

**EFFICACY OF *Trichoderma asperellum* SEED TREATMENT AND RIDOMIL®
APPLICATION IN MANAGING LATE BLIGHT (*Phytophthora infestans*) ON POTATO
(*Solanum tuberosum* L.)**

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**A Thesis Submitted to the Graduate School in Partial Fulfillment of the
Requirements for the Master of Science Degree in Crop Protection of Egerton University**

JUNE 2021

DECLARATION AND RECOMMENDATION

Declaration

This is my original work and has not been previously presented elsewhere for an award of degree or diploma.

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DEDICATION

This thesis is dedicated to my family; my dear wife, Maggy Ndeto, son, Ethan Mumo and daughter, Lakisha Ndanu.

ACKNOWLEDGEMENTS

I thank my academic supervisors' (Dr. Joseph Mafurah and Dr. Moses Nyongesa) for their mentorship and guidance throughout the study period. My special thanks goes to Center for Sustainable Agriculture and Agribusiness Management for funding and extra-ordinary support this research. The support of Centre Director KALRO Tigoni and Real IPM, Kenya in providing laboratory and consumables is highly appreciated. Special thanks go to Dr. Ann Njoroge for assistance in PCR work at the BeCA Hub ILRI Laboratories.

ABSTRACT

Late blight (*Phytophthora infestans*) is a major biotic stress in potato production worldwide. Blight management majorly depends on use of fungicides which presents economic and health concerns. The aim of this study was to determine the effect of seed potato treatment using *Trichoderma asperellum* in combination with Ridomil® (metalaxyl 4% + mancozeb 64%) to manage late blight on potato. Laboratory and field experiments were conducted to determine the efficacy and antifungal effects of *T. asperellum* on *P. infestans*. Seed (potato tubers or rooted cuttings) were pre-treated with *T. asperellum* via peridermal injection or dipping and followed by spraying ridomil® at intervals of 7, 14 and 21 days on emerged plants. Cost benefit analysis of the blight control options and analysis of metalaxyl residue in harvested tubers were also conducted. Costs and returns were first converted to per hectare prior to calculating marginal rate of return (MRR). In residue analysis, metalaxyl was extracted using soxhlet extraction and quantified using gas chromatography mass spectrometer. Results from *in vitro* trials showed that, whereas Ridomil® (2.5g/L) had the most inhibition effect on mycelial growth of *P. infestans*, *Trichoderma asperellum* at 66% concentration had a growth inhibition of 91.1% and reduced lesion size to 1.91 cm². *Trichoderma asperellum* at 100% concentration had 91.3% growth inhibition and lesion size of 1.89 cm². Under field conditions, results showed that no significant difference in amount of disease or yield from Ridomil® application at 7 and 14 day intervals on plants from seed pre-treated with *T. asperellum*. However, treatment by pericardial injection gave 1.24 t ha⁻¹ higher yield than treatment by dipping. Metalaxyl residue analysis showed that samples from plots with ridomil® sprays at 7 and 14 day intervals and samples from Limuru market exceeded the acceptable limit of maximum residue levels (0.08 mg Kg⁻¹). It is concluded that a combination of *T. asperellum* at 66% and 100% concentration with spray interval of 14 days had the highest returns. Seed tuber resulted in cost benefit ratio of 5.9 while rooted apical cuttings resulted in net loss. The use of *T. asperellum* in disease management can reduce fungicides application and their negative effects and also contribute to improved net farm income.

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LIST OF ABBREVIATIONS/ACRONYMNS

a.i	Active Ingredient
AFLP	Amplified Fragment Length Polymorphism
ATP	Adenosine Triphosphate
bp	Base pairs
CFU	Colony Forming Unit
CIP	International Potato Center
EBIS	Ethylene Bisiothiocyanate Sulphade
EEC	European Economic Community
EFSA	European Food Safety Authority
EU	European Union
FAO	Food Agricultural Organization
FAOSTAT	Food Agricultural Organization Statistics
GC-MS	Gas Chromatography Mass Spectrometer
ILRI	International Livestock Research Institute
ITS	Internal Transcribed Spencer
KALRO	Kenya Agricultural and Livestock Research Organization
KEPHIS	Kenya Plant Health Inspectorate Service
MRI	Manufacturer Recommended Interval
MRR	Marginal Rate of Return
MRR	Manufacturer Recommended Rate
MS	Mass Spectrophotometer
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
RFLP	Restricted Fragment Length Polymorphism
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
ppm	Parts Per Million
SSR	Simple Sequence Repeats
Taq	Thermal aquatics
USA	United States of America

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Potato has high potential in addressing food insecurity and low incomes experienced by small scale farmers. The crop yield per unit area and nutritional value are relatively higher compared to cereal grains (Azimuddin *et al.*, 2009). The global and Africa annual potato production is estimated to be 377 and 25 metric tonnes respectively. Kenya is ranked fifth in Africa with production of 1.35 metric tonnes annually (FAOSTAT, 2018). In Kenya potato average yield is 8 t ha⁻¹ compared to the potential of 40 t ha⁻¹. The Low production per unit area is attributed to poor seed quality, poor agronomic practices and diseases (Mumia *et al.*, 2018).

Late blight (*Phytophthora infestans*) is a devastating disease affecting potato production worldwide. The disease was associated with Irish potato famine in 1840s (Kroon *et al.*, 2012) and continues to be a major challenge in potato production globally (Mizubuti *et al.*, 2007). Global yield losses attributed to late blight is estimated to 16% (Tsahkna & Tamm, 2013) contributing to world annual economic losses valued at about 170 billion USD incurred as direct costs in disease management hence regarded as a threat to food security (Haverkort *et al.*, 2008; Penisi, 2010). The disease is mainly managed using multiple fungicide applications of up to 10 times per cropping season (Namanda *et al.*, 2004) resulting in increased cost of crop production consisting of chemicals and labour costs estimated at 12.5 billion USD. This excludes costs incurred in treating illness associated with the chemical effects on the field workers (Haverkort *et al.*, 2008; Svubure *et al.*, 2015). In addition to significant contribution to increased cost of production, multiple fungicide applications raise environmental and human health concerns (Nyankanga *et al.*, 2007).

In Kenya, potato yield losses attributed to late blight range from 20% to 100% depending on the prevailing weather conditions which contribute to low production of 8 to 10 t ha⁻¹ (Gildemacher *et al.*, 2009a). These challenges call for more efficient and eco-friendly practices in managing the disease. The success of *P. infestans* is attributed to its ability to mutate (Matson *et al.*, 2015; Njoroge *et al.*, 2019a). It attacks all parts of the plant and can destroy a whole field within one week when environmental conditions are conducive (Turkensteen *et al.*, 2000).

Use of host resistance is more economical and environmentally safe approach that initially involved use of R genes to confer resistance but has failed due to emergence of new strains that overcame the genes (Chen *et al.*, 2015). Identification and deployment of genes to confer durable

resistance has been reported to be effective when combined with fungicides (Haverkort *et al.*, 2016). Although resistant cultivars reduce early disease onset, they require timely protective fungicides to reduce disease severity (Anwar *et al.*, 2015). *Phytophthora infestans* has a huge genome (600 bp to 240 mp) with many repetitive sequences making it able to evolve rapidly, overcome resistance genes in resistant cultivars and develop fungicide resistance. The pathogen has also developed a short life cycle over the last decade enhancing continued spore production throughout the cropping season (Chen *et al.*, 2015). This has resulted in the need for increased application frequency to achieve reasonable blight control (Saville *et al.*, 2015).

Ridomil® (metalaxyl 8% and mancozeb 64%) is one of the most widely used fungicide in managing late blight (Kamau, 2017). The co-formulation offers both curative and preventive actions against *P. infestans* (Siddique *et al.*, 2016). Metalaxyl and mancozeb residues have been reported in tomato associated with late blight management using overuse of Ridomil® (Kamau, 2017). Exposure to fungicides has negatively impacted on farm workers and potato consumers. metalaxyl and mancozeb has been associated with respiratory effects and hematological defects (Al-amoudi, 2012). However, there is limited information on possibility of chemical residue dissipation in potato tubers as a result of increased Ridomil® application to manage late blight.

Bio-agents provide environmental friendly alternative for managing soil borne pathogens (Goufo *et al.*, 2017). Use of biological controls to manage crop diseases provide a sustainable option that can be exploited in modern agriculture to reduce inoculum level thereby decreasing disease severity and incidence as well as promoting human and environmental safety (Laila *et al.*, 2014). Beneficial fungi including *Trichoderma* are among the most preferred biological agents in managing crop diseases due to their ability to survive in soil under diverse environmental conditions (Lopez-bucio *et al.*, 2015). Bokhari (2009) found that *Trichoderma harzianum*, *T. viride*, *T. Konigii* and *T. reesei* reduced *Rotylenchulus reniformis* and *Meloidogyne javanica* severity in eggplant (*Solanum melongena*) in Saudi Arabia. Basahi (2014) showed that *Pseudomonas fluorescens* effectively controlled potato common scab and improved potato quality. *Bacillus subtilis*, *Trichoderma spp.* and *P. fluorescens* have been found to be effective in managing bacterial wilt (*Ralstonia solanacearum*) when combined with salicylic acid (Mohsin, 2016; Ramesh & Pandey, 2018). Chaouch (2016) and Stephan *et al.* (2005) in their separate research reported that *T. harzianum*, *T. viride* and botanicals could manage late blight on potato. In previous studies, *T. asperellum* has been reported to induce plant immunity (Hexon *et al.*, 2011)

and to act against plant diseases including *Pythium* and *Rhizoctonia* through mycoparasitism and enzymatic activities while promoting crop growth. In addition, Hermosa *et al.* (2013) also reported that *T. asperellum* contributed to improved potato yield by enhancing nutrients uptake. However, there are only few portions of beneficial micro-organisms that have been developed and applied to manage only specific crop pathogens.

The use of disease free seed potato including rooted apical cuttings has been proposed to have potential in contributing to disease management when incorporated in integrated disease management programme (Gildemacher, 2012). However, there is limited literature on the effect of rooted apical cuttings on late blight management and yield improvement

Biological controls have been found to induce slower influence on late blight (Yao *et al.*, 2016). Fungicides offer both preventive and curative mode of action (Tadesse & Tesfaye, 2018). In order to reduce the fungicides application while taking advantage of their fast action to improve the efficiency of the biological controls, screening their combination in both laboratory and field environment is vital. Cost benefit analysis of use of biocontrol and rooted apical cuttings is also vital in providing a reliable and economically sound disease management strategy. The present study therefore aimed at determining the efficacy of *T. asperellum* in managing late blight through pre-treatment of seed potato in order to minimize the frequency of Ridomil® applications and determine the cost benefits accrued to the practice and metalaxyl residue in potato tubers.

1.2 Statement of the Problem

Late blight (*Phytophthora infestans*) remains a key biotic factor contributing to significant potato yield reduction. Potato late blight infects all parts of the crop including the economical part (tuber) resulting in total crop loss when conducive weather conditions are prolonged. The disease problem is exacerbated by use of farm saved seed among farmers is practiced. Attempts to control the disease using synthetic fungicides has not only failed to yield appreciable results but also has led to increased cost of production and emergence of fungicide insensitive strains and negative effects on environment and human health. Emergence of fungicide resistant strains has led to reduced efficacy of the fungicides requiring farmers to increase frequency of application to manage the disease. This has led to increased costs and declining net farm income. Moreover, the approach could contribute to chemical residues in potato tubers raising health concerns for chemical applicators on the farms and potato consumers. This therefore necessitate search for affordable and

environmental friendly alternatives with potential of enhancing reduced chemical fungicide dependence to manage potato late blight efficiently.

1.3 Objectives

1.3.1 General Objective

To contribute to integrated disease management of late blight on potato (*Solanum tuberosum*) through use of a biocontrol in combination with fungicides to enhance increased potato production.

1.3.2 Specific Objectives

To determine:

- i. The antifungal effects of *Trichoderma asperellum* on *Phytophthora infestans* and its compatibility with fungicides *in vitro*
- ii. The efficacy of *Trichoderma asperellum* seed treatment and Ridomil® application interval on severity and incidence of late blight and yield of potato
- iii. Cost benefit in the management of late blight on potato through *Trichoderma asperellum* seed treatment and Ridomil® application.
- iv. The effect of Ridomil® application interval on metalaxyl residue in potato tubers.

1.4 Hypotheses

- i. *Trichoderma asperellum* has no antifungal effects on *Phytophthora infestans* and compatibility with fungicides *in vitro*
- ii. *Trichoderma asperellum* seed treatment and Ridomil® application interval has no effects on severity and incidence of late blight and yield of potato
- iii. Management of late blight on potato through *Trichoderma asperellum* seed treatment and Ridomil® application has no cost benefits
- iv. Ridomil® application interval has no effect on metalaxyl residue in potato tubers

1.5 Justification

Potato is one of the major food crop and source of income globally. Potato subsector in Kenya employs about 850,000 people directly as growers and 2.5 million indirectly along the potato value chain (Janssens *et al.*, 2013) contributing to poverty alleviation while enhancing food

security (Hirpa *et al.*, 2010). In Kenya, potato yield stands at 8 to 10 t ha⁻¹ compared with a potential of 40 t ha⁻¹ (Gildemacher *et al.*, 2009). The low production is attributed majorly to diseases with late blight being a significant contributing factor to crop loss (Son *et al.*, 2008). Late blight can cause up to 100% crop loss in major potato growing regions especially when favourable conditions are prolonged (Taylor *et al.*, 2013). The disease is primarily managed by field sanitation, development of host resistance and extensive use of fungicides that has often led to environmental problems, concerns on health of consumers and workers in the field as well as development of resistant strains. Emergence of resistant strains has resulted in short fungicide application interval of 5 to 7 days increasing the cost of production and possibly chemical residue accumulation in tubers. Use of biocontrols has been used singly to manage late blight to reduce minimize disease albeit ineffective when conditions conducive to the pathogen are prolonged. Within the context of disease-free planting material, use of biocontrols in combination with reduced fungicide application could increase their effectiveness in managing late blight on potato. This will result in reduced negative effects associated with fungicides and cost of production while improving on potato yield.

1.6 The Scope and Limitations

The study attempted to explore the effect of seed treatment with *T. asperellum* on fungicide application interval in effort manage late blight while reducing fungicide dependence. Effects of seed treatment using *T. asperellum* on Ridomil® spray regime was explored on crops from rooted apical cuttings and seed tuber. Cost benefit analysis and metalaxyl residue analysis was also conducted. However, the study did not establish reduction in toxicities resulting from oral, inhalation and dermal exposure nor mancozeb dissipation in pericardial layer of the tubers. It was observed that, plots treated with *T. asperellum* were greener than the untreated plots which was an unexpected finding. Rooted apical cuttings were more vulnerable to late blight than crops from seed tuber resulting to lower yields under field conditions. This was not explored in the present study and therefore future studies could attempt to explain the cause.

CHAPTER TWO

LITERATURE REVIEW

2.1 Potato Production

Potato (*Solanum tuberosum*) was first grown in Andes in southern Peru and north-west Bolivia between 5000 and 8000 BC before spreading to Europe via Spain in 16th century (Hawkes, 1992). It became a major staple food in Europe in 19th century and since then it has spread to other countries with variable environmental conditions due to its adaptability to different ecological conditions (Stevenson, 2005). Potato has become an important food crop worldwide in terms of consumption and industrial demand. Currently the tuber crop is ranked fourth in importance after maize, wheat and rice (FAOSTAT, 2018). Potato is a good source of carbohydrates, proteins, vitamins and minerals. It has gained industrial use in production of starch, crisps and chips due to increased urbanization worldwide (Rana, 2011).

While in the 1990s, potato was produced and consumed highly in Europe, North America and former Soviet Union, recently, China become a leading producer (99 million tonnes) followed by India (44 million tonnes) both accounting for about a third of world production. Consumption has also shown increment from 33.2 kg/capita/ year in 2008, 33.76 kg/capita/year in 2010 to 34.64 kg/capita/year globally (FAOSTAT, 2018). In 2007, potato was cultivated in 100 countries with a yield of 314 million tonnes and with an average consumption of 32.6 Kg per capita per year. Recently, FAOSTAT (2018) reported that the world potato production was 379 million tonnes from an area of 19,246,500 ha in 2016 while in 2015 production was 377 million tonnes from 18,978,600 ha. This suggests that increase in potato production is associated with expansion of more cultivated land. In Africa, potato production stands at 25 million tonnes from 1,767,960 ha with Egypt being the leading producer (5.2 million tonnes) followed by Algeria (5.0 million tonnes) while in Kenya the production stands at 1.3 million tonnes according to FAOSTAT (2016). Potato is targeted to play a crucial role in global food needs as it has higher returns per unit area than cereals (FAOSTAT, 2018).

In Kenya, potato has high potential of addressing the recurring food insecurity and improving farmers' livelihood due to its higher output per unit area. It is the second important food crop after maize in terms of volumes produced and consumption (Janssens *et al.*, 2013). The sub sector employs over 850,000 directly and 2.5 million people indirectly along the potato value chain. The tuber crop is mainly grown in highland altitude ranging from 1500 to 3000 m above

sea level although potato production has recently extended into new non-traditional areas such as Naivasha due to increased demand for the commodity (Mumia *et al.*, 2018). Potato growing regions are characterized by high population density leading to increased land subdivision affecting viable agricultural enterprise. However, potato fits perfectly in the small holder farms owing to its higher yield per unit area (Chindi *et al.*, 2017). According to a study conducted by Janssens *et al.* (2013), potato is mainly grown by over 500,000 small holder farmers who produce an average yield of 8 to 10 t ha⁻¹. Major potato producing counties include; Bomet, Elgeyo Marakwet, Embu, Nakuru Narok, Kiambu, Meru, Muranga, Nandi, Nyandarua, Nyeri, Uasin Gishu and Trans Nzoia (Mumia *et al.*, 2018).

Potato consumption in Kenya has been increasing due to rising urbanization that has resulted in growth of fast food industry. In fact, potato is currently the largest grown vegetable because it matures faster contributing to food and family income (Muthoni *et al.*, 2013). Gildemacher (2012) estimated that 81% of potato grown in Kenya is for sale. However, potato production in Kenya has not yet achieved its full production potential (40 t ha⁻¹) (Gildemacher *et al.*, 2009b). This is attributed mainly to unavailability of affordable certified seed and diseases majorly bacterial wilt, late blight and viruses. Yield losses due to diseases are 77% attributed to bacterial wilt, 67% arising from late blight and 12% attributed to viral infections (Kaguongo *et al.*, 2014). However, late blight continues to be the greatest yield reducing biotic factor due to its potential to spread to a larger region when conducive conditions are prolonged (Nyankanga *et al.*, 2007). It causes severe damage to foliage affecting assimilation process (starch accumulation) and to tubers leading to significant loss both in quality and quantity affecting tuber marketability and consumption (Agrios, 2005).

A recurrence of late blight epidemics in potato fields is majorly due to the use of farm saved seed that are often latently infected, inadequate deployment of cultivar resistance and overreliance on fungicides use that result in emergence of more virulent fungicide insensitive strains (Nyankanga *et al.*, 2007). In Kenya there is a number of varieties bred to suit farmers preferences but *Shangi* is currently the most widely grown variety despite it being susceptible to late blight (Kaguongo *et al.*, 2014; Mumia *et al.*, 2018). New biotypes have risen in the last decades, challenging available disease control practices (Pule *et al.*, 2013). Furthermore, seed shortage in Kenya has led to farmers sharing farm saved seed resulting in inoculum multiplication and spread over a wider area leading to increased epidemics. This result in soil infection that

become source of primary inoculum for more than four months (Kromann *et al.*, 2008). Sporangia from volunteer crop and soil also cause early disease attacks and increased epidemics requiring farmers to apply fungicides multiple times over the cropping season (Turkensteen *et al.*, 2000).

2.2 Potato Seed Sector in Kenya

As seed is the starting point of plant life, it becomes an important component in Agricultural systems. In Kenya, seed potato system consists of both informal and formal seed system (Gildemacher *et al.*, 2011). Gildemacher *et al.* (2009a) reported that less than 3% of total seed potato required comes from either direct or indirect quality controlled (informal system) seed multiplication while Kaguongo *et al.* (2014) indicated this formed only 6.9% in 2013. Use of farm saved seed is the major constrain facing seed potato production (Kamuyu, 2017). In East Africa countries including Kenya, nearly 75% of the potato fields are contaminated with phytopathogens due to continued use of latently infected seed. Seed shortage is worsened by few seed multipliers requiring farmers to incur high transport costs increasing that add to the high cost of production (Gildemacher *et al.*, 2009b).

Improving seed potato quality is the primary strategy in avoiding potato diseases to enhance increased yield and farmers' income (Hirpa *et al.*, 2010). In Kenya, there are some efforts promoted to improve seed potato system among smallholder farmers involving several technologies aiming at reducing the recurring seed shortages (Gildemacher, 2012). There is high demand for disease free seed that has prompted the use of farmers'-based technologies including positive selection technology and seed plot technique which were promoted in Kenya in 2004 by International Potato Center (CIP) to fill the seed deficit gap. These technologies led to 34% yield increase that corresponded to 340.8 US dollars (Gildemacher *et al.*, 2011). Currently, different propagating materials including intact whole tubers, minitubers, *in vitro* plantlets, rooted stem and apical cuttings are been promoted to improve on seed supply (Muthoni *et al.*, 2011).

Potato is propagated sexually (true potato seed) and asexually (vegetative). Although seed potato tuber is the most widely used propagative material, it is prone to latent infection and most likely spread of pathogen inoculum from one region to another affecting seed production especially through repeated cycling of seed (Frost *et al.*, 2013). The use of seed tuber in seed multiplication results in tuber infection ranging from 0.01% to 3% of the seed lot which is sufficient to initiate late blight epidemics in the subsequent season (Hussain *et al.*, 2013). This limits the lifespan of

superior variety (Mani *et al.*, 2014; Simmonds, 1997). Minitubers and microtubers provide opportunity for solving the recurrent seed shortage (Higgins *et al.*, 2017). Tissue culture is increasingly becoming an important technology in enhancing availability of seed for agriculture and industry to match the increasing demand for potato. The technology provides high quality disease free seed and with superior genetic and physiological uniformity and not limited by season (Batool *et al.*, 2014; Mohapatra & Batra, 2017). Apical cuttings which are produced through tissue culture technology using disease free ex-plant *in vitro* offer opportunity for rapid multiplication of seed (Mohamed & Alsadon, 2010; Singh *et al.*, 2019). The vegetative material therefore form an important technique in rapid and clonal multiplication of potato seed to solve shortages experienced in most Sub Saharan countries. El-Helaly (2012) reported that, potato seedlings from rooted apical cuttings could increase the rate of seed multiplication.

2.3 A Global Perspective on *Phytophthora infestans*

2.3.1 Strains and Distribution

Phytophthora infestans (Mont.) de Bary causes one of the most persistent diseases (late blight) of potato. Late blight was responsible for the Irish potato famine that resulted in deaths of more than one million people and emigration of 2.5 million in the mid-19th century (Yoshida *et al.*, 2013). Global distribution of late blight to other potato growing regions is thought to have arisen from European blight epidemics of 1840s and later during epidemics of late 1970s which were facilitated by contaminated tuber seed that carried the inoculum (Fry & Goodwin, 1997). Pathogen variability has been explored using mating type determination, metalaxyl sensitivity and use of molecular techniques including isozymes, Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Simple Sequence Repeats (SSR) and mitochondria haplotyping to monitor changes in population structure (Cooke & Lees, 2004). Guo (2009) and Haas *et al.* (2009) reported *P. infestans* variability about 7 years after introduction of resistant variety providing evidence that a resistant variety with R- genes alone cannot manage late blight epidemics.

Phytophthora infestans is heterothallic with two mating types (A₁ and A₂) (Flier *et al.*, 2003). If both mating type come into contact sexual reproduction occurs leading to formation of oospores (resting body) that are associated with more aggressive strains. Oospores are capable of surviving outside host and remain infective for a longer period (Turkensteen *et al.*, 2000).

Phytophthora infestans population continues to evolve and are highly clonal adapting to current management practices (Montarry *et al.*, 2010). Mating type A₂ was only restricted in Mexico and Andean mountains (centre of potato origin) until 1980's while A₁ is predominant worldwide in potato growing regions (Tooley *et al.*, 1986). Mating type A₂ has been reported in various parts of Europe, Asia and America. Dyer *et al.* (1993) provided evidence on mating type A₂ being outside Mexico in his first report. However, the old predominant *P. infestans* populations have been displaced by the new populations (Harbaoui *et al.*, 2014) in most potato growing regions worldwide. Pule *et al.* (2013) reported that only mating type A₁ was detected in most Sub Saharan countries precluding sexual reproduction and denoting absence of sub structuring probably due to limited importation and intensive trade in potato tuber in these countries. Migration of the pathogen is presumed to take two pathways; one in 1840s and second in 1970s (Chowdoppa *et al.*, 2013; Cooke *et al.*, 2012). Globally, migration of *P. infestans* has become evident thereby increasing the possibility of occurrence of a new population characterized by genetic diversity and more aggressive strains with shorter life cycle, adaptation to wider temperature ranges and insensitive to metalaxyl (Spielman *et al.*, 1991). *Phytophthora infestans* heterothallism enables completion of sexual cycle resulting in large genetic variation in the offspring and may lead to increased and rapid evolution of more aggressive and fungicide resistant strains (Cooke *et al.*, 2011).

