Error	21	578,90	27,57		
Total	31	12199,20			

Appendix 4.3.2: Analysis of variance for disease severity under micro-plot

Source	DF	SS	MS	F	P
REP	3	21,09	7,03		
TRT	7	2974,22	424,89	60,43	0,00
Error	21	147,66	7,03		
Total	31	3142,97			

Appendix 4.4: Analysis of variance for yield parameters

Source	DF	SS	MS	F	P
Plant height					
REP	3	331,09	110,365		
TRT	7	2883,97	411,996	4,50	0,0034
Error	21	1922,66	91,555		
Total	31	5137,72			
SPAD					
REP	3	2868,1	956,037		
TRT	7	2996,8	428,112	0,89	0,5323
Error	21	10114,1	481,625		
Total	31	15979			
SPAD					
REP	3	4127,1	1375,69		
TRT	7	6546,5	935,22	1,35	0,2767
Error	21	14541,4	692,45		

Total	31	25215			
SPAD					
REP	3	9225,6	3075,21		
TRT	7	6561	937,29	1,57	0,1992
Error	21	12541,9	597,23		
Total	31	28328,5			
NDVI					
REP	3	914,38	304,795		
TRT	7	2147,69	306,813	1,00	0,4576
Error	21	6433,24	306,345		
Total	31	9495,31			
NDVI					
REP	3	0,01563	0,00521		
TRT	7	0,05615	0,00802	0,49	0,8314
Error	21	0,34403	0,01638		
Total	31	0,4158			
NDVI					
REP	3	0,12116	0,04039		
TRT	7	0,1479	0,02113	1,43	0,2446
Error	21	0,30962	0,01474		
Total	31	0,57867			
SD					
REP	3	5,8437	1,94792		
TRT	7	12,9688	1,85268	0,68	0,689
Error	21	57,4062	2,73363		
Total	31	76,2188			
YIELD					
REP	3	4,594	1,5313		
TRT	7	124,469	17,7813	0,68	0,6883
Error	21	550,156	26,1979		
Total	31	679,219			