The Irish famine of 1840 was probably caused by *P. infestans* populations with mtDNA haplotype Ia genotype which were replaced by US-1(haplotype Ib genotype). US-1 were globally distributed before the second migration (May & Ristaino, 2004). Some Sub Saharan countries including Kenya, Uganda, Tanzania and South Africa were least affected by this migrations probably due to incomplete displacement of the US-1 (McLeod *et al.*, 2001).

In East Africa, late blight was first reported in Kenya in 1941 on imported seed from United Kingdom which spread to Uganda then to Congo and Tanzania in the following year (Natrass, 2016). Since, the regions were then dominated by single clonal lineage (US-1) *P. infestans* strains though different genotypes lineages were reported in Rwanda and Ethiopia (Gavino & Fry, 2017). In Kenya, US-1 lineage has been displaced by 2_A1 on tomato and potato which is more pathogenic and virulent than the US-1 (Njoroge *et al.*, 2019b).The displacement of US-1 by 2_A1 is attributed to its higher competitiveness ability (ability of lineage to thrive well while coexisting with another lineage) which is partly influenced by aggressiveness that cause quick spread and dominance. Competiveness in this lineage is characterized by greater sporangia production, earlier

release of zoospores, faster germination rate, efficient nutrient assimilation (Chapman, 2019) higher rate of lesion growth and short latent period (Suffert & Thompson, 2018).

2.3.2 Effects of Environmental Conditions on Reproduction of *P. infestans*

Optimum temperatures for *P. infestans* development and establishment ranges from 16 to 24 °C. The pathogen is mainly spread through sporangia formed on the foliage during epidemics which requires low temperature of about 10 °C and high relative humidity of above 90% (Skelsey *et al.*, 2010). Sporangia germination requires favourable temperatures ranging from 18 to 20 °C. However, at lower temperatures (4 to 10 °C) each sporangia will release 6- 8 zoospores (Maziero *et al.*, 2009). Sporulation requires high relative humidity (above 90%) and surface moisture. Under field conditions, sporulation may be inhibited by certain light wavelengths (low peak of blue light; peak 450 nm) during the day hence occurs at night when conditions favour sporangia formation (Fall *et al.*, 2015). Field conditions in most potato production regions experience temperatures ranging from 18 to 22 °C that favour oospores (produced by mating of type A1 and A2) formation with an incubation period of 7 to 14 days. Continuous illumination for a prolonged time leads to few or no oospore formation (Kromann *et al.*, 2008).

A zoospore forms germ tube that infects a healthy host to produce disease (Vargas *et al.*, 2008). Sporangia and zoospore establish only in presence of free water and therefore leaf wetness duration is critical. Sporangia may germinate directly (producing hyphae at 15 to 18 °C) or indirectly (release of zoospores at 4 to 12 °C). *Phytophthora infestans* may undergo both sexual and asexual reproduction allowing new genetic recombination resulting to new genotypes that cause a more severe epidemics (Gavino & Fry, 2017). Oospores formed from union of opposite mating types are capable of producing mycelia that multiply the pathogen causing new infection. Oospore mycelia is more tolerant to temperature and variations in relative humidity than sporangia and zoospores that are killed when exposed for 84 hours to temperatures above 25°C. In the absence of a water film for 5 mins at 95% relative humidity and 2.5 mins at 90% RH, sporangia lose viability accelerated by solar radiation exposure. Oospores become embedded in host plant tissue or soil that reduce their exposure to dry air. In addition, they possess thick walls that enable them to resist drying (Harnish, 1965). Therefore *P. infestans* survive and infect the crop in field (on-going cropping season) through sporangia (asexual reproduction) while, occasionally, through oospores (sexual reproduction) between cropping seasons (Whisson *et al.*, 2016).

2.3.3 Infection Process and Symptomatology

Zoospores may provide appressorium that is capable of breaching the epidermal cell wall and cuticle integrity followed by growth of hyphae inserting haustoria within the host plant tissues. *Phytophthora infestans* being a hemibiotroph first targets only the living tissues in which it draws nutrients from the apoplast and continues to thrive on the tissues that have died as it infects new healthy parts of the hosts. Formation and development of haustoria is followed by host tissue necrosis and emergence of sporangiophore with sporangia through the stomata. Sporulation is dependent on environment and genotype (Fry *et al.*, 2015) requiring high humidity but suppressed by light. However, light sensitive transcripts (cryptochromes) in the fungus regulate the sporulation (Xiang & Judelson, 2014). Release of sporangia that carry zoospores mostly occurs late at night because they are prone to desiccation (Granke *et al.*, 2009). Sporangia and zoospores have capability of inducing fresh infection on host plant. However, infection by sporangia germination takes longer than that caused by zoospore which remain motile for 22 hours at 5 to 6 °C. Zoospore encyst once they become non-motile and adhere on leaf surface, produce germ tube and germinate and cause infection within 2.5 hours followed by necrotic spot in the next 24 to 48 hours (Grünwald & Flier, 2005). Formed sporangia are dispersed in the morning when there is rise in temperature and fall in relative humidity (due to increased irradiation) that causes detachment from the sporangiophores on leaves. Detached sporangia are dispersed in water films because of their hydrophilic nature and may be transported over a short distance on soil aided by splashing of irrigation water or rain drops to leaves to initiate infection. Infected soil carries inoculum that is infective for more than 3 months (Johnson & Pathology, 2010; Turkensteen *et al.*, 2000). Sporangia may also be washed down to stem and tubers by rain or irrigation water. Disease development is highly correlated with relative humidity, timing of rains and overhead irrigation. In the presence of high moisture and wind, the infection is accelerated (Fry *et al.*, 2013).

Late blight appears as water-soaked spots with pale green lesions at the margins and tips of the leaf at initial stages of infection. The lesion rapidly turns to large black purplish necrotic spots and a white mycelia on the lower side of the leaf is clearly visible around the edges of the necrotic spots. The pathogen produces sporangia and zoospores as propagules that attack other parts of the plant including stems (Agrios, 2005). The stem and petiole become encircled by light to dark lesions that soon become weak and collapse. Under conducive conditions the whole crop becomes blackened (blighted) and eventually die within one week. This is followed by defoliation resulting

to spread of sporangia from the infected leaves or tubers to moist soil especially during rainy and/or irrigation. Infected tubers become hard, dry and firm and show reddish brown to purplish areas that extend to internal tissues (Sharma, 2014).

2.3.4 How *Phytophthora infestans* overcomes Potato Defense Mechanisms to Cause Disease

At biotrophic stage *P. infestans* survives on living tissues and therefore defeating host defense mechanism and maintaining its cell integrity is very key for the success of the pathogen. Plants respond to the pathogen by employing pathogen associated molecular patterns in pathogen recognition as the first layer of defense. *Phytophthora infestans* is able to avoid chemicals thereby reducing their efficacy (Dahlin *et al.*, 2017). The pathogen hyphae is usually shielded within the hosts tissues requiring good spray coverage which most farmers do not practice and perceive as economically unsustainable. *Phytophthora. infestans* has mechanism for pumping out of cells toxicant chemicals including metalaxyl using ATP-binding cassette transporters (Childers *et al.*, 2014) or slowing down chemical uptake through plasma lemma modification (Maridueña-zavala *et al.*, 2017). The pathogen also secretes p450 monooxygenases and glutathione conjugating enzymes to degrade chemical fungicides and protect it from phytoalexins (Pang *et al.*, 2015). Use of systemic fungicides that target specific sites such as metalaxyl on RNA synthesis has resulted in mutation of the target site which was observed after metalaxyl introduction in 1970s and fluazinam in 1992 (Randall *et al.*, 2014).

Phytophthora infestans produces defense blocking effectors by initiating effector triggered immunity that causes programmed plant cell death thereby overcoming pattern triggered immunity produced by the host plant (Silva *et al.*, 2019). Plants secrete enzymes in apoplast such as protease but are counteracted by enzymes inhibitors or suppressors produced by *P. infestans* (Raffaele *et al.*, 2010). Glycosides hydrolases are produced by the pathogen to degrade phytoalexins that interfere with salicylic acid signaling in the host (Bastian *et al.*, 2017). *Phytophthora infestans* lacks chitin in its cell wall therefore avoiding detection by the plant. Haustoria produced by the pathogen in mesophyll wall are very thin thus limit damage associated with molecular patterns thereby avoiding detection by the host defense systems (Lu *et al.*, 2012). Previously resistant genotypes based on dominant resistance (R) genes have succumbed to late blight due to development of clonal variation and adaptation to new resistance gene (Yoshida *et al.*, 2013).

2.3.5 Management of Late Blight on Potato

Integrated disease management that includes cultural, mechanical, biological, legislation and chemical methods is the best option in the management of the disease (Tsedaley, 2014). Volunteer crops, infected seed and soil borne oospores serve as initial pathogen primary inoculum and pathogen reservoir (Hannukkala *et al.*, 2007). Volunteer crop resulting from potato left overs in the field, inefficient harvesting techniques and too small sized tubers accelerate late blight attacks. Reduction of initial inoculum is the first step in management of late blight in potato field (Cooke *et al.*, 2011). Volunteer crops should be removed from field before planting while potato pile should be burned (Zwankhuizen *et al.*, 2000).

The use of synthetic fungicides to manage late blight is the most common practice among farmers. The chemicals are able to inhibit or reduce late blight development in plants by damaging the pathogen cells or affecting critical enzymes and processes (Hirooka & Ishii, 2013). Early fungicide application followed by 5 to 7 days interval is usually practiced especially where conducive weather conditions for *P. infestans* prevails and farm saved seed is used (Sharma & Saikia, 2013). However, fungicides dependence pose environmental and health concerns as well as increased cost of production. Introduction of metalaxyl in 1980 which has systemic mode of action was a success in managing *P. infestans* sporulation in foliage (Platt, 2009). However, metalaxyl resistance has been reported worldwide affecting its efficacy in managing late blight (Fontem *et al.*, 2005; Kumar, 2017).

Despite phenylamide high efficiency in some potato growing regions, resistant strains have also been reported in most potato growing regions (Majeed *et al.*, 2017a). Fungicide resistance is associated with the ability of *P. infestans* to mutate in their target site, over expression of target proteins, altering target biosynthesis pathway or change in cell composition (Avenot *et al.*, 2009).

However, *P. infestans* has been able to evade chemicals effects or reduce their efficacy (Dahlin *et al.*, 2017). The pathogen hyphae is usually shielded within the hosts tissues requiring good spray coverage which most farmers do not practice and perceive as economically unsustainable. *P. infestans* has mechanism for pumping out of cells toxicant including metalaxyl using ATP-binding cassette transporters (Childers *et al.*, 2014) or slowing down chemical uptake through plasma lemma modification (Maridueña-zavala *et al.*, 2017). The pathogen also secretes p450 monooxygenases and glutathione conjugating enzymes to degrade chemical fungicides and protect it from phytoalexins (Pang *et al.*, 2015). Use of systemic fungicides that target specific

sites such as metalaxyl on RNA synthesis has resulted in mutation of the target site which was observed after metalaxyl introduction in 1970s and fluazinam in 1992 (Randall *et al.*, 2014)

This prompted chemical companies to co-formulate metalaxyl with other fungicides molecules including mancozeb. During Euroblight 13th workshop special report, Nielsen and Isaac (2014) indicated that metalaxyl is effective for canopy blight control while mancozeb is effective for both canopy and tuber blight control hence the need for co-formulation. They proposed that, fungicide spraying regime should be according to need depending on the variety, infection pressure and weather condition to save on cost in managing late blight. They indicated that using this technique, fungicide use was reduced by 26%. Moreover, there is increasing pressure from European Union (EU) member states to promote non-chemical approaches in disease management by lowering chemical inputs (Nærstad *et al.*, 2007). Therefore the use of biocontrols could be an alternative strategy in offering prior and post control of late blight (Syed *et al.*, 2018).

Resistant varieties form a key component in late blight management because they reduce early disease attacks resulting to reduced fungicide application frequency (Rana *et al.*, 2013). There is evidence of evolving *P. infestans* leading to emergence of new and more virulent strains with the ability to break resistance gene in new introduced cultivar. In addition, cultivation of resistant cultivars is currently limited due to the overwhelming farmers' preference for certain varieties which are mostly susceptible to late blight (Mumia *et al.*, 2018). Other farmers combine resistant cultivar with the use of fungicide to manage potato late blight (Kefelegn *et al.*, 2012).

Liljeroth *et al.* (2016) in their studies reported that use of nutrition including phosphorous, potassium and calcium reduced late blight severity in potato. Higher calcium in potato foliage contribute to reduction in late blight infection (Seifu, 2017). Crop rotation and manipulation of planting date have also been suggested to reduce early late blight attacks through elimination of primary inoculum sources (Hannukkala *et al.*, 2007). Use of non-solanaceous crops in a cropping system for three seasons eliminate host crop including volunteer crops. However, Turkensteen *et al.* (2000) found that, oospores where sexual hybridization occurs, can remain viable for 3 to 4 years in soil under temperate conditions making short cycled crop rotation inefficient.

2.4 Ridomil®

Ridomil® (mancozeb 4% + metalaxyl 64%) manufactured by Syngenta (Switzerland), is one of the most widely used fungicide in managing potato late blight globally (Nyankanga *et al.*,

2007). Metalaxyl [N-(2,6-dimethylphenyl)-N-(methoxyacetyl) alanine methyl ester] belongs to phenylamide (acylalanine) group of systemic fungicides that were originally very effective in combatting fungi in the peronosporales that include late blight and mildews (Yigal & Moshe, 1982). The molecule is readily taken up by roots, leaves and green stems rapidly and cause fungitoxic effects only once it's in the tissue. Metalaxyl targets rRNA transcription thereby interfering with RNA synthesis. This target site specificity has led to development of resistant strain challenge (Thind *et al.*, 2001).

Mancozeb (dithiocarbamate) is a contact preventive fungicide that inhibits respiration of *P. infestans*. Mancozeb was first registered by Dupont, Delaware in 1950 which replaced the earlier discovered dithiocarbamates (zineb and nabam) and since then it has been used to manage a wide range of fungal diseases in over 120 countries registering annual sales of 740 million US dollars in 2007 globally. Mancozeb itself is not toxic to pathogens until it is exposed to water which then breaks into ethylene basisothiocyante sulphide (EBIS). Ethylene basisothiocyante sulphide is converted to ethylene bisisothiocyante in the presence of ultra violet light. The molecule interferes with sulphhydryl containing enzymes resulting in inhibition of six biochemical reactions in cytoplasm and mitochondria (Gullino *et al.*, 2010). Mancozeb does not penetrate plant cuticles but remains on the surface of the leaf for some days to inhibit *P. infestans* spore germination. The molecule has no curative but preventive mode of action because once the disease is within plant tissues its unable to interact with the pathogen (Wong *et al.*, 2001). The molecule needs to be applied at higher frequency of up to 5 to 7 day intervals to counteract the effects of weathering and plant growth which reduce protection offered by the chemical. This ensures prior protection of foliage before arrival of *P. infestans* spore on the leaf surface (Muhinyuza *et al.*, 2008). Co-formulation with metalaxyl offer a plausible strategy to manage late blight through systemic, curative (metalaxyl) and preventive options (mancozeb).

According to EFSA (2015) report, directive 91/414/EEC, the acceptable daily intake of metalaxyl is set at 0.08 mg Kg⁻¹ body weight per day and acute reference dose set at 0.5 mg Kg⁻¹ body weight. During the evaluation, 0.01 mg/Kg was used as limit of quantification on validated analytical methods. The report further concluded that, overuse of metalaxyl could lead to elevated residue levels. The half-life of metalaxyl disappearance from soil range from 5 to 35 days mostly obtained 10 cm from the soil surface. In 2005, mancozeb rate recommendation was set at 1.6 kg a.i ha⁻¹ but the current use of the molecule is 5 to 10 times higher per cropping season (EFSA,

2015). Multiple application of metalaxyl and mancozeb cause negative impacts on soil microbial activities (Muñoz-leoz *et al.*, 2011; Sukul, 2006) and potential biocontrols (Carriger *et al.*, 2007). Prolonged exposure to these chemical molecules result in acute and chronic illness including cancer, neurological disorders, liver and kidney damage and dermatitis (Lascombe *et al.*, 2014). Pesticides dissipation studies are essential to evaluate their impacts on environment and on non-target organisms including human beings when they enter food chain.

2.5 *Trichoderma asperellum*

Use of biocontrols and cultural practices provide a more environment friendly and effective method in managing late blight (Köhl *et al.*, 2019; Thomas-sharma *et al.*, 2016). Management of tuber infection and soil borne pathogen with biocontrols including plant extracts, natural enemies and micro-organisms have been studied (Maharjan *et al.*, 2010). *Trichoderma* species are free living endophytic and symbiotic fungi inhabiting most soils and root zones (Harman, 2005). The fungus comprises of many strains that have been used as biological agents to manage nematodes and various crop diseases due to their ability to activate multiple mechanisms that involve synthesis of hydrolytic enzymes including protease, glucanases, chitinases, esterases and lipases that degrade host's cell membrane and wall and antibiotics that include trichodermin and trichodermol (Schuster & Schmoll, 2010). Moreover, *Trichoderma spp.* also induce acquired systemic resistance in plants (Enkerli *et al.*, 1999).

The success of *Trichoderma spp.* as a biocontrol is further enhanced by its ability to compete for nutrients and space, modifying rhizosphere environmental conditions and employing defense mechanism (antibiosis) and mycoparasitism antifungal activities. The biological agent compete with nearby pathogens for nutrients through extended mycelial growth resulting in dissolution and finally death of the pathogen (Howell, 2007). Pathogen dissolution by *Trichoderma* is through cell wall degrading enzymes including glucanase, chitinase and protease which penetrate pathogen mycelium absorbing nutrients (Bailey *et al.*, 2008). These mechanisms may be activated singly or multiple depending on the environmental conditions and plant properties (Naglot *et al.*, 2015). *Trichoderma spp.* works well in temperatures ranging from 20 to 30 °C and produces conidiophores that are highly branched assuming a pyramid shape forming a concentric ring (Qiu *et al.*, 2017). *Trichoderma asperellum* also exhibits plant growth promotion mechanisms by altering plant hormone levels, iron acquisition through siderophore production and

atmospheric nitrogen fixation (Lugtenberg & Kamilova, 2009). *Trichoderma* lowers the levels of ethylene which inhibits plant growth when the plant is under pathogen induced stress using 1-aminocyclopropane-1-carboxylate deaminase (Farwell *et al.*, 2007). Herrera-t *et al.* (2019) reported enhanced germination, rapid flowering and vigorous increase in height in tomato treated with *Trichoderma*.

Foran (2016) evaluated several bacterial strains on late blight in potato. Recently, Caulier *et al.* (2018) reported that, *Bacillus spp.* and *Pseudomonas spp.* were effective in managing late blight. *Trichoderma viride*, *T. viridicatum* *T. harzianum* and *Myrothecium verrucaria* have been found to reduce late blight severity on potato in detached leaflet (Gupta, 2016). *Trichoderma asperellum* has been found to reduce *Pythium*, *Rhizoctonia* and *Fusarium* mycelial growth which infect potato (Raut *et al.*, 2014). To broaden its spectrum of action, efficacy of the biocontrol was explored on late blight through seed treatment in combination with foliar fungicide application in the present study. However, there is limited information on costs and benefits on the use of biocontrol in combination with fungicides to reduce application frequency to manage late blight as well as chemical residue that accumulate in potato tubers.

CHAPTER THREE

***IN VITRO* EFFICACY OF *Trichoderma asperellum* AGAINST LATE BLIGHT (*Phytophthora infestans*) AND ITS COMPATIBILITY TEST WITH FUNGICIDES**

Abstract

Late blight is one of the most devastating diseases of potato contributing to low yield globally. The disease is managed by extensive use of fungicides that has led to emergence of fungicide resistant strains resulting in chemical ineffectiveness and increased cost of late blight management. Biological agents offer a sustainable alternative in managing potato late blight. The objective of the study was to determine the efficacy of *Trichoderma asperellum* on *P. infestans* and its compatibility with fungicides. *Trichoderma asperellum* at 33% (3×10^6), 66% (7×10^6) and 100% (1×10^7 CFU/mL; g/L), Ridomil® (metalaxyl 4% + mancozeb 64%) and Mistress 72® (cymoxil 4% + mancozeb 64%) were plated alongside *P. infestans* on unamended Potato Dextrose Agar (PDA) and detached leaflet assay. *Trichoderma asperellum* compatibility with Ridomil® and Mistress 72® was conducted on PDA plates. Results indicated that Ridomil® and Mistress 72® inhibited growth of *P. infestans* mycelia in the PDA plates and establishment in detached leaflet assay. *Trichoderma asperellum* at 33% concentration had the lowest inhibitory score (38.0%) while at 66% (91.10%) and 100% (91.30%) concentrations were not significantly different ($P \leq 0.05$) on PDA. Lesion sizes were not significantly different at 66% (1.91 cm²) and 100% (1.89 cm²) of *T. asperellum* concentration. *Trichoderma asperellum* at 33% concentration (3.5 cm²) and untreated (3.55 cm²) had the largest lesion size and were not significantly different. Ridomil® and Mistress 72® had no significant effect on *T. asperellum* mycelial growth. Results suggest that *T. asperellum* at 66% and 100% concentrations were effective in managing late blight. Results further indicate that *T. asperellum* could be used in combination with fungicides for effective management of late blight on potato.

Key words: Detached leaf assay, *in vitro*, *Phytophthora infestans*, *Trichoderma asperellum*

3.1 Introduction

Demand for potato was projected to increase by 250% in 2020 with annual increase of about 3.1% (Scott *et al.*, 2010). Low potato production per unit area has been attributed to poor seed quality and diseases (Muthoni *et al.*, 2013). Late blight is greatest yield reducing biotic stress in potato production especially when the weather conditions are conducive (Were *et al.*, 2014). Potato late blight is experienced in all potato production regions worldwide. Management of the disease is solely by extensive use of fungicides that costs about 1 billion USD annually (Haverkort *et al.*, 2008). The disease can cause up to 100% yield loss on potato if unmanaged depending on weather conditions and variety susceptibility (Mariita *et al.*, 2016). Globally, late blight is responsible for 6.7 billion USD worth of potato yield loss annually (Nowicki & Majid, 2012) and therefore threatens food security (Cooke *et al.*, 2012). Late blight epidemic is accelerated by the adoption of farm saved seed by farmers as a result of potato seed shortage which is a major constrain in most Sub Saharan countries (Okello *et al.*, 2017). The disease can rapidly defoliate whole field within a week if unchecked resulting in tuber infection that lowers both tuber quality and quantity (Gigot *et al.*, 2009). *Phytophthora infestans* survives well in potato seed culls and piles (Johnson & Cummings, 2009) which serves as source of primary inoculum in the subsequent cropping season resulting in early epidemics (Runno-Paurson *et al.*, 2013). This often result in latent infection that produce viable sporangia that cause disease epidemics in the farmers' fields (Johnson & Cummings, 2013).

Ridomil[®] (metalaxyl 4% + mancozeb 64%) and Mistress 72[®] (cynamoxil 4% + mancozeb 64%) are among the most widely used fungicides in Kenya to manage late blight due to their curative, preventive and systemic modes of action (Nyankanga *et al.*, 2007). However, dependence on chemical application has raised environmental and human health concerns while emergence of fungicide resistant strains including metalaxyl insensitive isolates implies the loss of efficacy of the fungicides (Matson *et al.*, 2015). In addition, emergence of new strains of *P. infestans* that adapt to new chemical fungicides and host resistance (Njoroge *et al.*, 2019b) has led to reduced spray intervals and increased frequency of sprays. Use of biocontrols could offer the best sustainable and ecofriendly alternatives to chemical application (Yao *et al.*, 2016).

Biological agents including *Trichoderma spp.*, *Bacillus spp.* and *Pseudomonas fluorescens* and plant extracts have been explored in managing late blight on Solanaceous plants (An *et al.*, 2010; Chowdappa *et al.*, 2013; Kabir *et al.*, 2013). *Trichoderma spp.* is one of the most studied

fungus that is widely used in the management of plant diseases. The fungus is known to induce systemic disease resistance in plants as well as offer prior protection by activating enzymes that degrade cell walls in the pathogen (Yao *et al.*, 2016). Saravanakumar *et al.* (2016) reported that suppression mechanisms of *T. harzianum* on *P. infestans* through competition, antibiosis, promotion of crop growth and mycoparasitism while Wu *et al.* (2017) reported enzymatic activities against plant and soil borne pathogens. Various studies have shown that *T. asperellum* could manage a number of plant diseases. Carrero-carr *et al.* (2016), Kipngeno *et al.* (2015), and Patel and Saraf (2017) reported that *T. asperellum* could manage Fusarium wilt in Tomato, Verticillium wilt in olive and Pythium in tomato respectively. However, there is limited information on the efficacy and potential of *T. asperellum* to manage late blight on potato to broaden the bioagents application to manage crop pathogens.

The use of biocontrols is yet to be fully exploited in managing late blight, because synthetic fungicides act faster than biocontrols against disease causing agents (Xu *et al.*, 2011). The fast action of fungicides against plant pathogens can be effectively tapped and combined with biocontrols to reduce fungicides. However, little is known on the possibility of combining biological controls with fungicides in the management of *P. infestans* on potato to reduce amount of chemicals applied. Therefore, the objective of the study was to determine the antifungal activity and efficacy of *T. asperellum* on *P. infestans* *in vitro* in detached leaflets assay and to assess compatibility of Ridomil® and Mistress 72® with *T. asperellum*.

3.2 Materials and Methods

3.2.1 Isolation, Culturing and Bulking of *P. infestans* Inoculum

Thirty freshly blighted potato leaves (Plate 1a) samples were randomly collected from a potato crop at Kenya Agricultural Livestock and Research Organization (KALRO), Tigoni. The leaves were surface sterilized by soaking in 70% ethanol for 1 minute, rinsed with distilled water to remove excess ethanol and then air dried for 5 minutes. The surface sterilized leaves were placed in a petri dish lined with wet (two drops of distilled water) serviettes to maintain humidity for survival of the pathogen. The petri dishes were incubated at 18 °C for 24 hours to stimulate sporulation. Mycelia was carefully picked using sterilized hypodermal syringe without touching the leaf tissue and inoculated on pea agar amended with antibiotics rifampicin 50 µg/mL. The inoculated pea agar petri dishes were incubated at 18°C for 5 days and then sub cultured to increase

inoculum quantity. Mycelial was obtained from the pure culture by scrubbing off using a sterilized spatula and put in 3 eppendorf tubes containing 10 mL of sterilized distilled water. This was vortexed for 2 minutes using electric vortex (model VM-1000 of MRC Laboratory equipment company) at 3000 revolution per minute (rpm), filtered through sterilized four layered muslin cloth and the suspension incubated for 4 hours at 4 °C to induce sporangia to release zoospores. Inoculation was performed by placing 20 µl droplets of *P. infestans* sporangia suspension on healthy leaves (abaxial side) (Plate 1 b).

Identification and pathogenicity test was done on healthy potato seedling and tuber slices of *Shangi* varieties using Koch's postulates (Forbes, 1997). Briefly, inoculum bulking was performed on *Shangi* on tuber slices. Tubers were cleaned with sterilized distilled water and dipped in 70% ethanol for 10 seconds. They were rinsed with distilled water and then air dried on the laboratory benches for 15 minutes. The tuber slices of 0.4 cm thickness (Plate 1 c) were cut transversely using sterilized surgical blades. From the inoculated leaves samples (Plate 1 b), a piece of leaf with *P. infestans* lesion and healthy part was cut and placed on plastic dishes (15 x 12 x 5 cm) and incubated at room temperature 18 ± 2 °C for 4 days in laboratory benches. The tuber slices were placed on the infected leaf piece in the plastic dishes and incubated for 7 days at room temperature (18 ± 2 °C) on laboratory benches (Forbes, 1997; Nyongesa, 2014). Mycelia growth (Plate 1 d) on the upper side of tuber slice was carefully picked using sterilized hypodermic needle while avoiding touching the tuber and placed in 30 eppendorf tubes containing 15 mL of sterilized distilled water. The suspension was vortexed for 2 minutes using electric vortex and then incubated for 4 hours at 4 °C to enhance sporangia and zoospore formation. The suspension was filtered through double folded cheese cloth and put in one litre bottle which was incubated for 4 hours at 4 °C. This was used in detached leaflet assay and field experiment.

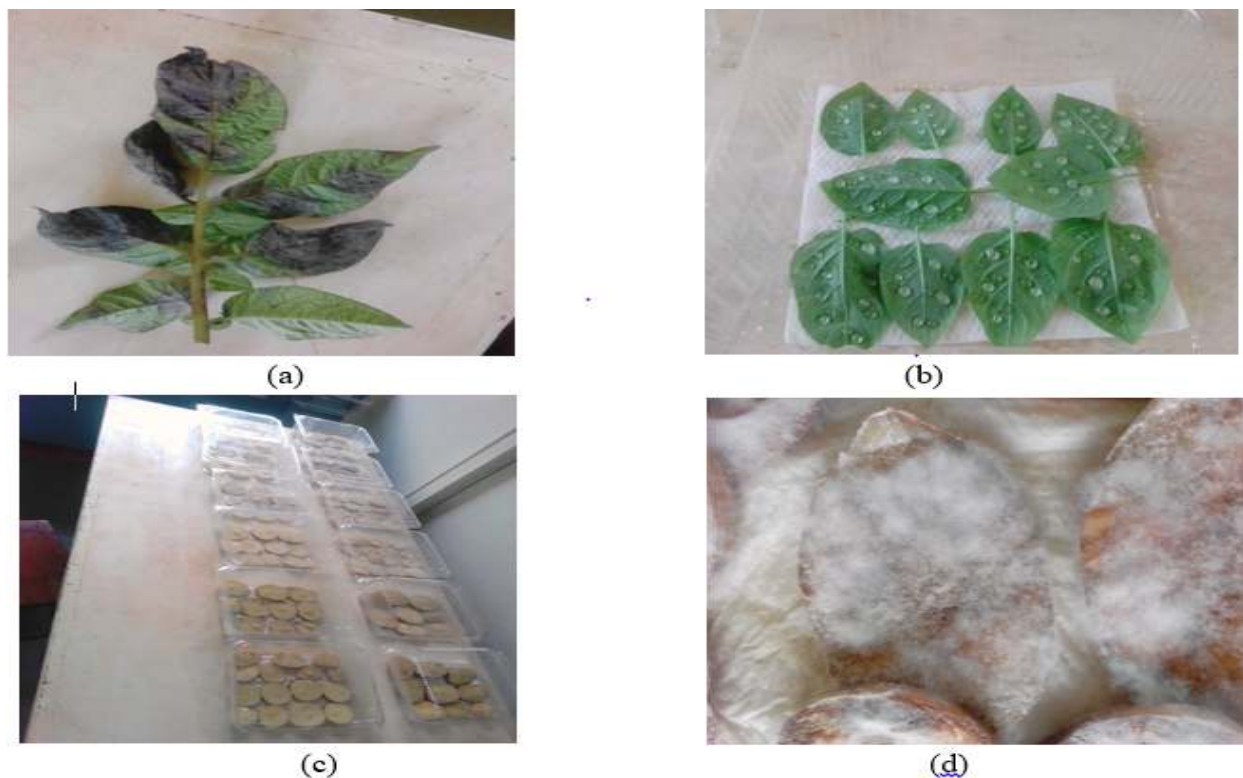


Plate 1: Isolation and bulking of *P. infestans*. (a) Freshly blighted leaves, (b) inoculated leaf tissue, (c) potato slices with *P. infestans* suspension for pathogen bulking (d) mycelial growth on potato slices.

3.2.2 Amplification of DNA from *P. infestans* using Polymerase Chain Reaction (PCR)

To confirm that the isolate used in this study was *P. infestans* a molecular test was conducted in laboratory at Biosciences of Eastern and Central Africa (BecA) Hub, International Livestock Research Institute (ILRI), Nairobi, Kenya. DNA samples were extracted from mycelia obtained from infected tuber slice (5 samples) as shown above in Plate 2 d, leaf tissues (2 samples) and pure culture of *P. infestans*. Extraction of DNA was performed using sodium hydroxide extraction method (Wang *et al.*, 1993). Each sample was placed in eppendorf tubes and subsequently 90 μL of 0.5 M NaOH added to each tube. The contents were ground using hand held plastic pestle in a mortar until the samples liquefied and immediately 3 μL of the solution from each tube was transferred to new tubes containing 300 μL of 100 mM Tris buffer, pH 8.0. DNA in the samples was amplified in PCR reactions. Negative control (non-template control) was included. Polymerase Chain Reactions were performed using AccuPower Taq PCR 2 \times master mix (Bioneer), 3 μL DNA sample as template and Internal Transcribed spacer (ITS₅)

(GGAAGTAAAAGTCGTAACAAGG) as forward primer and a species specific primer *pinf2* (CTCGCTACAATAGCAGGCGTC) as the reverse primer (Njoroge *et al.*, 2019b), PCR conditions were set as follows: initial denaturation at 96 °C for 2 minutes followed by 35 cycles consisting of denaturation at 96 °C for one minute, annealing at 55 °C for one minute, extension at 72 °C for two minutes and a final extension at 72 °C for 10 minutes followed in that order. Agarose gel electrophoresis of 1% was performed containing 0.5 mgL⁻¹ of ethidium bromide. To prepare the gel, extracted samples were loaded in the wells alongside, test *P. infestans* DNA (positive control), while negative control and 100 base pairs DNA ladder (Bioneer cat. No. D-1030) were loaded in to the wells to the right as described by Njoroge *et al.* (2019b). Visualization of successful amplicons was done in agarose gel electrophoresis.

3.2.3 Antifungal Bioassay through Co-inoculation of *T. asperellum* and *P. infestans*

Co-culture was conducted to determine the inhibition of *P. infestans* caused by the *T. asperellum* as described by Fatima *et al.* (2015). *Trichoderma asperellum* pure spores were obtained from Real IPM Company, Kenya and their viability confirmed on Potato Dextrose Agar (PDA). Petri dishes (9 cm) containing PDA were inoculated with 0.5 × 0.5 cm *P. infestans* mycelial plug cut using sterilized surgical blades and incubated at 18 °C for 48 hours. This was followed by introduction of *T. asperellum* suspensions prepared as follows: 0.1 g of *T. asperellum* spores powder were weighed and placed in falcon tube containing 10 mL of distilled water and adjusted to 1.0 × 10⁷ CFU/mL (100%) using hemocytometer. *Trichoderma asperellum* at 66% (7.0 × 10⁶ CFU/mL) and 33% (3.0 × 10⁵ CFU/mL) concentrations were achieved by varying the 0.1 g of *T. asperellum* with the respective percentage followed by adjustment using hemocytometer. This was mixed with 500 g of coarse sorghum grains and incubated at room temperatures (18 ± 2 °C) for 3 days to initiate sporulation. A suspension of 20 µL was pipetted into PDA plates bearing *P. infestans* about 1 cm away. Positive control plates were prepared from Ridomil[®] (metalaxyl 4% + mancozeb 64%) at rate of 2.5g/L and Mistress 72[®] (cynamoxil 4% + mancozeb 64%) at rate of 2 g/L and 20 µL droplet inoculated separately on the mycelia of *P. infestans*. Negative control consisted of plates with *P. infestans* alone. The plates were then incubated at room temperature (18 ± 2 °C) under alternating lighting of 12-hour light and 12-hour darkness for 7 days (Goufo *et al.*, 2017). The treatments were laid in completely randomized design with three replications. The experiment was repeated two times and observations were made using optical microscope on 3rd,

5th and 7th days after inoculation. Inhibition of the test *T. asperellum* and the controls were determined by the percentage of mycelial growth inhibition calculated by the formula below as described by Yao *et al.* (2016):

$$I = \frac{R1-R2}{R1} \times 100 \dots \dots \dots \text{I}$$

Where:

I represent percentage reduction of growth (inhibition) of the fungi, R1, diameter of radial growth of pathogenic fungus in control plates in mm and R2 diameter of radial growth of *P. infestans* in presence of *T. asperellum* concentrations in mm.

3.2.4 Antagonistic Effects of *T. asperellum* on *P. infestans* in Detached Leaflet Assay

Healthy leaflets which were approximately equal in size (6 cm long × 4.5 cm wide) from middle canopy were detached from apical cuttings (6-7 weeks old) in glasshouse using sterilized scalpel. The leaflets were washed with sterilized distilled water and their bases covered with moist cotton wool to reduce desiccation (Goufo *et al.*, 2008). *Trichoderma asperellum* concentrations and positive controls (Mistress 72[®] and Ridomil[®]) suspensions in a shallow dish were applied by dipping the leaves for 2 seconds on the abaxial side. The leaves were then placed upside down (abaxial surface up) in 20 cm (length) × 18 cm (width) × 6 cm (depth) plastic dishes lined with a wet serviette paper to maintain humidity (6 leaflets per dish). *Phytophthora infestans* suspension was adjusted to 1 × 10⁴ zoospores/mL using hemocytometer and 40 μL was applied on the abaxial side of the leaflets using micropipette. The negative control included leaves inoculated with *P. infestans* alone. The plastic dishes while open were placed in laminar flow hood for about 5 minutes to air dry the leaflets and then incubated at room temperatures (20 ± 2 °C) for 24 hours. The treatments were laid in completely randomized design with three replications and measurements on lesion size taken after 3 days and then once after every two days for two weeks. The lesion size was measured and area calculated using the formula below as described by (Goufo *et al.*, 2017):

$$S = \frac{\pi(L+W)^2}{4} \dots \dots \dots \text{II}$$

Where S, L and W represents area, length (cm) and width (cm) of the lesion for each detached leaflet respectively. π = 3.14

3.2.5 Compatibility of *Trichoderma asperellum* with Ridomil® and Mistress 72®

Potato dextrose agar (unamended) was inoculated with 20 µL of *T. asperellum* suspension adjusted to 1×10^7 CFU/mL. The plates were incubated at 20 °C for 7 days. The mycelial plug of *T. asperellum* from the pure culture was scrubbed off using a sterile spatula and placed in 10 ml of distilled sterilized water in eppendorf tube. A droplet of 40 µL (1×10^7 sporangia/mL) of the suspension was inoculated on fresh PDA plates and incubated for 48 hours. Likewise, a droplet of 40 µL from Ridomil® (2.5g/L) and Mistress 72® (0.5 g/L) suspension was applied on the developing *T. asperellum* mycelia in PDA plates. The plates were then incubated at 20 °C for 7 days. The experiment was laid out in completely randomized design with 4 replications. Mycelial growth was observed and measured under optical microscope at 3, 5 and 7 days after inoculation.

3.3 Data Analysis

Statistical model was

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij} \dots\dots\dots\text{III}$$

Where Y_{ij} , μ , τ_i and ε_{ij} refer to variable response, overall mean, effect due to treatment i^{th} and random error due to response from i^{th} treatment of sample j^{th} respectively.

Data on percentage inhibition of *P. infestans* by *T. asperellum* and lesion size on detached leaf assay (first transformed using the square root ($x + 0.5$)) (Fontem *et al.*, 2005) were subjected to analysis of variance (ANOVA) using Statistical Analysis System (SAS) version 8.2. Treatment means were separated using Tukey's Honest Significant Difference (HSD) whenever analysis of variance showed significant difference ($P \leq 0.05$) among the treatment means.

3.4 Results

3.4.1 Confirmation of *P. infestans*

Phytophthora infestans sporangia viewed under stereoscope microscope and image (Plate 3a) captured using Optika software version 3.2 showed lemon shaped sporangia (Plate 2a) that germinated directly by producing germ tube when temperature range was 10 to 18 °C which is the characteristic feature of the pathogen (Matson *et al.*, 2015). Polymerase Chain Reaction (PCR) revealed expected band size of 600 base pairs (bp). The band length of the tested samples used in the present study (Plate 2b) matched the positive control.

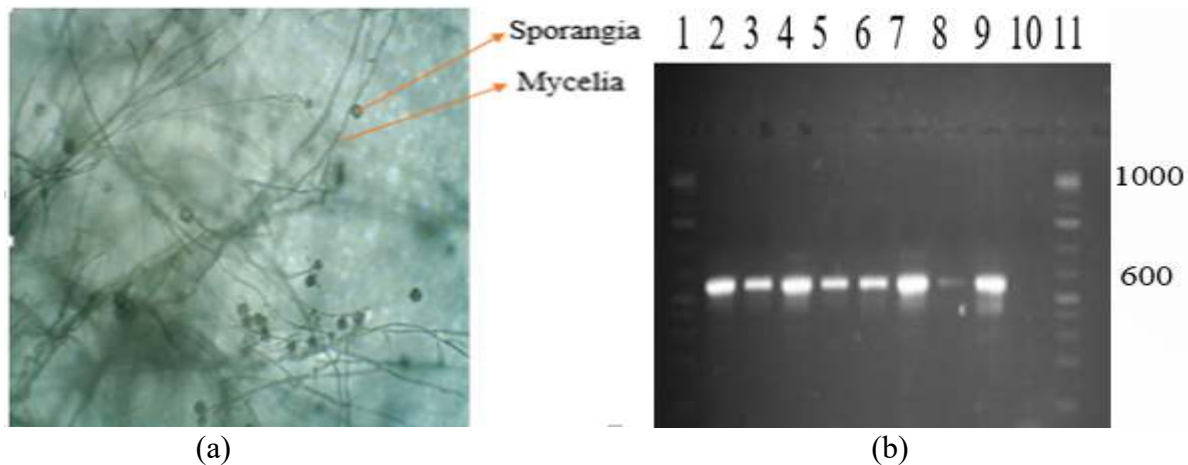


Plate 2: Identification of *P. infestans* (a) Lemon shaped sporangia and mycelia of *P. infestans* under compound microscope and (b) amplified PCR products from *P. infestans* DNA mycelium obtained from infected tuber and leaf (2 to 8), pure culture (positive control) of *P. infestans* (9) and negative control (10) while 1 and 11 is the ladder.

3.4.2 Antifungal Bioassay

The antagonistic activity of *T. asperellum* against *P. infestans* was observed in *T. asperellum*-*P. infestans* co-culture but depended on the biocontrol inoculum concentration. *Trichoderma asperellum* at 66% and 100%, positive control significantly ($P \leq 0.05$) inhibited *P. infestans* mycelial growth *in vitro* (Plate 3). Mycelial growth inhibition was clearly observed on the third day reaching 89.7% and 89.3% but showed no observable change in growth after the fifth day on plates treated with Ridomil® and Mistress 72® respectively. Higher *T. asperellum* mycelial growth rate at 66% (64 mm) and 100% (65 mm) than that of *P. infestans* (42 mm) in pure culture was observed from third day after inoculation. *Phytophthora infestans* plates treated with *T. asperellum* concentrations at 66% and 100%, Ridomil® and Mistress 72® were not significant different ($P \leq 0.05$) by third day after inoculation. *Trichoderma asperellum* concentration at 33% gave the lowest inhibition of 37.3, 46.0 and 38.0 mm in co-culture by 3, 5 and 7th day respectively after inoculation compared to 66% and 100% concentration (Table 1). Mycelial growth of *P. infestans* continued even in presence of *T. asperellum* at 33% concentration. In the dual culture between *P. infestans* and 33% *T. asperellum* had the lowest mycelial growth than any other treatment. In pure cultures, *P. infestans* had a higher mycelial growth than *T. asperellum* at 33% concentration but lower than that of *T. asperellum* at 66% and 100% concentration. In addition, 66% and 100% *T. asperellum* concentration co-culture showed a plateau at day three while for

33% dual culture showed continuous growth. An increase in *T. asperellum* inoculum concentration from 33% to 100% resulted in enhanced growth restriction of pathogen from 3rd to 7th Day after inoculation (Table 1)

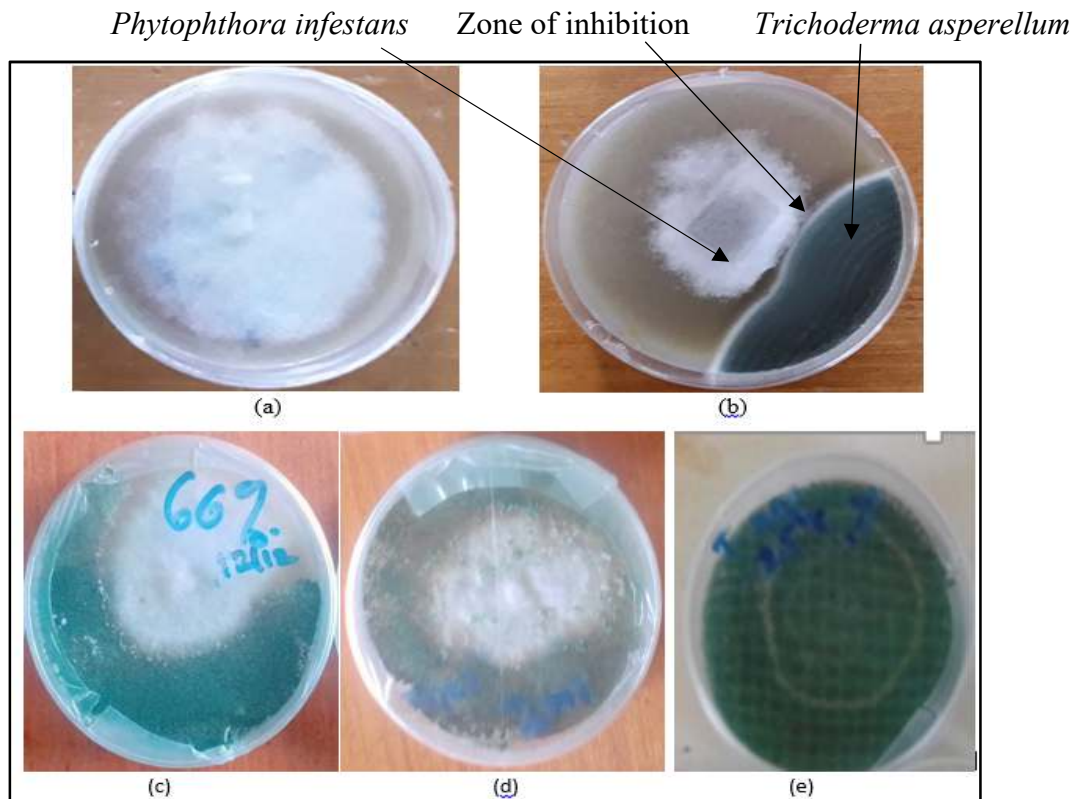


Plate 3: The antifungal effects of *T. asperellum* on *P. infestans* in dual culture; (a) pure culture of *P. infestans*, (b) *T. asperellum* concentrations at 33%, (c) 66% (d) 100% (e) pure culture of *T. asperellum* after 5 days.

Table 1: Effect of *P. infestans* growth inhibition (percentage) induced by *T. asperellum*

Treatment	Radial growth inhibition (mm) across Days After Inoculation		
	Three	Five	Seven
Ridomil®	80.30a	89.70a	100.0a
Mistress 72®	79.50a	89.30a	100.0a
<i>T. asperellum</i> 100%	80.00a	88.30a	91.30a
<i>T. asperellum</i> 66%	80.0a	88.00a	91.10a
<i>T. asperellum</i> 33%	37.3b	46.0c	38.0c
R ²	0.993	0.996	0.995
CV%	6.63	4.24	4.82
HSD	11.12	7.24	7.97

Means followed by same letter in each column are not significantly different ($P \leq 0.05$)

3.4.3 Determination of Antagonistic Effects *T. asperellum*-*P. infestans* in Detached Leaflet Assay

Trichoderma asperellum, Ridomil® and Mistress 72® influenced late blight lesion size in detached leaflet assay. Lesion size was progressively reduced in day three by 4%, 14% and 16% and by 1%, 47% and 49% in 11th day after inoculation by *T. asperellum* at 33%, 66% and 100% concentrations respectively (Table 2). There was no significant difference ($P \leq 0.05$) in lesion size associated with *T. asperellum* at 66% (1.91 cm²) and 100% (1.89 cm²) concentrations at 11 days after inoculation. Lesion expansion was curtailed in detached leaflets treated with Mistress 72® and Ridomil® (Plate 4). During the first 5 days after inoculation, *T. asperellum* was not significantly different from negative control (*P. infestans* alone) but thereafter the treatment of *T. asperellum* at 66% and 100% concentrations reduced lesion size significantly as compared to *T. asperellum* at 33% and the negative control.

Table 2: Effect of *T. asperellum* applied at different concentrations on lesion size (cm²) of potato late blight on detached leaf assay

Treatment	Lesion size at Days after inoculation				
	3	5	7	9	11
<i>P. infestans</i>	1.73a	2.23a	2.82a	3.05a	3.55a
<i>T. asperellum</i> 33%	1.66a	2.12a	2.62a	2.94a	3.50a
<i>T. asperellum</i> 66%	1.49a	1.66a	1.78b	1.89b	1.91b
<i>T. asperellum</i> 100%	1.46a	1.62a	1.72b	1.86b	1.89b
Mistress 72 [®]	0b	0b	0c	0c	0c
Ridomil [®]	0b	0b	0c	0c	0c
R ²	0.813	0.950	0.989	0.986	0.996
CV%	5.55	4.65	3.25	4.22	2.892
HSD	0.249	0.251	0.206	0.287	0.215

Means followed by the same letter within the same column are not significantly different at $P \leq 0.05$

Phytophthora infestans necrotic lesions

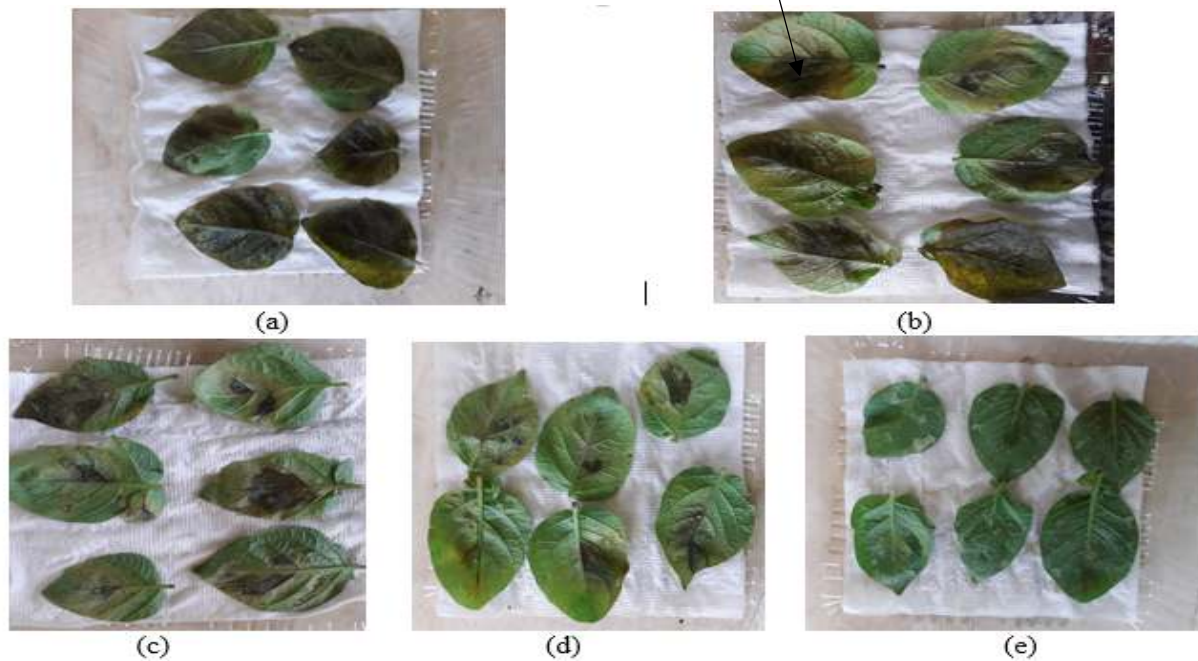


Plate 4: Detached leaf assay at day five after inoculation *P. infestans* (a) negative control, (b) *T. asperellum* at 33%, (c) 66%, (d) 100% concentration (e) Ridomil[®] inoculated on healthy detached leaves

3.4.4 *Trichoderma asperellum* Compatibility with Ridomil® and Mistress 72®

Trichoderma asperellum pure culture colony grew on Potato Dextrose Agar (PDA) forming a white concentric rings that changed to green as it matured after 5 days. Establishment and development of *T. asperellum* was not inhibited by Ridomil® and by Mistress 72® *in vitro*. *Trichoderma asperellum* mycelia continued to grow on PDA over the incubation period in presence of Ridomil® and Mistress 72®. Radial mycelial growth for pure *T. asperellum* culture, *T. asperellum* + Ridomil® and *T. asperellum* + Mistress 72® were not significant different. However, Mistress 72® showed some *T. asperellum* mycelial growth suppression than Ridomil® across all days after inoculation (Figure 1).

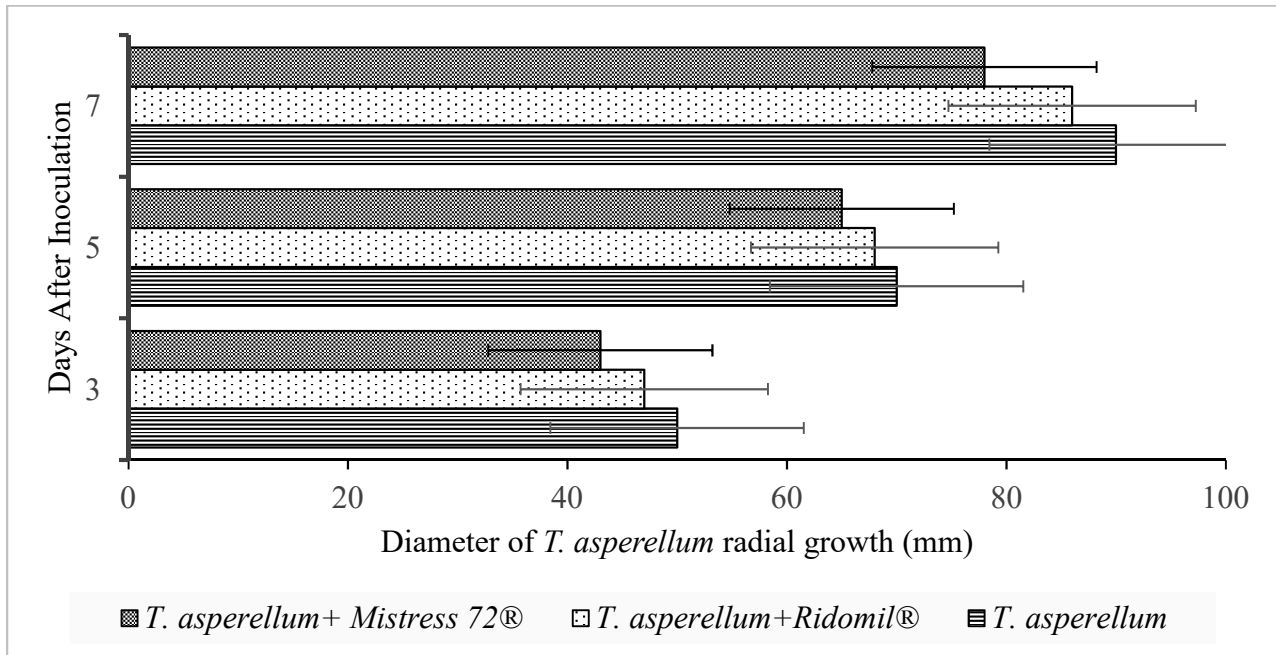


Figure 1: Effect of Ridomil® and Mistress 72® growth inhibition on *T. asperellum* *in vitro*

3.5 Discussion

Phytophthora species are difficult to identify based on morphology because they lack clear physical and morphological distinguishing characteristics under different culture conditions (Skelsey *et al.*, 2010). There are over 120 known species of *Phytophthora* which infect different host plants (Hardham, 2007). The species develop distinct cellular structures that can only be confirmed by PCR technique after visualization under microscope. This ensures that the study is conducted with the right organism. *Phytophthora infestans* produce lemon shaped sporangia which

are unique to them. Pure culture of *P. infestans* from plate media agar is less pathogenic than a sample collected from infected host tissues. Therefore infecting host tissues with pure culture of the pathogen first and then harvesting the mycelia is effective for artificial inoculation (Jupe *et al.*, 2013).

The use of biological agents to suppress plant diseases including *P. infestans* has been reported in previous studies (Fatima *et al.*, 2015; Miles *et al.*, 2012; Syed *et al.*, 2018). The results of the present study indicate that *T. asperellum* is aggressive and pathogenic to *P. infestans* but this inhibitory action is underpinned by the biocontrol inoculum concentration. These findings are consistent with Kipngeno *et al.* (2015) results who reported the efficacy of *Bacillus subtilis* and *T. asperellum* on *Pythium aphanidermatum* on tomato (*Lycopersicon esculenta*). Positive results were also reported by Bahramisharif and Rose (2018), Fatima *et al.* (2015), Istv (2014), Widmer (2014) and Yao *et al.* (2016) in their studies in effort to manage potato diseases using biocontrols. However, there is need to explore new biological agents from the vicinity of host plant that may have a better biocontrol activity against phytopathogens or even their spectrum of action to enhance effective disease control. Therefore, this study attempted to explore the effects of *T. asperellum* on *P. infestans* to widen the biocontrol's action spectrum against pathogens.

The treatments with *T. asperellum* at 66% and 100% concentrations had a higher sporulation and competing ability filling up the PDA plates faster than *P. infestans*. The faster growth observed in *T. asperellum* led to overgrowing on the *P. infestans* mycelia overwhelming the pathogen. High rate of sporulation and colonization observed in the biocontrol compared to the pathogen are the key important traits for excellent biocontrol (Xu *et al.*, 2011). Zone of inhibition was observed between the two fungi which could be attributed to the effect of diffusible products released by the *T. asperellum* that suppressed further growth of the *P. infestans*. The continual growth of *P. infestans* mycelial in presence of *T. asperellum* at 33% concentration indicated that concentration of biocontrols is an important factor in their action against disease causing microorganisms. Similar results were reported by Patel and Saraf (2017) on efficacy of *T. asperellum* against *Fusarium oxysporium* on tomato that caused decrease in wilting severity by 85%. The results suggests that, *T. asperellum* mycoparasitism could manage late blight of potato. Sharma *et al.* (2017) reported *Trichoderma* spp. mycoparasitism and competition mode of action against phytopathogens. The inhibition zone (white mycelial growth) observed indicated release of metabolite products by the biocontrol (Widmer, 2014). The antagonistic activity of *T.*

asperellum against *P. infestans* revealed mycoparasitism of *P. infestans* as indicated by the presence of white mycelial between the two fungi confirms results of Itachi *et al.* (2007). Similar mycoparasitic actions were observed in *T. viride* antagonistic activities against *P. infestans in vitro* (Ephrem *et al.*, 2011) The absence of close inhibition zone observed in dual culture associated with 33% *T. asperellum* - *P. infestans* interaction suggests that the biocontrol concentration was low and therefore unable to release sufficient metabolites to overcome similar products released by the pathogen. This was consistent with Sharma (2011) study who reported Fusarium wilt - *Trichoderma* chemical signal interactions. Furthermore, *T. asperellum* at 33% concentration increase in mycelial growth was low compared to other treatments in the co-culture possibly due to ‘tag of war’ involving metabolites that have to be secreted first and in sufficient amounts before being released by the biocontrol and pathogen against each other where the strong one overcome the other. Leonetti *et al.* (2017) and Naglot *et al.* (2015) reported that salicylic acid signaling pathway and enzymatic activities respectively have to be initiated before mycelial growth in presence of developing pathogen. This suggests that for a biocontrol to be effective it should have high reproduction capacity and exhibit strong continuous release of metabolites that cause antibiosis, cell wall degradation and mycoparasitism to overwhelm the crop pathogens as reported by Wu *et al.* (2017).

Trichoderma asperellum at 66% and 100% mycelia growth was directed towards the *P. infestans* indicating chemotropism towards the pathogen as observed by Sharma (2011). However, the present study reported the antifungal activities of *T. asperellum* against *P. infestans* being slow regardless of its concentration allowing the pathogen to partially grow compared to Ridomil® and Mistress 72® treatments that effectively inhibited *P. infestans* growth. The effectiveness of Ridomil® and Mistress 72® could be attributed to the fact that their fast action and ability to act by targeting specific region of the pathogen (Sharma & Saikia, 2013).

The trend in slowed increase in lesion size from day three to eleven after inoculation indicate that *T. asperellum* are slow acting just like other biocontrols as reported by Lal (2017). *Trichoderma asperellum* at 66% and 100% concentration inhibited late blight lesion increase while Ridomil® and Mistress 72® did not allow lesion establishment. *Trichoderma asperellum* at 33% concentration lesion size was similar to that of *P. infestans* alone (control) providing further evidence that concentration of the biocontrol is a key factor. Even though in the first 5 days after inoculation, lesion development was not significant different between *P. infestans* and *T.*

asperellum concentrations and among *T. asperellum* concentrations, *T. asperellum* at 66% and 100% concentrations in the long run managed the disease lesion reducing further increase. This further demonstrates biocontrols have slow action against phytopathogens. The biocontrol needs to mobilize resources and establish first before tackling the pathogen and detect pathogen signals to secrete enzymes, pathogenesis related proteins and metabolites that take time giving the pathogen advantage to establish and grow if they delay or if produced in insufficient amounts. By 7th day after inoculation, the observed antifungal activity could be attributed to a faster growth (competition) of the *T. asperellum* compared to *P. infestans* and release of secondary metabolites (defense mechanisms) by the biocontrol (Amin *et al.*, 2010). Schuster and Schmoll (2010) reported presence of cell wall degrading enzymes including the glucanases released by *Trichoderma* spp. that degrade *P. infestans* cells affecting their growth. This study further provides evidence that a biocontrol concentration is an important characteristic in biological agents for them to be effective whose literature is limited. Thus, it appears *T. asperellum* at 66% concentration could be adopted to manage *P. infestans* under field conditions to reduce the overuse of fungicides that could led to emergence of new strains that tend to be aggressive and require increased fungicide application (Childers *et al.*, 2014) to be effective. This pose threat to environment, human population and increased cost of production (Cooke *et al.*, 2011). This study provides evidence that a biocontrol concentration is an important characteristic in biological agents for them to be effective which was missing in literature.

In Ridomil[®] and Mistress 72[®] and *T. asperellum* compatibility experiment, the biocontrol radial mycelial growth was not inhibited by either Ridomil[®] or Mistress 72[®]. Thus, the results indicated possibility of combining *T. asperellum* with synthetic fungicides to effectively manage late blight. Aparecida *et al.* (2018) reported that *T. asperelloides* reduced *Sclerotinia minor* growth more when combined with Azoxystrobin while Agbeniyi *et al.* (2014) reported that *T. asperellum* was able to reduce cacao pod rot severity when combined with mancozeb fungicide. However, Mistress 72[®] had some suppression on *T. asperellum* mycelial than Ridomil[®] which need to be explored in future studies. The two fungicides have been co-formulated with mancozeb in similar concentration. In addition to mancozeb, Ridomil[®] has metalaxyl while Mistress 72[®] has cynamoxil. This suggests cynamoxil may have inhibition aspects if its concentration was increased while *T. asperellum* could tolerate metalaxyl better. Further studies should determine effect of cynamoxil on beneficial organisms especially where its concentration is increased.

CHAPTER FOUR
EFFICACY OF *Trichoderma asperellum* SEED TREATMENT AND RIDOMIL®
APPLICATION INTERVAL ON SEVERITY AND INCIDENCE OF LATE BLIGHT
AND YIELD OF POTATO

Abstract

Seed potato with latent infection of *Phytophthora infestans* is implicated in initiation and transmission of late blight early in cropping seasons. Field experiments were conducted to determine the efficacy of *Trichoderma asperellum* through seed treatment and foliar application of Ridomil® on late blight. Ridomil® was applied at an interval of 7, 14 and 21 days interval separately on crops from rooted apical cuttings and whole seed tuber which had been pre-treated with *T. asperellum* at 33% (3.0×100^6), 66% (7.0×100^6) and 100% (1.0×100^7 CFU/mL) concentrations. A separate experiment was also conducted to compare seed treatment by pericardial injection versus dipping. Results revealed that 7 and 14 days spray interval were not significantly different in terms of yield and late blight severity. Yield was reduced by 77% in treatments associated with 21 days interval in comparison to 7 days interval. Rooted apical cuttings had 7.4 % higher disease severity that led to 2.3% lower yield than crop from seed tubers. Sprout emergence and survival in untreated plots was reduced by 32% and 21% on seed tuber and apical cuttings respectively. Disease severity and yield observed on untreated and *T. asperellum* at 33% concentration plots did not differ significantly ($P \leq 0.05$). *Trichoderma asperellum* at 66% and 100% concentrations were not significantly different and reduced disease severity by 26%, and 27% while increasing yield by 30% and 29% respectively relative to untreated control. Pericardial injection had 7.8 % higher disease score than dipping. Combination of *T. asperellum* at 66% and 100% concentration with 7 and 14 days spray interval provided better late blight management. The results suggest that seed treatment by dipping in *T. asperellum* at 66% and 100% concentration could increase fungicide application interval by 7 days to manage late blight on potato while improving on yield.

Key words: Apical cuttings, Efficacy, Ridomil®, Seed treatment, *Trichoderma asperellum*

4.1 Introduction

Late blight is an important disease of potato that reduces potato yield significantly threatening food security globally. The fungus infects all parts of the potato plant including foliage, stem and tuber (Keil & Benker, 2010). Tuber and stem infection has been attributed to emergence of new aggressive *P. infestans* strains that defoliate and kill the crop within a short time if no timely interventions are made (Kiiker *et al.*, 2018). Moreover there are few synthetic chemicals that can control late blight once it infects the stem and tuber (Runno-paurson *et al.*, 2014). In East Africa, potato seed shortage is a recurring problem facing potato farmers. This has led to high seed and transport cost even though Gichuru and Dijk (2013) showed that farmers were willing to buy certified seed. Seed shortage has led to adoption of farm saved seed including buying potato seed from neighbours or from the market that could be latently infected (Gildemacher *et al.*, 2011). In addition, production and multiplication of certified seed in Kenya and most Sub Saharan countries is based on use of seed tubers which are multiplied in the field increasing the risk of seed tubers acting as carrier of inocula to the next season (Yilmaz, 2017). Seed multiplication using seed tuber also results in small sized tubers and volunteer crop left in soil in the field that harbour *P. infestans*. Resulting early infections also require fungicide applications to start early in the season (Zwankhuizen *et al.*, 2000).

Latent infection causes increased outbreaks of late blight early in the cropping season that require farmers to adopt short interval fungicide application regimes (Liljeroth *et al.*, 2016; Subhani, 2016). In addition, new clones of *P. infestans* that are resistant to fungicide and adaptive to newly released resistant varieties has worsened the situation (Cooke *et al.*, 2011). Crops severely affected by late blight result in production of sporangia that cause tuber blight when the infected crop is defoliated during wet periods (Leesutthiphonchai *et al.*, 2018). Most fungicides used to manage tuber blight are ineffective or have little to no activity against *P. infestans* especially when applied after the disease has already established (Champouret *et al.*, 2018; Inglis *et al.*, 1999; Leesutthiphonchai *et al.*, 2018).

Use of seed tuber contaminated by the pathogen accelerates late blight severity and therefore use of disease free seed including apical cuttings could contribute to reduction *P. infestans* inoculum and spread from one farm to another (Vleeshouwers *et al.*, 2011). Therefore seed health forms a key factor in determining the crop yield (Thomas-sharma *et al.*, 2016; Webster *et al.*, 2017). Gildemacher *et al.* (2009a) reported that, there is need for improving the quality of

seed system in Eastern Africa to fill the seed deficit gap. Rooted apical cuttings technology enhances rapid multiplication of disease free seed material (Kaur *et al.*, 2017). The technology has the potential to increase seed supply when combined with modern seed potato production technologies including aeroponics, hydroponics, stem cuttings to produce disease free materials (Muthoni *et al.*, 2011). However, there is limited literature on the contribution of rooted apical cuttings to management of late blight and yield improvement under field conditions.

Integrated disease management programme incorporating biological agents could offer sustainable alternatives in managing late blight to enhance yield that match demand for the product (Forrer *et al.*, 2017; Pacilly, 2018). *Trichoderma spp.* is the most widely used biological agent globally to manage various crop diseases. The fungus is known to grow very faster than the phytopathogen enforcing a competitive action as well as secreting cell wall degrading enzymes that inhibit growth and survival of the pathogens (Ravensberg & Urbaneja, 2018). *Trichoderma* species have been studied by various scientists in managing potato diseases (Ha, 2010; Harman *et al.*, 2004; Howell, 2007; Sharma *et al.*, 2017; Wu *et al.*, 2017). *Trichoderma harzianum*, *T. atroviride* and *T. viride* have been found to manage crop diseases including late blight on potato. Fatima *et al.* (2015) reported that, *T. harzianum* reduced late blight epidemics on potato and tomato. When *T. viride* was applied on tomato, it reduced late blight severity while improving potato production (Chaouch, 2016).

Trichoderma asperellum also has biofertilizer properties in addition to its ability to induce systemic plant disease resistance against *Pythium*, *Fusarium* and *Rhizoctonia* species soil borne pathogen. Recently, Guzmán-guzmán *et al.* (2017) found that, when applied on plant foliage, *T. asperellum* could induce plant defense mechanisms and promote growth. However, efficacy of *T. asperellum* through seed treatment prior to planting to prevent or delay late blight epidemics has not been explored. Moreover, biocontrols alone are ineffective in managing late blight on potato because they are slow acting, and prefer certain conditions that favour their establishment (Lal *et al.*, 2017) while synthetic fungicides have a fast mode action and target specific sites in the pathogen (Andrew *et al.*, 2010). Use of fungicides as a sole strategy also faces inefficiency challenges especially where emergence of new *P. infestans* have being reported. Therefore, combining biocontrols and disease free seed with reduced fungicide application could be a reliable and sustainable option in managing late blight.

The present study aimed at establishing effective *T. asperellum* seed treatment concentration against late blight in order to increase Ridomil® application interval on rooted apical cuttings and tuber seed of potato. This was expected to will substantially reduce the cost of production, minimize risks to human health (reduced exposure and chemical handling) and the environmental (reduced seepage to water sources and biodiversity) and counter emergence of fungicide resistant strains while improving potato production and income among farmers.

4.2 Materials and Methods

4.2.1 Description of Study Site

The study was conducted at Kenya Agricultural and Livestock Research Organization (KALRO) Tigoni, in Limuru, Kiambu County from September 2018 to August 2019. The Centre is located at latitude 10° 9' 22" south and longitude 36° 4' 72" east. The area experiences a bimodal rainfall pattern with average rainfall of 1800 mm per annum, temperature ranging from 10°C to 25°C and at altitude of 2300 m above sea level (Jaetzold *et al.*, 2006). Weather experienced in this area provides conducive conditions for late blight development at any stage of potato growth. The experiment was conducted in a field which had not been cropped with potato for the previous three years.

4.2.2 Production of Potato Rooted Apical Cuttings

In vitro plantlets of tissue cultures of *Shangi* variety were produced at the plant tissue culture laboratory at KALRO Tigoni. Cultures were maintained on Murashige and Skoog medium (Murashige, 1962) that contained sucrose (3%, w/v) and agar (Technical number 2) in culture bottles. The *in vitro* plantlets (Plate 5a) were sub cultured at regular interval of 21 days and placed in growth chamber maintained at 18 °C under light intensity 42 mol m⁻² s⁻¹ provided by fluorescent lamps in 18 hours photoperiod regime. To harden off, the *in vitro* plantlets (8-10 cm) were transferred to horticultural germination trays containing peat moss (Plate 5b) in the glass house and covered with polythene paper to enhance uniform humidity. The plantlets were transferred into 300 g cups containing a mixture of sand and coco peat (1:1) after 7 days. Rooted apical cuttings were developed by cutting the apical part (primordial leaf + 2 leaves) of the ex- tissue culture plants. Rooting hormone (indole butyric acid) was applied at the cut end of the stem and planted *in vitro* tray containing peat moss. This was placed in a grid covered with polythene bag

in glasshouse for 10 days. The seedlings were then transferred into the cups (Plate 5c) described above and placed on the bench in the glasshouse to enhance acclimatization and hardening off for about 3 weeks before field transplanting.



Plate 5: Production of apical cuttings (a) *in vitro* plantlets established in culture bottles, (b) *in vitro* plantlets on horticultural germination trays, (c) established rooted apical cuttings

4.2.3 Experimental Materials and Land Preparation

Rooted apical cuttings aged 4 weeks with a height of 20 cm were produced at KALRO Tigoni's rapid multiplication facility while sprouted whole tuber seed of about 45 mm grade size (basic seed class) were provided by the Seed Program of KALRO Tigoni Centre. The variety is one of the most widely grown in Kenya because it is excellent for French fries and table consumption (Mutunga, 2014). The variety yields 35 to 40 t ha⁻¹ and takes 3 to 4 months to mature. It is susceptible to late blight and moderately drought tolerant. Ridomil® was obtained from local agro-stockist. The fungicide is one of the widely used fungicide in Kenya (Taylor *et al.*, 2013). Primary and secondary cultivation was conducted during dry periods (September 2018 and March 2019) before planting to remove weeds and break soil clods on fallow land.

4.2.4 Seed Treatment with *T. asperellum*

Whole tubers were cleaned using tap water to remove soil, rinsed with distilled water and surface sterilized by dipping in 5% sodium hypochlorite for 15 seconds. The tubers were rinsed with sterilized distilled water to remove excess alcohol and air dried in the shade for 15 minutes. Suspensions of *T. asperellum* at 33% (3.0 x 10⁶ CFU/mL), 66% (7.0 x 10⁶ CFU/mL) and 100% (1 x 10⁷ CFU/mL) concentration of Manufacturer Recommended Rate (MRR) as described in chapter

three were used to inoculate the seed. Mistress 72[®] (2 g L⁻¹; positive control) suspension was also prepared. From each standardized suspension, 3 litres were prepared in a bucket which was changed after every seed treatment. Cleaned intact tubers were placed in a netted bag and dipped in the *T. asperellum* concentration for 15 seconds. The tubers were air-dried, incubated in wooden store for 24 hours and then inoculated with *P. infestans* by dipping in a zoospore suspension adjusted to 4×10^4 zoospores/ml. Similarly rooted apical cuttings were cleaned with distilled water and inoculated by dipping for 15 seconds. The inoculated rooted apical cuttings were placed in sterilized distilled water and incubated for 24 hours in diffused lit wooden store (18 ± 2 °C) to allow uptake of the biocontrol.

4.2.5 Experimental Design and Planting

Treatments were laid in randomized complete block design in split split plot arrangement with three replications. Fungicide application regime (unsprayed, 7, 14 and 21 days interval) and seed type (apical cutting and tuber seed) were main plot and subplot respectively while seed treatment suspensions (untreated, mistress 72[®] and *T. asperellum* at 33%, 66% and 100% concentrations) were randomized within the subplots. The sub-sub plots measured 2.1 m \times 2.1 m with crop spacing of 0.75 m \times 0.3 m and a path of 1.5 m width between sub plots and 2 m between the main plots to avoid fungicide drifts. At onset of rains, tuber seed was planted first and rooted apical cuttings planted 24 hours later. Diammonium Phosphate (DAP) at 500 kg ha⁻¹ was applied and mixed with soil in planting furrows. Calcium Ammonium Nitrate (CAN) was applied at rate of 440 kg ha⁻¹ four weeks after transplanting.

4.2.6 Field Inoculation and Fungicide Application

Artificial inoculation of the whole field was performed using zoospores suspension adjusted to 4×10^4 zoospores/ml using hemocytometer. Inoculation was conducted in the evening hours using a calibrated hand sprayer at a rate of 150 ml of sporangia solution per m². This was done at 18 Days After Emergence (DAE) applied to the external rows to enhance uniform disease spread and infection. Overhead irrigation was done a day before inoculation and in the next two days after inoculation in the morning and late evening to induce *P. infestans* infection. Ridomil[®] application was initiated upon appearance of the first late blight symptoms four days after pathogen inoculation which depicts strategy used by the farmers. The fungicide spray regimes were;

unsprayed (negative control) 21 days interval applied on 22, 43 and 64 DAE, 14 days interval applied on 22, 36, 50 and 64 DAE and 7 days interval (manufacturer recommended frequency) applied on 22, 29, 36, 43, 50, 59, 66 and 73 DAE. The intervals correspond to the growth phases of the potato plant where at 10 to 35 DAE represent establishment stage, 36 to 50 DAE represent vegetative phase, 51 to 65 DAE correspond to flowering stage and tuber initiation, 66 to 84 DAE correspond to tuber enlargement while 85 to 110 DAE represent maturation (Rykaczewska, 2015). The knapsack sprayer was calibrated prior to every spraying of fungicide application regime so as to deliver spray volume of uniform discharge. Spray drifts to neighbouring plots was prevented using a polythene paper. Furthermore, data was collected only in the inner rows.

4.3 Effect of *T. asperellum* seed Inoculation Method on Late Blight and Yield

To determine an efficient seed treatment method, a parallel experiment was conducted to compare seed dipping with pericardial injection. A suspension of *T. asperellum* concentration at 100%, mistress 72[®] (2 g L⁻¹) and *P. infestans* suspension was prepared. The surface sterilized whole tubers were inoculated with 45 µL of each test preparation separately by peridermal injection at the apical end about 1 cm away from developing sprout using a 100 µL hypodermic syringe and needle. Tubers in the control treatment were inoculated with 45 µL of *P. infestans* suspension (adjusted to 4 × 10⁴ zoospores/mL using hemocytometer) only (Chaouch, 2016). The tubers were incubated in netted bags in wooden store on a rack at 15-20 °C for 24 hours. Afterwards the tubers were inoculated with *P. infestans* at 4 × 10⁴ zoospores/mL by peridermal injection and incubated again at room temperature 18 ± 2 °C for 24 hours prior to planting (Hollywood, 2014). Another set of intact tubers were placed separately in netted bags and inoculated by dipping in the treatments' suspensions for 15 seconds and then stored in wooden store on a rack at 15-20 °C for 24 hours.

Ridomil[®] spray regime was applied as described above. The treatments (Negative, *T. asperellum* at 100% concentration and mistress 72[®] treated seed, treatment method and spray regime) were laid in a randomized complete block design in split split plot arrangement with 3 replications. Spray regime and treatment method (peridermal injection or dipping) were the main plot and sub plot respectively while seed treatment agent was randomized within the sub plots.

4.4 Data Collection

Data on weather were collected daily from KALRO Tigoni weather station from November 2018 to July 2019 located about 100 m away from the trial site. Emergence count was taken 3 weeks after planting while apical cutting survival rate was taken two weeks after transplanting. Sprout, stem and foliage infection symptoms were observed weekly. Stem and branch count were conducted 21, 35 and 45 days after emergence on three pre-tagged plants per plot. Rooted apical cuttings produce several branches from a single stem while seed tuber give rise to several stems. Late blight severity and incidence were taken on weekly basis starting 16 DAE. Severity was evaluated on the basis of the proportion of diseased foliage on a scale of 0 to 5 where 0 = healthy, 1 = one fresh lesion (small circular water soaked spot), 2 = up to 25% lesion plus foliar blight, 3 = up to 50% lesion, necrotic, foliar and stem blight, 4 = up to 75% lesion, necrotic, foliar and stem blight and slight defoliation and 5 = 100%; defoliation (Yuen & Forbes, 2009). The results were summarized using the formula below to convert weekly disease scores to Area Under the Disease Progress Curve (AUDPC).

$$PDS = \frac{\Sigma \text{ individual numerical rating}}{\text{Total number of plants assessed} \times \text{maximum score in the scale}} \times 100 \dots \text{IV}$$

AUDPC was calculated using the below formula;

$$AUDPC = \sum_{i=1}^{n-1} \left(\frac{y_i + y_{i+1}}{2} \right) \times (t_{i+1} - t_i) \dots \dots \dots \text{V}$$

Where y_i , t_i , and i^{th} represents assessment of disease (percentage) at i^{th} observation, time (days) at i^{th} observation and i^{th} represent total number of observation respectively (Simko & Piepho, 2012). Disease incidence data (number of plants showing late blight symptom in each plot) were collected and converted to percentage disease incidence (PDI) using the formula below;

$$PDI = \frac{\text{Number of diseased plants}}{\text{Total number of plants assessed}} \times 100 \dots \dots \dots \text{VI}$$

At maturity, potato tubers were harvested from the inner rows of each plot and inspected for tuber blight symptoms. Tubers were graded as ware (>60 mm), seed (30 to 60 mm) and chatt (< 30 mm) grades according to KALRO Tigoni potato grading system. The tubers in each grade

group were counted and weighed using weighing scale and converted to tonnes per hectare. Tubers that seemed symptomatic and asymptomatic (10 samples) were cut transversely and incubated at 22 - 23 °C for three weeks and inspected every third day for late blight symptoms to determine latent infection and estimate yield losses. The data were summarized using the formula below;

$$\text{Tuber infected \%} = \frac{\text{Total no. of infected tuber harvested from the plot}}{\text{Total number of harvested tubers}} \times 100 \dots \text{VI}$$

4.5 Data Analyses

Statistical model was;

$$y_{ijk} = \mu + S_i + \beta_j + S\beta_{ij} + F_k + SF_{ik} + \delta_{ij} + A_l + FA_{kl} + \gamma_{ijk} + T_m + FT_{km} + AT_{lm} + FAT_{klm} + \epsilon_{ijklm} \dots \text{VII}$$

y_{ijk} - Observations due to i^{th} , j^{th} and k^{th} factor, μ - overall mean effects, S_i - effects due to i^{th} season, β_j - effects due to j^{th} block, $S\beta_{ij}$ - effects due to i^{th} season and j^{th} block, F_k - effects due to k^{th} Ridomil® application regime, SF_{ik} - effects due to i^{th} season and k^{th} Ridomil® application regime, δ_{ij} - main plot error effects, A_l - effects due to l^{th} seed source, FA_{kl} - effects due to k^{th} Ridomil® application regime and l^{th} seed source, γ_{ijk} - sub plot error, T_m - effects due to $T. asperellum$ seed treatment, FT_{km} - effects due to k^{th} Ridomil® application regime and m^{th} $T. asperellum$ seed treatment rate, AT_{lm} - effects due to l^{th} seed source and m^{th} $T. asperellum$ seed treatment rate, FAT_{klm} - effects due to k^{th} Ridomil® application frequency, l^{th} seed source and m^{th} $T. asperellum$ seed treatment rate and ϵ_{ijklm} - sub-sub plot error effects

Statistical comparison was based on standard error on means to assess interactions between or among fungicide regimes, seed type and $T. asperellum$ rate. Emergence and branch count, yield and yield components, percentage disease severity, AUDPC and percentage disease incidence data collected across the two seasons were analyzed using Statistical Analysis System (SAS) software version 8.2. Whenever Analysis of Variance (ANOVA) indicated significant differences ($P \leq 0.05$), Tukey's Honest Significant Difference (HSD) was used to separate treatment means.

4.6 Results

4.6.1 Effect of Weather on Late Blight Epidemics and Yield

Contrasting weather conditions were experienced during short and long rain season that influenced late blight development. The number of wet days in the short rain season were 21 and

temperatures were generally warm (24.5 °C) resulting in low Relative Humidity (RH) of 64% while in the long rain season there were 62 wet days and generally cool temperatures (20 °C) and high RH (77%). The result was, a high disease severity of 100% and 52% in the long and short rain season, respectively, in the unprotected plots (Figure 2) compared to plots protected with positive controls that had a high disease severity of 70% and 18% in the long and short rain season (Figure 3). In the short rain season, disease concentrated on the lower foliage but this was not the case during the long rain season as the disease infected every part of the canopy as result of prolonged conducive conditions for late blight development. There was a positive correlation between disease incidence and severity (Figure 4).

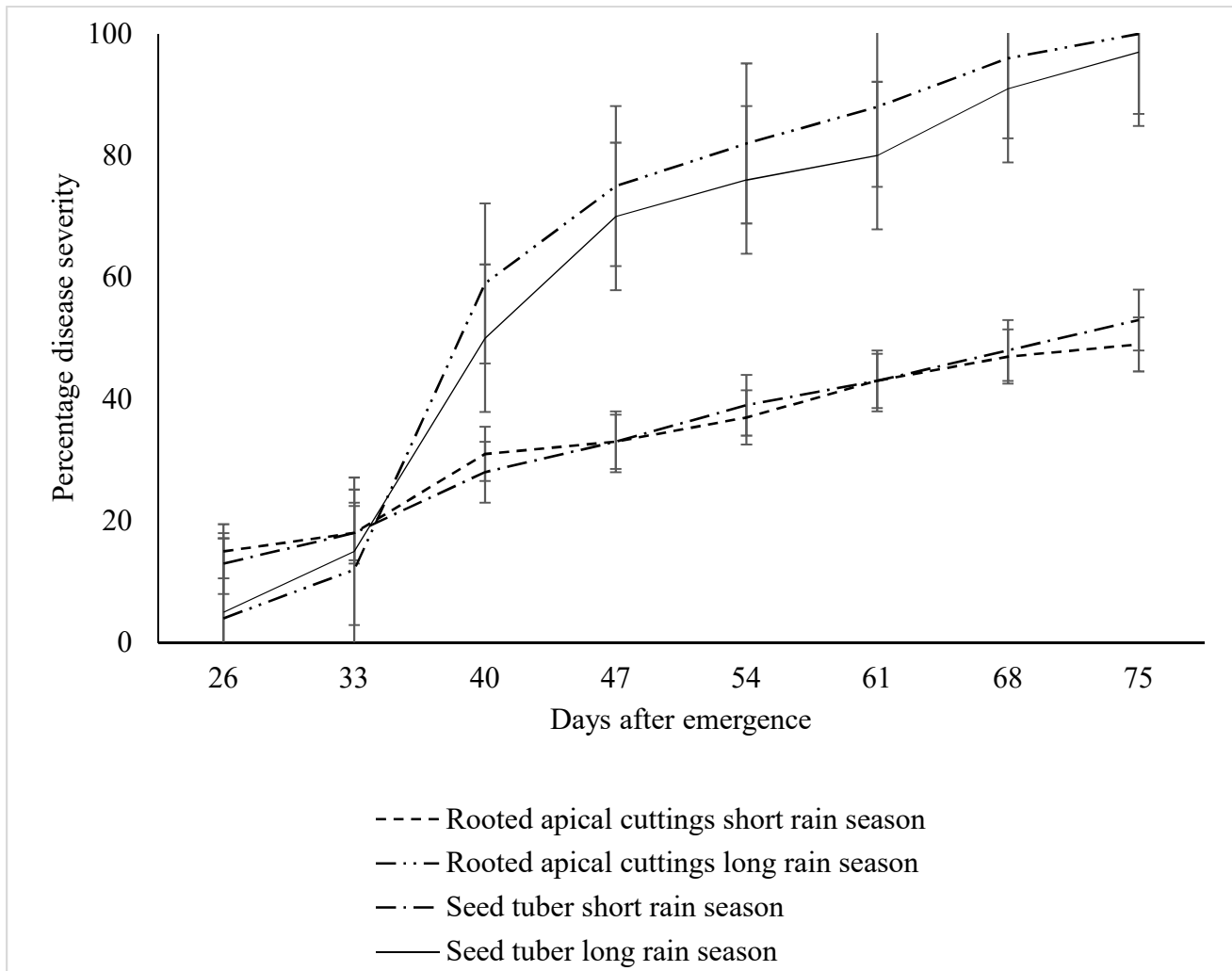


Figure 2: Effect of season on unsprayed untreated planting materials

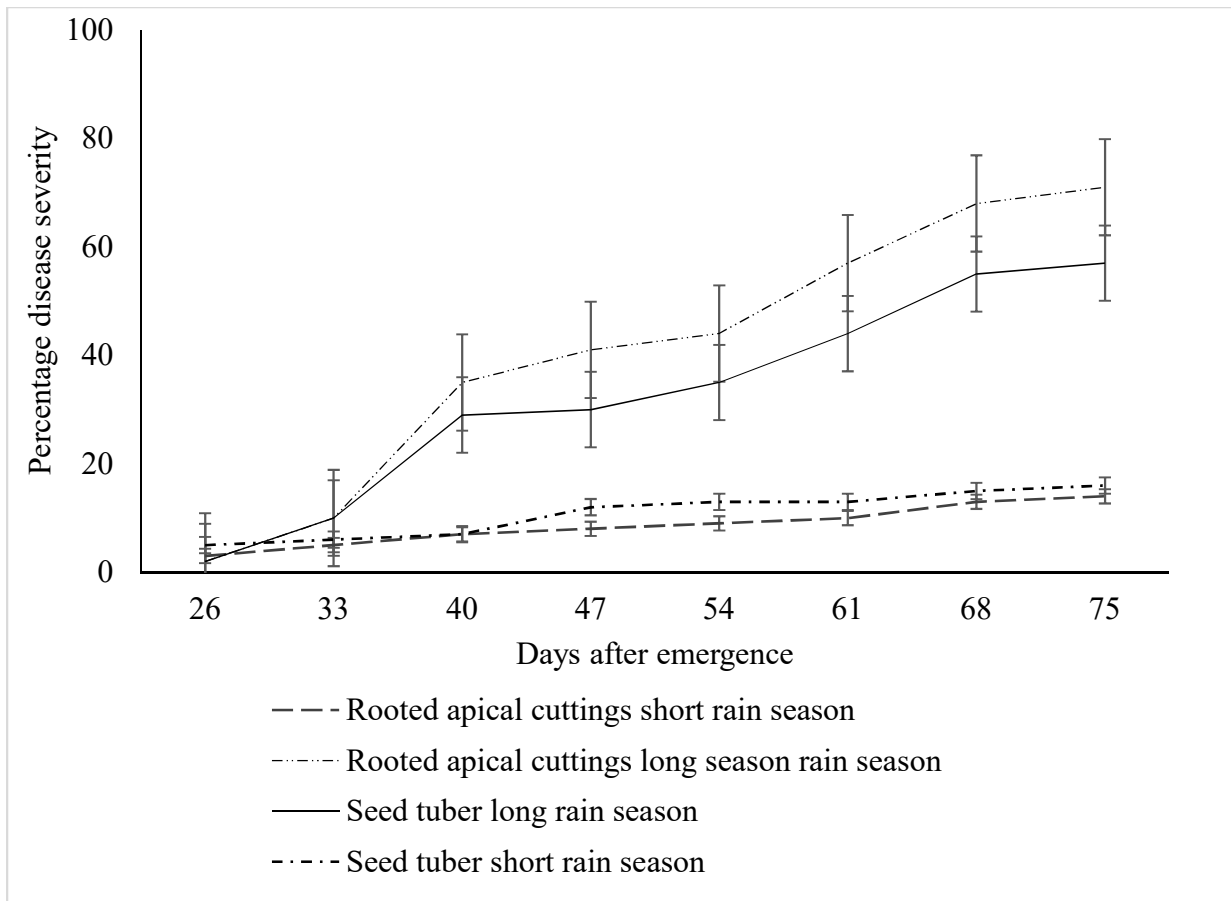


Figure 3: Effects of season on planting materials treated with Mistress 72[®] and sprayed with Ridomil[®] at 7 days interval

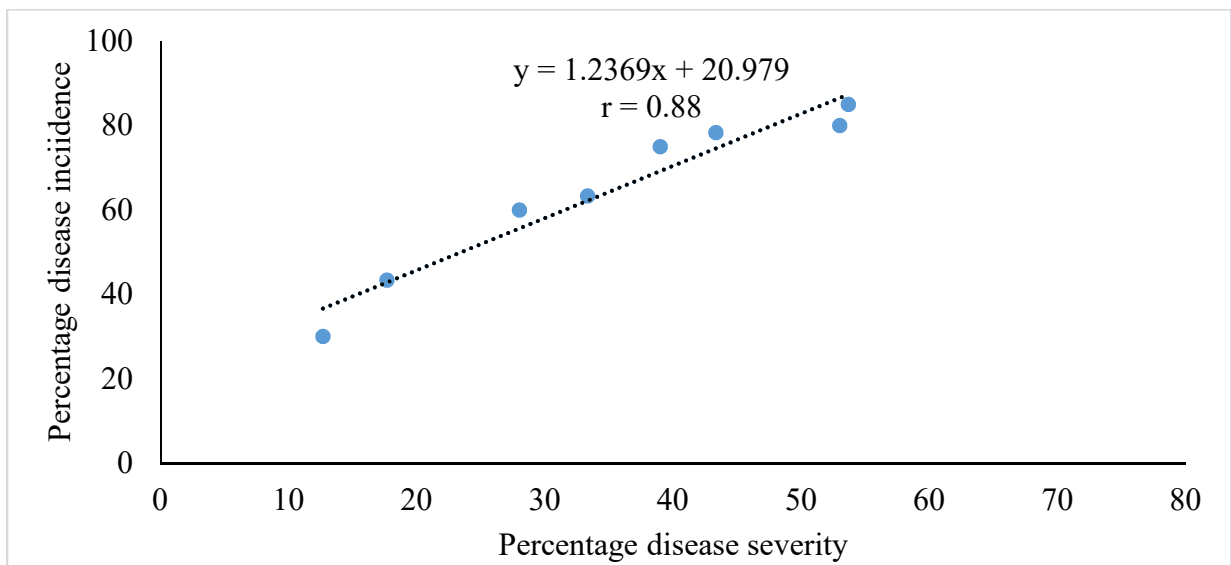


Figure 4: Correlation between Percentage Disease Incidence (PDI) and Percentage Disease Severity (PDS) across days after emergence for unsprayed untreated tuber seed.

4.6.2 Effects of Spray Regime, Seed Type and Seed Treatment and their Interactions on Late Blight and Yield

First late blight symptoms were observed 7 Days after artificial field inoculation on untreated check which began from lower canopy and progressed upwards. Yield and AUDPC data shows that season, fungicide regime, seed type and seed treatment, and their interactions except for seed by seed treatment interaction and regime by seed type by seed treatment interaction (3-factors interaction), were significant and therefore, contributed to the management of late blight and yield improvement. The seasonal differences led to late blight severity and yield variation where higher disease severity and lower yield were observed in the long rain season compared to short rain season. The variation in disease score resulted in additional yield of 39% in the short rain season above yield observed in the long rain season. Percent disease severity across days after emergence, AUDPC and ultimate yield significantly differed among Ridomil® application intervals, *T. asperellum* concentrations and between seed type. There was no significant difference among 21, 14 and 7 (positive control) days spray intervals in the first 49 DAE. In the later crop growth stages, disease severity on plots sprayed at 7 and 14 days interval regimes were not significantly different in the cropping seasons. The highest disease severity and lower yield were observed in unsprayed plots followed by those sprayed at 21 days interval (Table 3). Season influenced late blight severity on planting materials and ultimate yield where rooted apical cuttings had lower severity and higher yield than crops from seed tuber in the short rain season and contrary, higher disease score and lower yield than the latter in the long rain season. Disease severity progress on rooted apical cuttings was higher than crops from seed tuber in long rain season than short rain season. This led to 8.94% higher yield in crops from rooted apical cuttings than one from seed tuber in the short rain season and conversely, 10.03% higher yield in crops from seed tuber than one from rooted apical cuttings in the long rain season (Table 4). *Trichoderma asperellum* had significant effect on late blight and yield but the response dependent on the biocontrol's concentration. There was a strong negative correlation ($r = 0.75$) between *T. asperellum* concentration and disease severity (Figure 5). *Trichoderma asperellum* at 66% and 100% concentration and Mistress 72® were not significantly different which gave better disease control compared to untreated plots. The untreated seed had the highest disease score (AUDPC) and lowest yield followed by those treated with *T. asperellum* in both seasons (Table 5). Seed treatment with *T. asperellum* at 66% and 100% and Mistress 72® reduced late blight severity upsurge rate. This

resulted in reduction in late blight severity by 36% and 38% in the plots associated with *T. asperellum* at 66% and 100% while contributing to augmented yield by 20% and 21%, respectively, compared to untreated plots.

Season and fungicide application regime interaction were significant in both seasons resulting in better disease control using fungicide in the short rain season than in the long rain season. In fungicide spray regime and seed type interactions, lower disease severity and higher yield occurred in plots protected with 7 and 14 days spray intervals combined with seed tuber compared with any other combinations (Figure 6). Improved disease control that resulted in higher yield was observed in crops protected with *T. asperellum* at 66% and 100% concentrations and mistress 72[®] plots combined with 7 and 14 days spray intervals (Table 6). Higher disease and yield lower score was observed in the untreated plots than one with pre-treated seed crop in all three fungicide application regimes including 7 day interval (recommended by manufacturer).

Table 3: Effects of fungicide spray regime on late blight severity (AUDPC) and Yield (t ha⁻¹)

Regime	Short rain season		Long rain season	
	AUDPC	Yield (t ha ⁻¹)	AUDPC	Yield (t ha ⁻¹)
Unsprayed	1412.80a	5.88a	3242.10a	4.38a
21 days interval	1063.70b	21.02b	26.70b	8.81b
14 days interval	854.10c	23.99c	2201.41c	20.36c
7 days interval	806.70c	24.01c	2173.92c	20.40c
HSD ($P \leq 0.05$)	50.25	0.85	71.45	0.59
CV (%)	7.23	6.73	4.12	6.57

Values followed by similar letters in the same column indicate that the treatments were not significantly different ($P \leq 0.05$)

Table 4: Effects of planting materials on late blight severity (AUDPC) and Yield (t ha⁻¹)

Planting materials	Short rain season		Long rain season	
	AUDPC	Yield (t ha ⁻¹)	AUDPC	Yield (t ha ⁻¹)
Apical cuttings	1036.3a	19.24a	2691.82a	12.94a
Seed tubers	1023.18b	18.21b	2451.98b	14.03b
HSD ($P \leq 0.05$)	26.93	0.32	38.30	0.32
CV (%)	7.23	6.73	4.12	6.57

Values followed by similar letters in the same column indicate that the treatments are not significantly different ($P \leq 0.05$).

Table 5: Effects of *T. asperellum* concentration on late blight severity (AUDPC) and yield (t ha⁻¹)

<i>Trichoderma asperellum</i> rates	Short rain season		Long rain season	
	AUDPC	Yield (t ha ⁻¹)	AUDPC	Yield (t ha ⁻¹)
Untreated	1486.75a	14.84a	2862.58a	12.58a
33% <i>T. asperellum</i>	1472.46a	15.00a	2791.75a	12.76a
66% <i>T. asperellum</i>	789.38b	21.06b	2410.79b	13.91b
100% <i>T. asperellum</i>	750.42b	21.34b	2399.92b	14.02b
Mistress 72 [®]	749.29b	21.38b	2394.46b	14.15b
HSD ($P \leq 0.05$)	59.00	1.02	85.01	0.70
CV (%)	7.23	6.73	4.12	6.57

Values followed by similar letters in the same column indicate that the treatments are not significantly different ($P \leq 0.05$)

Table 6: Effects of seed treatment in combination with fungicide application regime in managing late blight

Season	<i>T. asperellum</i> rates	AUDPC Means ± Standard Error (SE)				Yield (t ha ⁻¹) Means ± Standard Error (SE)			
		unsprayed	21 days	14 days	7 days	Unsprayed	21 days	14 days	7 days interval
Short rain	untreated	1868±29.23a	1435±53.39b	1403±51.30b	1241±78.53c	0.73±0.06a	11.01±1.75b	20.9±0.85c	22.77±0.80d
	33%	1718±36.37a	1455±47.25b	1319±38.55c	1164±85.12d	0.86±0.06a	11.28±1.01b	20.9±0.69c	21.0±0.87c
	66%	1233±43.09a	780±22.47b	597±24.68c	540±55.34c	1.21±0.10a	15.98±0.49b	25.9±0.51c	25.9±0.45c
	100%	1120±14.15a	769±19.84b	578±23.87c	527±48.45c	1.28±0.12a	16.39±0.56b	26.1±0.48c	26.2±0.5c
	Mistress 72	1125±23.39a	781±27.22b	581±19.01c	516±65.43c	1.25±0.12a	16.19±0.56b	26.1±0.56c	26.3±0.4c
	CV%			7.19			6.92		
Long rain	untreated	3568±42.97a	2905±56.46b	2627±83.84c	2450±115.67d	0.51±0.11a	7.74±0.37b	18.04±0.65c	19.51±0.54d
	33%	3511±45.03a	2859±77.50b	2536±86.63c	2361±86.65c	0.64±0.09a	8.00±0.28b	18.71±0.70c	19.84±0.54c
	66%	3049±44.76a	2542±65.25b	2024±115.15c	2027±109.64c	0.89±0.05a	9.26±0.17b	20.70±0.69c	20.54±0.56c
	100%	3055±39.77a	2521±67.93b	2008±123.72c	2015±107.11c	0.84±0.05a	9.46±0.28b	20.77±0.73c	20.90±0.50c
	Mistress 72	3026±40.25a	2522±64.05b	2012±120.56c	2017±116.38c	0.79±0.06a	9.59±0.28b	20.77±0.70c	20.96±0.57c
	CV%			4.09			11.85		

Values followed by same letter within the same row indicate treatments do not differ significantly ($P \leq 0.05$)

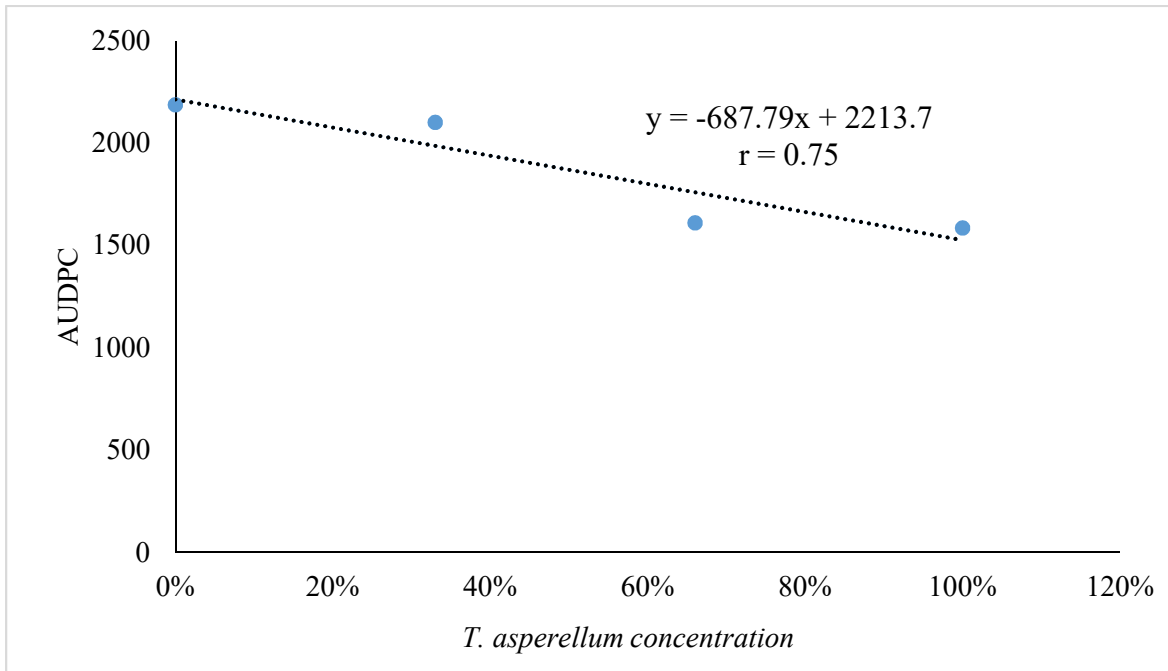


Figure 5: Correlation between *T. asperellum* concentration and amount of late blight as measured by area under disease progress curve.

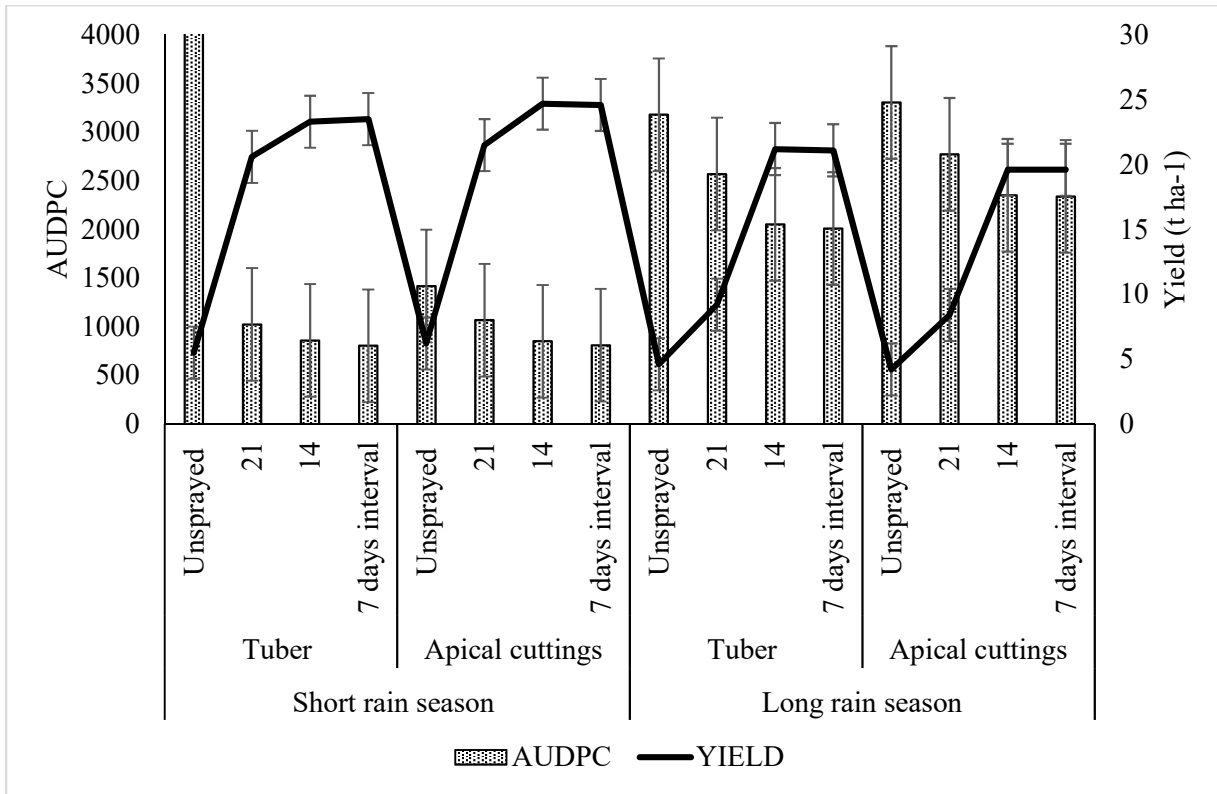


Figure 6: Effect of spray regime and planting material interaction on late blight severity and yield

4.6.3 Effect of Seed Inoculation on Seed Tuber Emergence, Rooted Apical Cuttings Survival

Seed tuber took 18 days to emerge after planting but in the 5th week they were about the same height demonstrating vigorous growth in tuber seed. However, apical cuttings flowered 15 days earlier than tuber seed crop. Seed tuber inoculation with *P. infestans* caused foliage infection appearing first on the base of the stems before leaves were infected about 7 DAE with untreated plots scoring the highest disease incidence. The symptoms afterwards progressed up the crop canopy. Emergence in seed tuber was affected significantly by *P. infestans* inoculation in untreated check resulting in reduced plant population. The lowest emergence score was observed in untreated treatments scoring 68% followed by *T. asperellum* at 33% concentration with 79% emergence. Emergence was not significantly different ($P \leq 0.05$) among *T. asperellum* at 66% (96%) and 100% (100%) concentrations and Mistress 72[®] (97%) treated seed. It was observed that seed tubers decayed before they emerged or sprouts died shortly after emergence in the untreated check. There was no significant difference in survival rate among rooted apical cutting plots inoculated with *P. infestans* only (83%) (Untreated check), *T. asperellum* at 33% (92%), 66% (96%), 100% (100%) concentration and Mistress 72[®] (96%) (Figure 7).

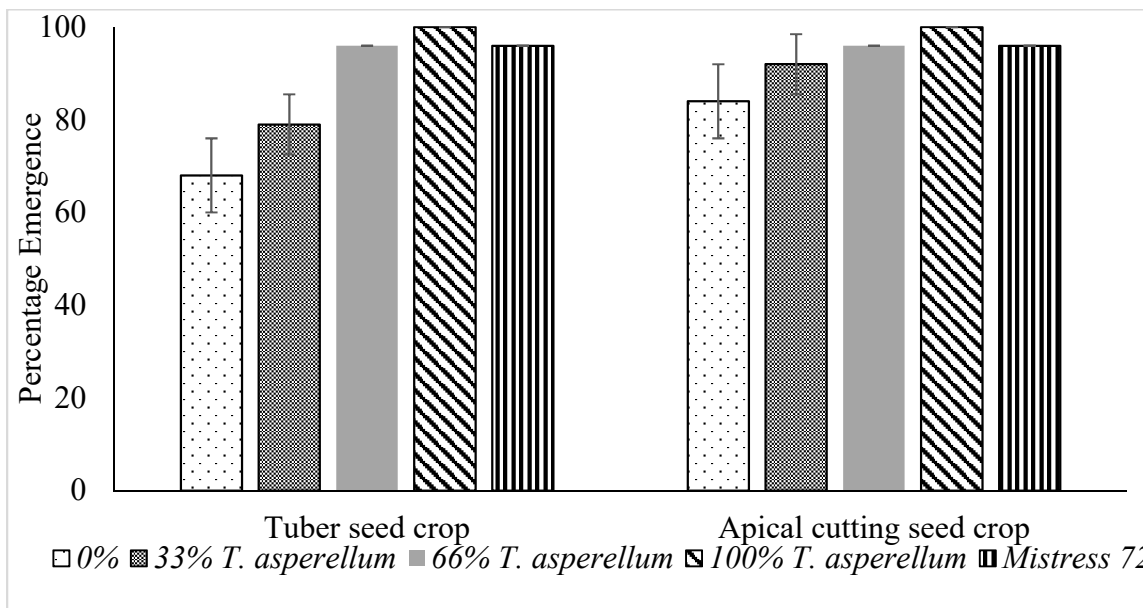


Figure 7: Effect of seed inoculation on percentage seed tuber emergence and rooted apical cuttings survival rate

4.6.4 Effect of Seed Treatment on Stem and Branch Count

Seed treatment contributed to increase in stem count by double and increased branch count by four-fold compared with the negative control. There was a decline in branch count in the untreated check and crop treated with *T. asperellum* at 33% concentration at 45 DAE compared to what was recorded at 21 DAE. Seed treatment with *T. asperellum* at 100% and 66% concentration resulted in higher hill, branch and stem count relative untreated seed. (Figure 8).

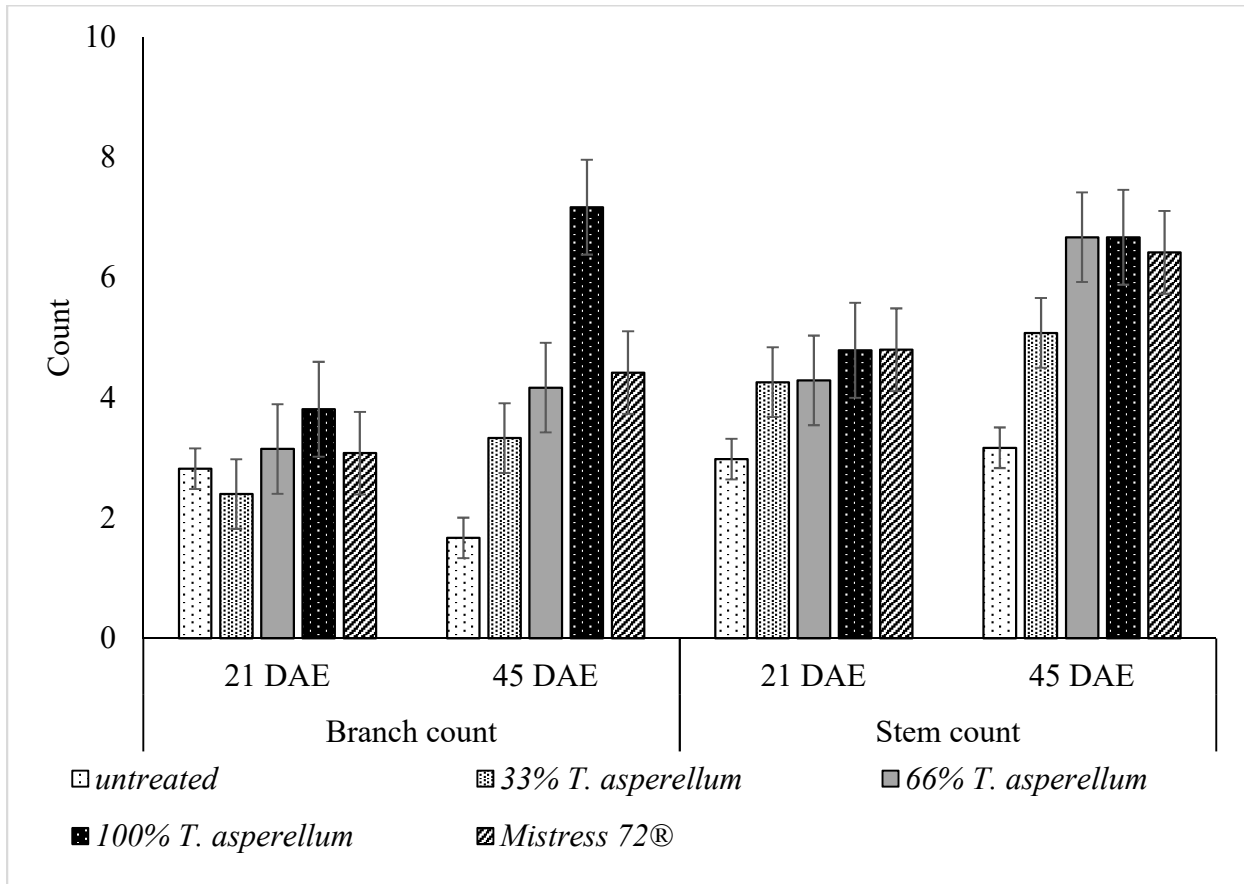


Figure 8: Effect of seed treatment on stem and branch count

4.6.5 Effects of Late Blight on Tuber Infection

Tuber infection was only observed during long rain season on unprotected plots which been treated by dipping or peridermal injection separately. Randomly sampled symptomatic and asymptomatic tubers from each plot were cut transversely (Plate 6a) and incubated for 4 days at 18 °C. Mycelial growth of *P. infestans* on the pericardial layer (Plate 6b) and sporangia observed through microscope for confirmation before employing Koch's postulates. Higher tuber infection

was observed on untreated and *T. asperellum* at 33% concentration plots than one treated with 66% and 100% *T. asperellum* concentrations and mistress 72®. This contributed to reduced potato yield from the allied plots (Figure 9)

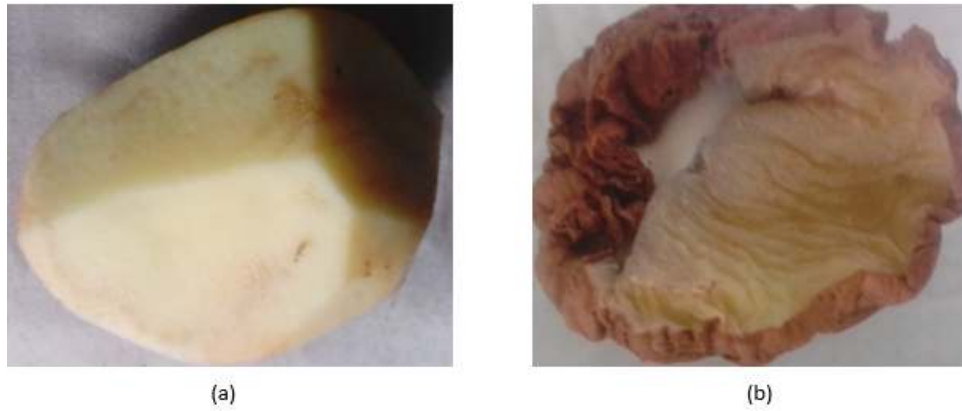


Plate 6: Tuber infected with late blight before incubation (a) and after 3 days incubation (b)

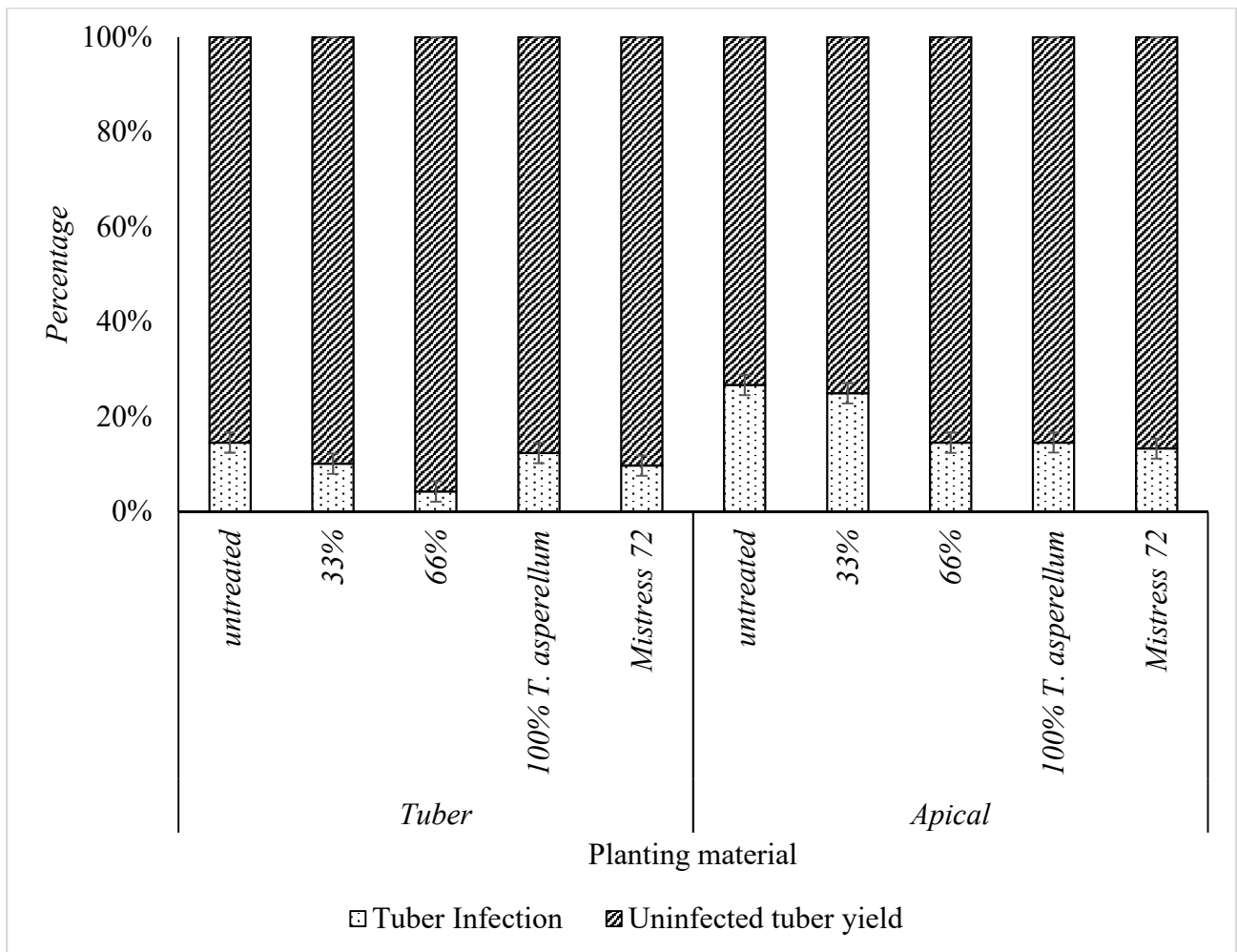


Figure 9: Proportion of tuber infection to uninfected tuber yield

4.6.6 Effect of Seed Treatment Method on Late Blight Development and Yield

Season, fungicide spray regime, seed treatment method and seed treatment rate were significant on late blight severity and yield. Regime and seed treatment interaction was not significant. In both seasons, seed dipping recorded higher disease progress than pericardial injection although they were not significantly different (Figure 10). Conversely pericardial injection had 7.73% higher yield compared to dipping. Results observed in plots protected with spray regime, seed treatment concentration and their interaction followed the same trend as described in experiment one above. Seed treatment with *T. asperellum* and mistress 72® by pericardial injection gave the highest disease severity and yield compared the same treatments pretreated by dipping (Figure 11).

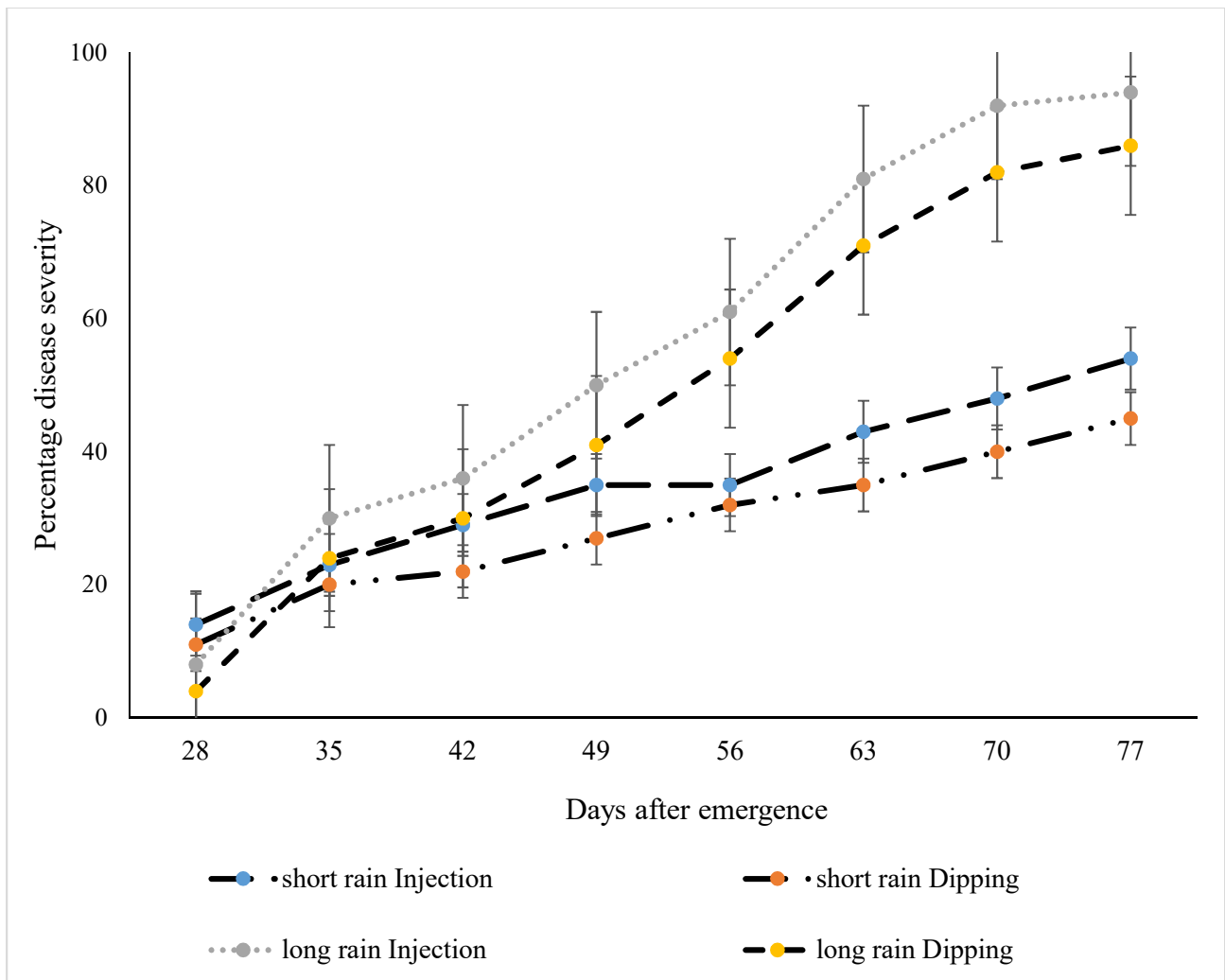


Figure 10: Effect of season and seed treatment method on late blight development across days after emergence.

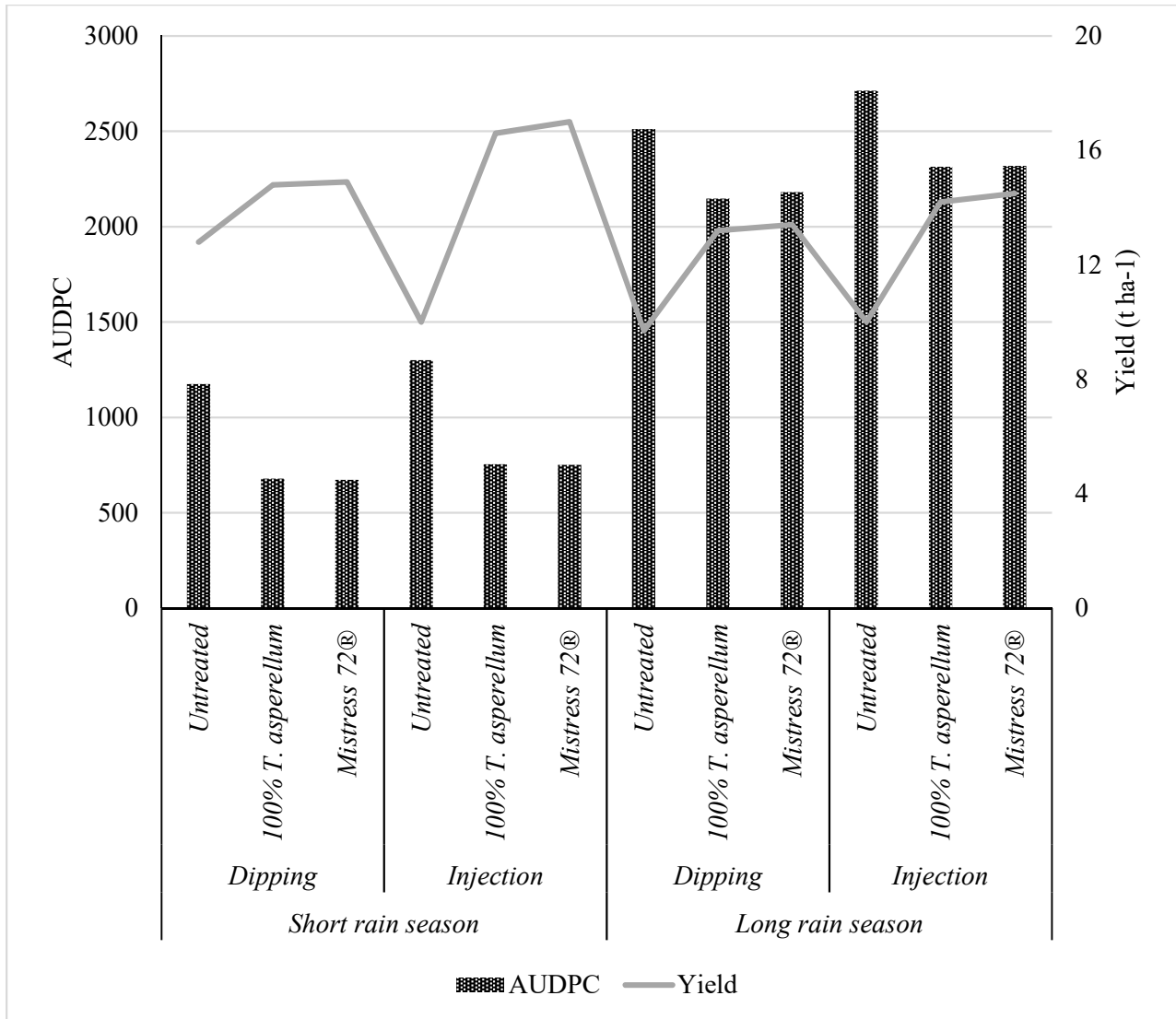


Figure 11: Effect of treatment method and seed treatment rate interaction on late blight severity and yield

4.7 Discussion

Interest in the use of beneficial micro-organisms has continued to increase over time. Of all the described beneficial fungi, only few have been progressed to commercial application as a result of limited selection and efficacy evaluation strategies. Whereas efficacy studies on biocontrol agents in general have been conducted *in vitro*, field screening under conditions with diverse soil microbial communities could provide an effective and reliable predictor. Field based screening of several *Trichoderma* spp. isolates for late blight control showed antagonistic activity against *P. infestans* albeit inefficient reduction of disease severity (Yao *et al.*, 2016). In the present

study, efficacy of *T. asperellum* when used in combination with metalaxyl based fungicide formulation (Ridomil®) was evaluated under field conditions to estimate the accruing biological efficiency.

A higher amount of disease observed on crops from seed tuber as compared to crops from rooted apical cuttings in the short rain season could be attributed to latent infection as primary source of inoculum in asymptomatic seed tubers. Indeed, disease free materials including rooted apical cuttings have been found to contribute to reduction in disease epidemics (Tsoka *et al.*, 2012). Contrariwise, rooted apical cuttings were more vulnerable to late blight than crops from seed tuber in the long rain season. Although the study did not aim to establish the relative vulnerability of rooted apical cuttings, we speculate that the dense canopy of crops from rooted apical cuttings could have predisposed the crop to late blight. Lack of food reserve unlike in seed tuber and weak rooting system at establishment phase may have also slowed adaptation to field conditions in rooted apical cuttings contributing to their vulnerability. Moreover, a strong rooting system influences nutrient acquisition including calcium and potassium which are vital in disease resistance (Ismail *et al.*, 2012; Seifu, 2017).

Optimal use of *T. asperellum* in seed treatment reduced seed decay and sprout infection thereby improving crop emergence. In cases of sprout death, this occurred shortly after emergence in untreated plots, suggesting transmission of *P. infestans* from latently infected seed to developing sprouts (Johnson & Cummings, 2009; Gigot *et al.*, 2009). Disease reduction meant more healthy plants as a result of escape as well as antifungal activities including mycoparasitism, antibiosis, enzymatic activities (chitinase, glucanase and proteinase) and induced plant defence instigated by *T. asperellum* against *P. infestans* are implicated (Howell, 2007; Naglot *et al.*, 2015; Wharton *et al.*, 2012). Further, the apparent increase in plant height, stem and branch count can be explained by enhanced nutrients uptake (Blauer *et al.*, 2013; Fontelle *et al.*, 2011; López-bucio *et al.*, 2015; Sundaramoorthy & Balabaskar *et al.*, 2013; Vinale *et al.*, 2008). The inability of the *T. asperellum* at 33% concentration to suppress the *P. infestans* implies that the bio-control was out-competed at this low concentration (Bahramisharif & Rose, 2019). A higher starting concentration ensures quick and profuse sporulation that contributes to competition against the pathogen (Shoresh *et al.*, 2010).

In regards to deployment of the metalaxyl based synthetic fungicide against *P. infestans*, closer spray intervals of 7-and 14-day intervals provided satisfactory late blight management. At

21 day spray intervals, fungicide sprays were insufficient to manage late blight especially during prolonged wet weather conditions. It is our view that in longer spray intervals, degradation of the fungicide's formulation compromised the efficacy (Evenhuis *et al.*, 2017; Paparu *et al.*, 2014). In addition, when the product dosage is below optimum, then the pathogen establishes faster to produce more disease (Rojas *et al.*, 2013; Sharma & Saikia, 2013).

A delineation of the effects due to each factor or interaction of factors assessed in disease reduction under this study is as follows. Firstly, during prolonged wet weather conditions, rooted apical cuttings, fungicide and seed treatment as essential disease measures singly provided unsatisfactory control of late blight. In contrast, use of either optimal fungicide spray intervals, cropping using seed tuber or seed tuber treatment individually reduced the amount of disease. Secondly, when considered separately, seed tuber pre-treatment with *T. asperellum* without crop spray with Ridomil® was ineffectual against late blight. Furthermore, crops from in control plots planted with seed pre-treated with *T. asperellum* yet unsprayed with the fungicide, were completely destroyed prior to tuberization. The effect of *T. asperellum* on the late blight could have been temporal to protect the potato crop from late blight (Sharma *et al.*, 2017). Therefore, simultaneous use of the *T. asperellum* at optimal concentration with 7- or 14-day fungicide spray interval provided the most suppression of the disease. The synergistic activity achieved by this combination ensured that the biocontrol reduced infection of late blight early in the cropping season while ridomil® protected the foliar or killed *P. infestans* mycelia already attached on the leaves (Aparecida *et al.*, 2018; Lal *et al.*, 2017).

The technique of treating seed with the biocontrol influenced disease control. Seed treatment by dipping produced better control of late blight compared to treatment by pericardial injection. We proffer that dipping seed in the bio-control suspension assures coating of the tuber surface and therefore increases the chance to counteract *P. infestans* (Flier *et al.*, 2001). As *P. infestans* infects host plant by establishing penetration peg into the host plant tissues, an intact seed tuber provides a natural barrier to *P. infestans* haustorial penetration during infection. Also, by virtue of precedence, dipping allows *T. asperellum* to penetrate and colonize internal tuber tissues and/or triggering induced disease resistance to hinder the pathogen from establishing (Aydın, 2019). Considering routine application of the biocontrol agent on a commercial scale, seed treatment by dipping is more efficient and cost effective compared to pericardial injection. The need for large scale seed tuber treatment can be resolved by exploring automated dipping as a

method of treatment. The practical needs for smallholder farming systems devoid of technical expertise and large capital investment can be addressed through availing pre-treated packages of seed ready for planting. This eliminates the challenges of determining the appropriate suspension concentration if seed treatment were to be done on-farm.

In view of the foregoing, the study shows that fungicide application cannot be avoided. However, fungicide usage could be reduced sustainably by combining with biocontrols to offer prior protection against late blight infection early in the cropping season in an integrated disease management approach.

CHAPTER FIVE
COST BENEFIT IN THE MANAGEMENT OF LATE BLIGHT ON POTATO
THROUGH *Trichoderma asperellum* SEED TREATMENT AND RIDOMIL®
APPLICATION

Abstract

Late blight management is one of the costly biotic stresses in potato production with the ultimate expense being on fungicides and their application costs. The use of disease free seed and biological controls could be efficient late blight management alternatives that have recently gained interests to minimize fungicides dependence. However, there are limited data on the costs and benefits accrued to the use of disease free seed, biological control and fungicides as well as their combination. The objective of the study was to determine costs and benefits accrued to the efficacy of *Trichoderma asperellum* seed treatment and Ridomil® application in managing late blight on crops from rooted apical cuttings and seed tuber. Costs benefit analyses were conducted on rooted apical cutting and seed tuber pre-treated with *Trichoderma asperellum* at 33%, 66% and 100% concentration by either pericardial injection or dipping and then foliar sprayed with Ridomil® at interval of 7, 14 and 21 day interval separately. Results showed that rooted apical cuttings planting took 8 more days than seed tuber. Seed treatment with *T. asperellum* at 66% concentration gave higher Marginal Rate of Return (MRR) than 100% concentration at 14 days interval. Higher returns were observed from tuber seed crop giving a net benefit ratio of 1.06 while apical cuttings recorded a net loss. Seed dipping was four times profitable than injection. The findings suggest that seed treatment at 66% and 100% *T. asperellum* concentration by dipping in combination with Ridomil® application at 14 days interval significantly reduced late blight epidemics and improved on yield and consequently increased net farm income.

Key words: Apical cuttings, Costs and benefits, Seed treatment, *Trichoderma asperellum*

5.1 Introduction

Increase in human population is often accompanied by decrease in agricultural land as result of land sub divisions and urbanization that require sustainable food production practices (Godfray & Charles, 2010). Cognizant of this challenge, development of new and improvement of existing technologies is essential for more efficient food crop production practices (Foley *et al.*, 2005). Potato is one of the crops with a potential of filling up the predicted food gap attributed to increase in population. The tuber crop is one of the most important food crop in terms of nutrition, socio-economics and generation of job opportunities globally (Mahima & Mahesh, 2017) and therefore plays a key role in food and nutritional security (Devaux *et al.*, 2014) which is the major concern globally. The tuber crop is grown mainly in the highlands where land subdivision is common because the crop matures earlier, has higher yield per unit area and with more nutritive value than cereal crops (Dersseh *et al.*, 2016). However, potato yield experienced globally is associated with biotic stresses (Guchi, 2015).

Late blight is probably the major potato yield reducing biotic stresses worldwide (Savary *et al.*, 2012) which has tremendous effect on the cost of potato production (Hu *et al.*, 2012). The pathogen causes economic losses by inducing tuber rot that affect a whole lot of seed in store or after planting and causes early tuber and foliar blight (Kirk *et al.*, 2010). High rainfall and low temperature experienced in potato growing regions favour increased rate of late blight development requiring multiple fungicide application per cropping seasons (Majeed *et al.*, 2017b). Management of late blight has therefore become a costly necessity in potato producing regions especially during the long rains when conducive environmental conditions are prolonged (Bengtsson *et al.*, 2014). Moreover, use of farm saved seed and emergence of fungicide resistant strains that are more aggressive with high mutation degree and ability to co-evolve with the host has led to adoption of more shorter interval spray regimes (Kamoun *et al.*, 2015; Runno-Paurson *et al.*, 2013). Increased usage of pesticides pose human health (Zhang *et al.*, 2017) and biodiversity concerns (Muñoz-leoz *et al.*, 2011). In addition, dependence on fungicides has led to increased cost of managing late blight than any other input (Sharma & Saikia, 2013). Disease free seed including use of rooted apical cuttings and minitubers reduce pathogen inoculum (Kamuyu *et al.*, 2017). However, profit in the use of rooted apical cuttings in managing late blight and yield improvement has not been explored. Rooted apical cuttings are produced through tissue culture which is a costly venture.

Use of disease free seed, cultural practices including removal of volunteer plants (Gigot *et al.*, 2009), use of biological controls (Syed *et al.*, 2018) form a sustainable integrated pest management programme (Xu *et al.*, 2011). Host resistance has been ineffective due to the rapid evolution of *P. infestans* which has overcome race specific resistance (Forbes, 2012). Combining host resistance with the fungicides has not yielded significant results in reducing fungicides application frequency (Ritchiea *et al.*, 2018). Therefore use of biocontrol including *Trichoderma* species to lower the costs associated in managing this devastating disease while enhancing a more effective and environmental friendly potato production practices (Kumar *et al.*, 2015; Miles *et al.*, 2012). The biological control suppress phytopathogens and promote plant growth (Keswani *et al.*, 2014). Studies have shown that treating seed prior to planting with a biocontrol reduces seed decay and introduction of primary inoculum into field (Somani & Arora, 2010; Wharton *et al.*, 2007). The practice can substantially delay onset of late blight early in the cropping season by preventing sprout infection attributed to latent infection thereby lowering fungicide application frequency. Powelson *et al.* (1999) reported that Curzate (cymamoxil 4% and mancozeb 64%) was effective in managing late blight of potato through seed treatment while Wharton *et al.* (2012) found that inoculation of seed with *Bacillus subtilis* and *T. harzianum* before planting reduced late blight epidemics by 54.4% and 86% respectively through seed piece treatment. However, there is limited literature on costs and benefits associated with seed treatment and fungicide application as well as their combination to manage late blight.

The aims of the study were to determine the costs and benefits associated with seed treatment in combination with optimal fungicide application intervals in relation to rooted apical cutting and seed tuber of potato production. Returns obtained from crops grown from rooted apical cuttings and seed tuber were calculated. Dipping and pericardial injection net benefit ratio was also determined. The results provide important information on managing late blight effectively with minimal costs as well as improving potato production.

5.2 Materials and Methods

5.2.1 Yield Assessment

Crops from the field experiment described in chapter four were dehaulmed at maturity and tubers from each plot harvested separately. The tubers were graded into ware (>60 mm), seed (30 – 60 mm) and chatt (>30 mm) class system and afterwards counted and weighed as described in

chapter 4. Plot yield (kg plot⁻¹) was converted to tonnes per hectare before performing cost benefit analysis. Yield was evaluated on marketable tuber grade (>30 mm).

5.2.2 Economic Analysis

Partial budgeting was used to determine differences in costs and revenues among the treatments (*T. asperellum* seed treatment concentrations fungicide regimes, seed type and their combinations and seed treatment methods). Costs applied uniformly to all treatments were not considered (Halloran *et al.*, 2013). Related costs that varied among treatments included planting, seed inoculation and fungicide application labour, seed and seed transport, Ridomil[®], knapsack hire and *T. asperellum*. Seed and ware grade prices were based on current pricing of potatoes when packed in 50 kg and 100 kg bags at KALRO Tigoni and local Limuru market (farm gate price) respectively. All costs and revenues were converted to per hectare which was used to calculate percent marginal rate of return in partial budgeting. Ridomil[®] and onion net roll of 600 m/ha was purchased from Limuru agro stockiest. Hypodermal syringes and needles were purchased from local pharmaceutical shop which were sold in packets containing 12 pieces (2 packets/ha).

Planting labour for seed tuber and rooted apical cuttings was determined by allocating workers (3 men and 3 women aged between 30 to 52 years) code A to F to avoid biased data and each planted 5 plots (replications) of 3 m × 3 m. Time spend on inoculation of the seed with *T. asperellum* using 4 workers in each seed type was averaged. A stop watch was used to record time spend by each worker per plot. Planting of seed tubers was done first followed by rooted apical cutting the following day to ensure the worker's energy was about the same level. Planting duration was converted to man days per hectare before transformation. Labour costs were based on Kiambu County Labour Wages scheme while transport costs were averaged from data collected from transporters during Limuru market day which was converted to Kenya shilling per tonnes per kilometre.

5.3 Data Analyses

Data on time spend to plant per plot and inoculation of seed with *T. asperellum* were first transformed using log₁₀ transformation while yield was converted to tonnes per hectare before subjecting to analysis of variance using Statistical Analysis System (SAS) software version 8.2. Treatments means were separated using Tukeys Honest Significant Difference. Economic analyses

were conducted using partial budgeting in Kenya Shillings (KES). Treatment or treatment combination which gave the highest marginal rate of return was classified as the economical alternative.

Marginal rate of return was calculated using the below formula;

$$\text{MRR} = \frac{\text{DNI}}{\text{DIC}} \dots\dots\dots \text{VIII}$$

Where, MRR is the marginal rate of return, DNI is the difference in net income compared with the control (change in net benefits (Net benefits from new technology minus net benefits from control) and DCI is the difference between input cost compared to control (Change in total variable costs (Total variable cost of new technology minus control)).

To compare seed treatment methods and seed type costs and benefits, the ratio of the net benefits (Gross margin) to total variable costs was calculated using the below formula. Treatment that showed the highest ratio was regarded as more economical.

$$\text{Cost Benefit ratio} = \frac{\text{Net benefit}}{\text{Total variable costs}} \dots\dots\dots \text{IX}$$

5.4 Results

5.4.1 Costs and Revenues

Through extrapolation of data from the study, 44,440 rooted apical cutting and 45 bags of seed tuber are required to plant per hectare. Seedlings were purchased singly at a cost of KES 10 while a bag of seed tuber was purchased at KES 3,000. There was no significant difference ($P \leq 0.05$) in time spend on planting among the workers both in rooted apical cuttings and seed tuber despite their age differences. Average planting time spent on rooted apical cutting and seed tuber per hectare differed significantly. Planting of rooted apical cuttings and seed tuber in one hectare took on average, 6 and 14 man days working 8 hours per day respectively. More time was taken in planting rooted apical cuttings than seed tuber by each worker (Figure 12). Inoculation of 4 bags of seed tuber by dipping took one man day which is equivalent to $1\frac{1}{4}$ man days ha^{-1} while pericardial injection took one man day per 500 tubers (approximately $\frac{1}{2}$ bag) translating to 90 man days ha^{-1} . A total of 3360 rooted apical cuttings inoculation with the biocontrol took one man day translating to 13 man days per hectare. A rate of KES 300 per man day was adopted in planting, seed treatment and other field management practices except fungicide application. From Chapter

four results, *T. asperellum* suspension at 33%, 66% and 100% resulted in 82.5 g, 165 g and 250 g per hectare respectively.

The biocontrol was packaged into 200 g, 100 g (KES 600) and 50 g which were sold at 1,160), 600 and 350 Kenya shillings respectively. A total of 16 g ha⁻¹ pure spores of *T. asperellum* was used for pericardial inoculation. Ridomil[®] was purchased at Kes 3000 per kilogram and applied at a rate of 2.5 kg ha⁻¹. A total of in 8, 4 and 3 ridomil[®] applications were observed which resulted in 20, 10 and 7.5 kg ha⁻¹ of ridomil[®] from 7, 14 and 21 day interval regime respectively. A packet of syringe and a roll of onion netted bag was purchased at KES 320 per packet and KES 2000 per roll respectively.

Labour for fungicide application was KES 400 per man day corresponding to 4 man days/ha while knapsack hire according to report from Ministry of Agriculture Limuru, Kiambu data was KES 50 per hectare per day. According to transport costs data collected from Ministry of Agriculture Limuru, Kiambu and private transporters on transport costs, it costs on average KES 15 per tonne per kilometre for transporting seed tuber while rooted apical cuttings box containing 1200 cuttings from Stockman Rozen (Commercial business identity) transported by G4S courier costs Kes 12 per box per kilometre. Farm gate prices for ware and seed potato were KES 2,000 for 100 kg bag and 3,000 KES for 50 kg bag respectively. Average yield (t ha⁻¹) observed in the experiment was adjusted by 10% before cost benefit analysis to approximately match field yield obtained by farmers (Muchiri *et al.*, 2017).

Unsprayed associated treatments yield was lower than the farmers' average yield (8 t ha⁻¹) (Gildemacher *et al.*, 2011) and therefore, were not considered in the economic analysis (Namanda *et al.*, 2004). Seed treatment with *T. asperellum* at 33% was not considered in the analysis since it was ineffective in managing late blight as observed in Chapter four Potato prices for both ware and seed remained constant in both seasons.

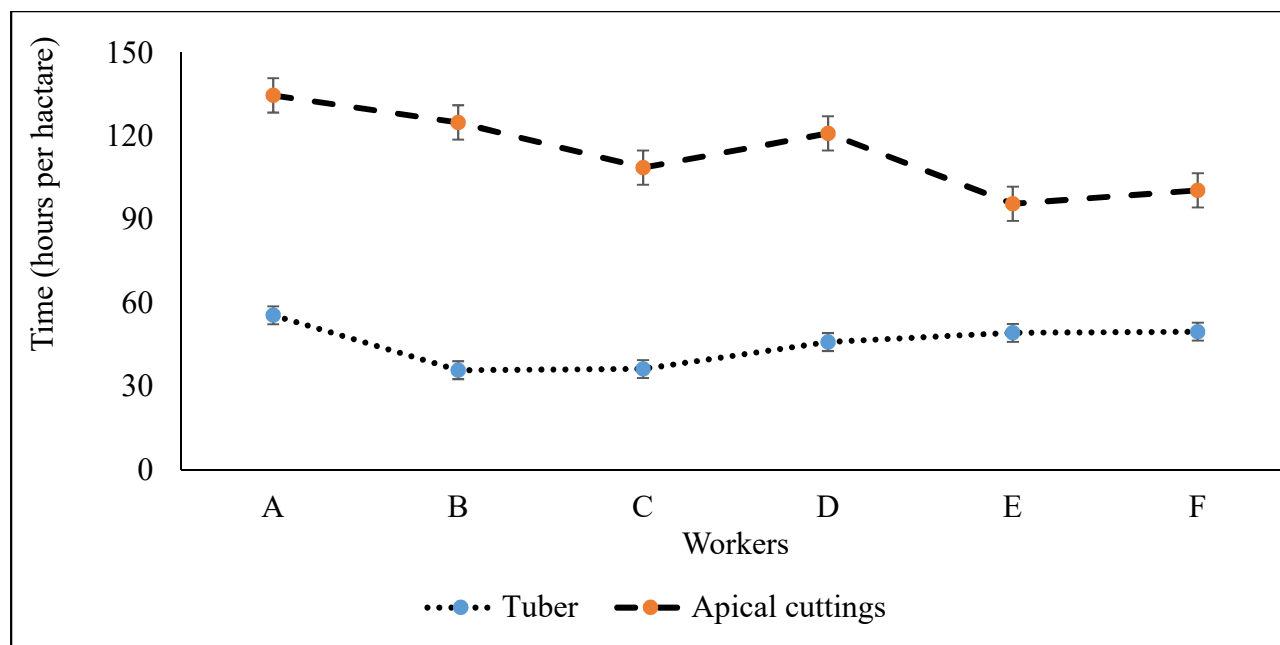


Figure 12: Time spend by each worker in planting tubers and apical cuttings seed

5.4.2 Fungicide Spray Regime and Seed Treatment Concentration

There was an increase in tuber size associated with disease control compared to unprotected plots. Harvest from the unsprayed plots consisted of 78% chatt size grade while fungicide application at 7, 14 and 21 days interval comprised chatt grade proportion of 0.91%, 2.04% and 2.43% the total yield respectively. The standard program was not significantly different in terms of yield from 14 day spray interval (Figure 13). Untreated and 33% *T. asperellum* yield composed of a large proportion of chatt size compared to *T. asperellum* at 66% and 100% concentration (Figure 14).

Partial budget indicated that 7, 14 and 21 day spraying interval regime had positive marginal rate of return and short rain season had a higher marginal rate of return than long rain season. The highest marginal rate of return was obtained from 14 days that had 8.15 and 7.53 during short and long rain season. Seasonal variations resulted in marginal rate of return percent differences between 21 and 7 day spraying interval. Spraying interval of 21 days interval had higher MRR of 5.20 during short rain season than 7 day interval that had 4.06 but during long rain season, 7 day interval had MRR of 3.60 while 21 days interval had 3.17. *Trichoderma asperellum* at 66% concentrations had a higher MRR in all spray fungicides regimes than *T. asperellum* at 100% concentration during short rain season but during long rain season it was vice versa (Table

9). Therefore, the highest returns were obtained from *T. asperellum* at 66% and 100% concentration when combined with 14 days interval.

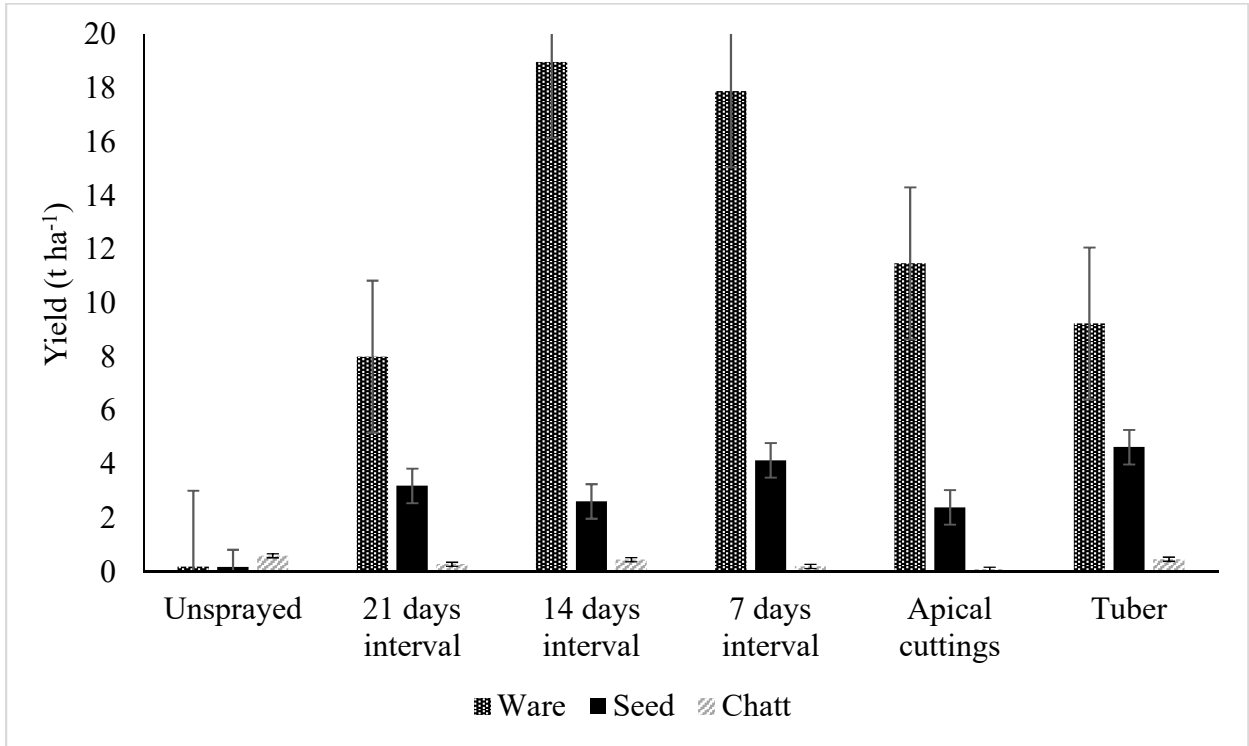


Figure 13: Effect of spray regime on potato grades

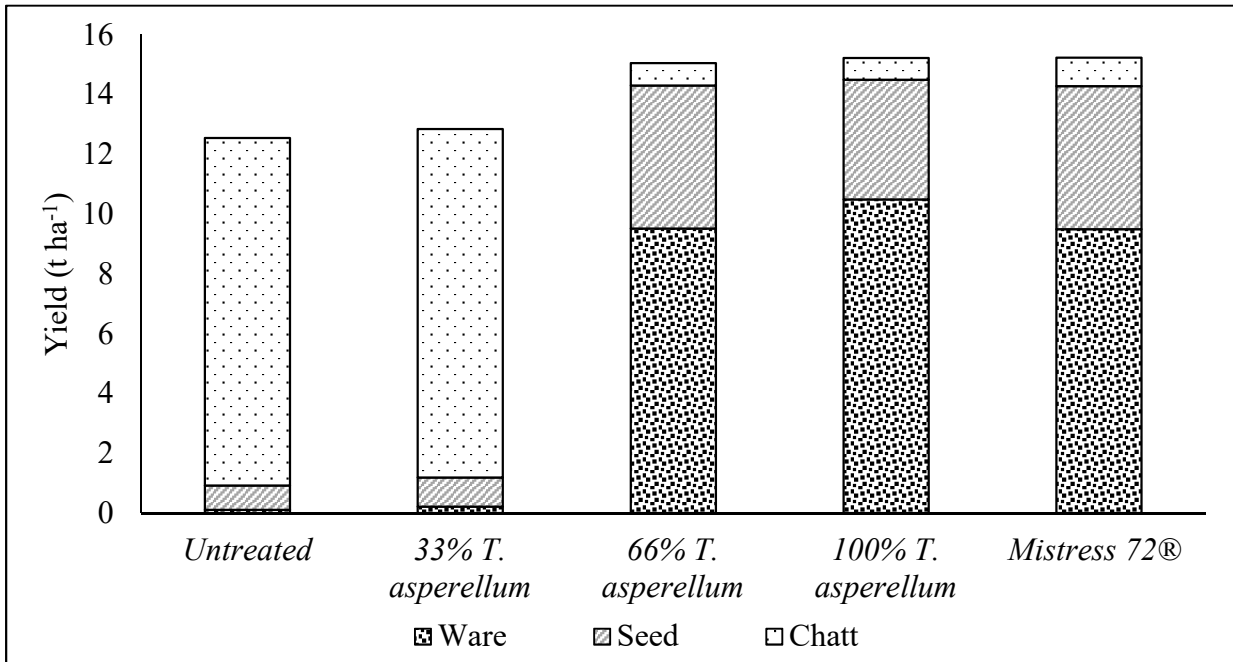


Figure 14: Effect of *T. asperellum* concentrations on potato tuber grade

Table 7: Cost benefit analysis (Kes ha⁻¹) on fungicide regimes and tuber seed treatment with *T. asperellum* by dipping in combination with ridomil® application

VARIABLE	Unsprayed		7 days interval		14 days interval			21 days interval		
	0%	0%	66%	100%	0%	66%	100%	0%	66%	100%
Sales										
SR yield (t ha ⁻¹)	0.83	21.77	24.97	25.14	19.74	24.85	25.06	10.45	16.79	16.88
SR Adjusted yield (t ha ⁻¹)	0.75	19.59	22.47	22.63	17.77	22.37	22.55	9.40	15.11	15.19
LR Yield (t ha ⁻¹)	0.60	19.63	21.43	21.69	18.24	21.43	21.83	7.07	9.52	9.92
LR adjusted Yield (t ha ⁻¹)	0.54	17.67	19.29	19.52	16.41	19.29	19.65	6.36	8.57	8.93
Gross field benefits SR(KES)	15,000	391,800	449,400	452,600	355,400	447,400	451,000	188,000	302,200	303,840
Gross field benefit LR(KES)	10,800	353,400	385,800	390,400	328,200	385,800	393,000	127,200	171,400	178,600
Costs (KES)										
<i>Trichoderma</i>	0	0	1,160	1,510	0	1,160	1,510	0	1,160	1,510
4 Knapsacks hire	0	1,600	1,600	1,600	800	800	800	600	600	600
Ridomil®	0	60,000	60,000	60,000	30,000	30,000	30,000	22,500	22,500	22,500
Labour (KES)										
Seed treatment	0	0	3,300	3,300	0	3,300	3,300	0	3,300	3,300
Spraying	0	12,800	12,800	12,800	6,400	6,400	6,400	4,800	4,800	4,800
Total costs (KES)	0	74,400	78,860	79,210	37,200	41,660	42,010	27,900	32,360	32,710
Net benefits for SR(Kes)	15,000	317,400	370,540	373,390	318,200	405,740	408,990	160,100	269,840	271,130
DNI SR(KES)		302,400	53,140	55,990	303,200	87,540	90,790	145,100	109,740	111,030
DCI (KES)		74,400	4,460	4,810	37,200	4,460	4,810	27,900	4,460	4,810
SR MR		4.06	11.91	11.64	8.15	19.63	18.88	5.20	24.61	23.08
Net benefits LR (KES)	10,800	279,000	306,940	311,190	291,000	344,140	350,990	99,300	139,040	145,890
DNI LR(KES)		268,200	27,540	32,190	280,200	53,140	59,990	88,500	39,740	46,590
DCI (KES)		74,400	4,460	4,810	37,200	4,460	4,810	27,900	32,360	32,710
LR MRR		3.60	6.26	6.69	7.53	11.91	12.47	3.17	1.23	1.42

Where DNI, DCI and MRR represent Difference in net income, Difference in input cost and Marginal rate of return percentage respectively while SR and LR represent short rain and long rain respectively. 0%, 66% and 100% are *T. asperellum* concentrations. Unsprayed, 7, 14 and 21 represent fungicide spray interval.

5.4.4 Comparison between Rooted Apical Cutting and Seed Tuber Crop

Crops from rooted apical cuttings had a higher ware grade yield of 11.48 t ha⁻¹ than seed grades which recorded a yield of 2.4 t ha⁻¹. Crops from seed tuber recorded yield of 9.24 t ha⁻¹ and 4.64 t ha⁻¹ for ware and seed respectively. Crops from seed tuber had a net benefit ratio of 1.06 and 0.72 during short and long rain season respectively while rooted apical cuttings recorded a net loss (Table 10). The cost of rooted apical cutting, transport and planting labour were 80%, 70% and 72% respectively higher than similar costs associated with crops from seed tuber (Figure 15).

Table 8: Cost benefit analysis on rooted apical cuttings and crop from seed tuber marketable grade size

VARIABLE	Short rain season		Long rain season	
	Tuber	Apical cuttings	Tuber	Apical cuttings
Yield (t ha ⁻¹)	15.62	15.99	13.09	11.98
Adjusted yield (t ha ⁻¹)	14.06	14.39	11.78	10.78
Gross field benefit (KES)	281,200	287,800	235,600	215,000
Costs				
Seed (KES ha ⁻¹)	135,000	444,440	135,000	444,440
Transport (KES/hectare volume/Km)	68	444	68	444
Labour (KES ha⁻¹)				
Planting	1800	4,200	1800	4,200
Total variable costs (TVC)	136,868	449,084	136,868	449,084
Net benefits (NB)	145,000	-161,284	98,732	-234,084
(NB ÷TVC)	1.06	Loss	0.72	Loss

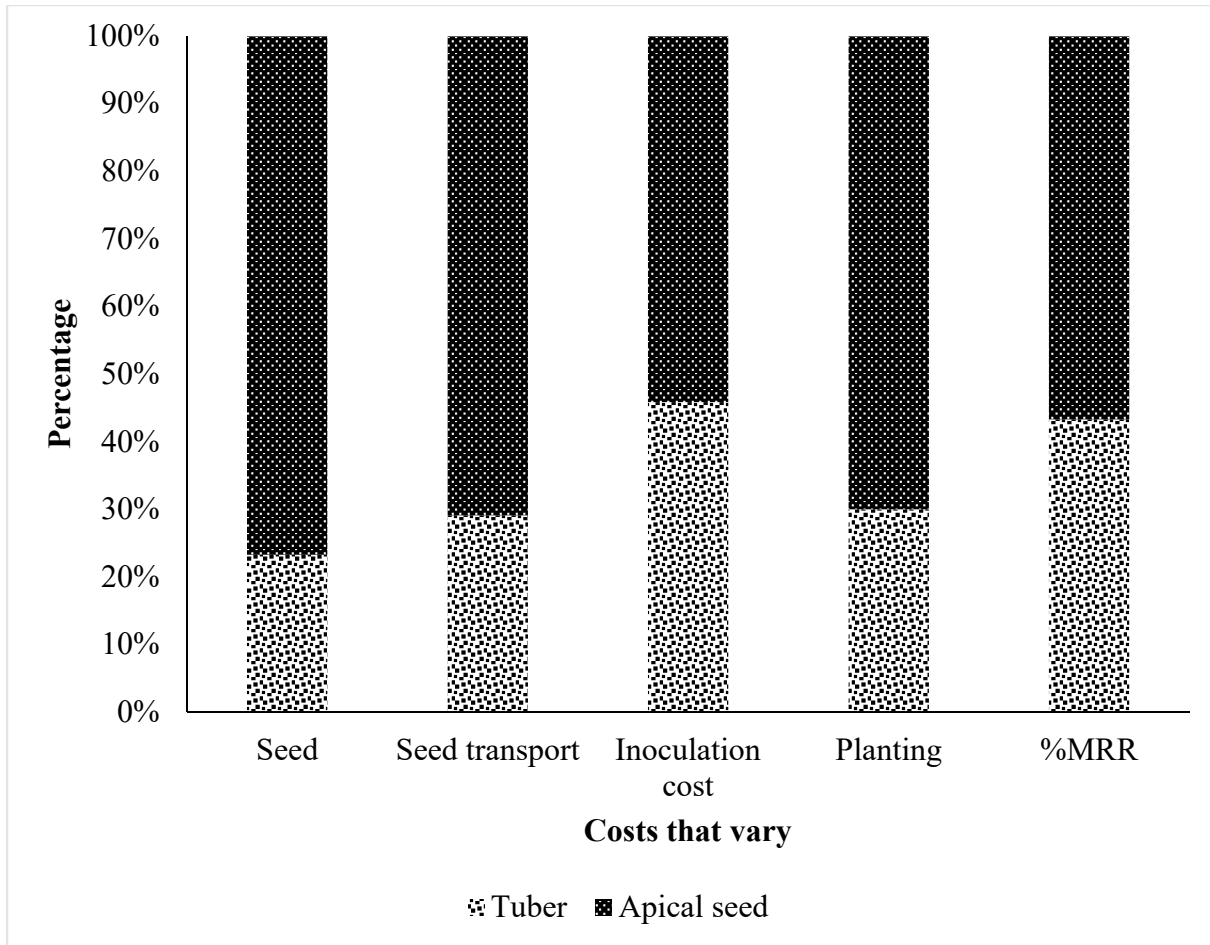


Figure 15: Costs that vary and percentage marginal rate of return between tuber and rooted apical cutting in seed production

5.4.5 Comparison between Peridermal Injection and Dipping Tuber Seed Treatment

Seed dipping had a higher cost benefit ratio than pericardial injection even though the latter had higher yield. Seed treatment labour contributed to 98% of the total variable cost in pericardial injection method of seed treatment as compared to dipping that had seed treatment labour taking 63% proportion of the total cost (63%) (Table 11).

Table 9: Cost benefit analysis (Kes ha⁻¹) on pericardial and dipping seed treatment methods

Variable	Short rain season		Long rain season	
	Injection	Dipping	Injection	Dipping
Yield (t ha ⁻¹)	15.47	12.13	14.48	13.09
Adjusted yield (t ha ⁻¹)	13.92	10.92	13.03	11.78
Gross field benefits (Kes)	278,400	218,400	260,600	235,620
Costs (Kes)				
Hypodermal syringe	640	0	640	0
Onion bag	0	2000	0	2000
Labour				
Seed treatment	27,000	3,375	27,000	3,375
Total variable cost	27,640	5,375	27,640	5,375
Net Benefit	250,760	213,035	232,960	230,245
(NB ÷TVC)	9.07	39.63	8.43	42.84

5.5 Discussion

Efforts in developing integrated disease management has been directed at minimizing excessive fungicides usage. Numerous strategies have been studied including a combination of nutrition with fungicides (Mosota *et al.*, 2017), use of biological controls (Kohl *et al.*, 2019) and disease free seed materials (Wang *et al.*, 2017). However, the profitability of the new practice or combination of the strategies has rarely been explored. The present study determined the cost benefit accrued as a result of *T. asperellum* seed treatment, Ridomil® application regimes and their combinations. The study further explored on the benefits associated with the use of disease free seed material (rooted apical cuttings) and seed treatment methods (Dipping or pericardial injection) to enhance improved net farm income. Partial budgeting using marginal rate of return is a simple method in determining profitability of new technology or combination of practices using additional costs and returns which could be easily understood by farmers (CIMMYT, 1998). The method has been used by several scientists including Carroll *et al.* (2009), Kassa and Bayene (2001) and Kumar *et al.* (2015).

Severe disease outbreaks as a result of prolonged favourable conditions (Whisson *et al.*, 2016) result in small tuber size grade affecting marketable grade due to complete defoliation which

ultimately affect farm returns (Bussan *et al.*, 2007). Similar results were observed in the present study in unsprayed treatments. Yesuf and Desta (2015) reported that defoliation by late blight led to 52.94% yield decline in unsprayed. Deahl and Inglis (1993) findings reported that reduction in yield is associated with loss of leaves loss due to defoliation due to increased late blight severity. Fungicide application at 14 and 7 days effectively suppressed *P. infestans* while seed treatment with *T. asperellum* offered prior protection which was also reported by Komy *et al.* (2015) resulting in higher additional yields than unprotected plots. Pre-treatment contributed to maximum tuberization and enlargement resulting in more marketable tubers. The observation was also in agreement with the results obtained by Hijri (2016) who found that seed inoculation with arbuscular mycorrhiza resulted in 10% yield increment.

The highest MRR was observed in treatments sprayed at 14 days interval in the long rain season despite yield not being significantly different from 7 day spray interval. Fungicide application at 21 and 7 day spray interval was affected by seasonality which also differed in late blight management and yield gained. Higher MRR was attributed to the additional yield obtained as a result of better disease control. Late blight management using fungicide application at 21 day spray interval during short rain season resulted in additional yield higher than during long rain season which was able to pay the additional costs than what 7 day spray interval managed to recompense resulting in higher marginal rate of return. Ridomil[®] application at 14 day spray interval reduced costs of purchasing Ridomil[®] and application labour by half relative to 7 day spray interval regime leading to higher returns. Namanda *et al.* (2004) reported increase in yield as a result of fungicide application on a weekly basis but observed higher total variable costs associated with the weekly spray interval compared to biweekly spray interval which resulted in saved costs of Ridomil[®] (KES 3000) and fungicide application associated costs (KES 2,600). This increased net benefits accrued to 14 days interval but was greatly dependent on weather conditions. Flannery *et al.* (2004) also reported cost saving of KES 34,200 per hectare from reduction of fungicide application on genetically modified potato.

Rooted apical cuttings produced through tissue culture has been proposed to have a potential of reducing the seed supply gap using rapid multiplication technology while managing disease incidences (El-Helaly, 2012). However, little has been documented on their cost benefit over conventional propagation method that involves use of seed tuber. In the present study, results suggest that apical cuttings had higher yield during short rain season than tuber seed crop. This is

against Benz (1995) findings who reported higher yield in tuber seed crop than apical cuttings. However, the greatest proportion of the costs was attributed to purchase of the seedlings and planting labour resulting in net loss. The study findings suggest that, the use of rooted apical cuttings either for seed or ware production without recycling the seed would result in net loss. Higher gross profit in the crops from seed tuber was attributed to higher yield against lower cost of seed and planting labour compared to rooted apical cuttings. During long rain season, survival rate of rooted apical cuttings also contributed to lower yield as affecting plant population per unit area (Johnson & Cummings, 2013) which led to higher net loss.

The results of the present study suggest that seed pre-treatment contributed to improved returns. Seed treatment with *T. asperellum* at 66% and 100% concentration both had a positive MRR higher than 100% as well higher net benefits than the untreated crops. The findings are in contrast with Hijri (2016) findings who reported seed treatment did not improve net income. Seed treatment with *T. asperellum* at 100% and 66% concentration had a variable MRR between long and short rain season spray regime. This could be attributed to higher efficiency in late blight management in crops treated with *T. asperellum* at 100 % than *T. asperellum* at 66% during long rains when the conducive conditions for *P. infestans* were prolonged increasing disease severity than during short rains. Improved disease control with *T. asperellum* at 100% concentration resulted in increased yield though not significantly different from *T. asperellum* at 66% concentration, the additional yield was able to pay for the additional costs associated with the treatment thereby giving a higher marginal rate of return. During short rain season, additional yield observed in *T. asperellum* at 66% concentration treatment gave higher marginal rate of return than *T. asperellum* at 100% concentration. This indicates that, even though the two treatments yield was not significantly different, using *T. asperellum* at 100% concentration could be more beneficial and advantageous where conditions conducive for late blight are prolonged. On the other hand, fungicide application in combination with seed treatment had a higher marginal rate of return compared to crops from untreated seed sprayed with Ridomil[®] and unsprayed crops. Seed treatment and fungicide application contributed to additional yield which resulted in improved returns consequently reducing the number of fungicide applications in the cropping season. Similar results were observed by Carroll *et al.* (2009) who found that border treatment resulted in reduced insecticide application on aphids (*Myzus* species).

Even though pericardial injection contributed to higher yield compared to dipping which was also observed by Hollywood (2014), the latter had higher cost benefit ratio as a result of lower seed treatment labour than the former which was not established by the worker. Seed treatment through dipping method reduced the cost of seed treatment by 87.5%. Seed treatment by either dipping or pericardial injection contributed to disease management and improved yield and therefore cost benefit analysis was essential in determining the most cost effective method of seed treatment.

Combination of fungicide application with seed treatment in addition to improved net income owing to minimized fungicide application which could further result in reduced pesticides exposure and chemical residues in the soil environment and crop products. Rooted apical cuttings though gave a net loss, they are superior in terms of health status and can be used to multiply seed in the subsequent seasons to realize their benefits. Alternatively, rooted apical cuttings could be purchased at lower prices of up to 2 Kenya shillings per seedlings if mass production is achieved but the harvested tubers sold as seed grade at a higher price than seed tuber as they can be replanted for several times. This would result would increase net income among potato farmers.

CHAPTER SIX
EFFECT OF RIDOMIL® APPLICATION INTERVAL ON METALAXYL
RESIDUE IN POTATO TUBERS

Abstract

Globally, Ridomil® (metalaxyl 4% + mancozeb 64%) is one of the most extensively used fungicide to manage late blight on potato. Emergence of fungicide insensitive strains has frequently led to the need for multiple sprays during a cropping season raising human health concerns. The objective of the study was to determine the effect of Ridomil® application intervals on metalaxyl residue in potato tubers. Freshly harvested samples were obtained by harvesting from randomly selected plants within plots sprayed separately with Ridomil® (2.5 g L⁻¹) at 7 (Manufacturer Recommended Regime (MRR)), 14 and 21 day spray intervals. Similarly fresh tuber samples were randomly collected from Limuru market and Wakulima market in Nairobi during market day. Metalaxyl residue was extracted from fresh potato tuber samples 48 hours after harvesting using Soxhlet method and quantified using Gas Chromatography Mass Spectrometer (GC-MS). Percentage metalaxyl recovery of 98.8% was observed. Results showed that plots sprayed at 7 day spray interval and samples collected from Limuru market recorded 1.69 and 0.09 mg Kg⁻¹ respectively which were higher compared to the acceptable limit (0.08 mg Kg⁻¹) proposed by European Food Safety Authority (EFSA) of European Union. Samples from Wakulima market and those obtained from plots sprayed with ridomil® at 21 day spray interval gave metalaxyl residues below the limit of detection level 14 day spray intervals. These results suggest that application of Ridomil® at short intervals could endanger the health of potato consumers. The findings are relevant to issues regarding occupational safety for farm workers as well as food quality concerns for potato consumers in Kenya. Further, the findings of this study support argument for safe alternatives in late blight management including utilization of integrated pest management practices that emphasize reduced fungicide application.

Key words: Application interval, Carbon disulphide, Ridomil® residue, Soxhlet

6.1 Introduction

Global human population is expected to reach 50 billion people by 2050 requiring 70% food increase to keep pace with the growing demand for food. The current food demands, in the face of 21st century challenges, has led to unsustainable practices to improve on food production so as to feed the ever increasing world population (Savary *et al.*, 2017). Increase in food production faces a number of challenges including reduced land size exerting pressure on existing agricultural systems (Saravi & Shokrzadeh, 2011). Efforts to increase food production has been directed to dependence on agrochemicals including fertilizers, insecticides and fungicides (Hirooka & Ishii, 2013). Potato has been identified as a key crop in enhancing food security and farm income worldwide (Devaux *et al.*, 2014). The tuber crop is consumed by over one billion people globally (Geremewe, 2019). However, potato is one of the most pesticide demanding agricultural crops because it is also prone to diverse biotic stresses. These biotic stresses includes late blight as one of the major causes for low yields globally (Majeed *et al.*, 2017a) and effort to manage the pathogen is solely through agrochemicals (Carputo *et al.*, 2005).

Use of fungicides has also led to increased yield due to their high efficiency in managing plant diseases (Kuai *et al.*, 2017). However, extensive use of pesticides to manage biotic stresses has led to nearly 38 billion USD spent on the chemicals each year (Pan-Germany, 2012). Gianessi and Reigner (2005) reported that without fungicides to protect vegetable crops, yield losses of 50 – 95% could occur. Recently Ogawa *et al.* (2011) found that, the use of Isotonil[®] resulted in additional rice yield due to increased effectiveness in leaf blast control. Chemical fungicide overuse to manage late blight has become a major practice globally (Lamichhane, 2017; Wesseling *et al.*, 2001) leading to accumulation of chemical residues into soil and food system implying a threat to nature and ecosystem (Palma *et al.*, 2014). In addition, the chemical residues pose human health problems especially if they enter the food chain (Rahman *et al.*, 2014) and biodiversity deterioration due to their ability to remain in soil for quite some time implying the need for chemical residue evaluation (Hussain *et al.*, 2009). The emergence of new fungicide resistant *P. infestans* strains has instigated an increase in fungicide use (Kromann *et al.*, 2009) and concomitant short spray intervals to manage late blight (Majeed *et al.*, 2017b). The effect of this has been an increase in exposure to chemical spray drifts and possibly accumulation in tubers that could pose health hazards to farm workers and consumers (Juraske *et al.*, 2009; Popp *et al.*, 2013).

Bio-accumulation of pesticide in higher trophic level food chain has been associated with several acute and chronic illnesses due to prolonged exposure (Noyes *et al.*, 2009). Moreover fungicide drifts cause unintentional poisoning to farm workers and consumers (Lee *et al.*, 2011). The level of toxicity depends on the length and frequency of fungicide exposure which in severe cases of long term exposure cause coma and death (Pan-Germany, 2012). Mostafalou and Abdollahi (2013) reported chronic human diseases affecting nervous, reproductive, cardiovascular and respiratory system as a result of long term exposure to fungicides. Pesticide residues have also been associated with cancer including brain cancer, renal cell cancer and prostate cancer (Cocco *et al.*, 2013; Heck *et al.*, 2011; Hou *et al.*, 2012; Lee *et al.*, 2011; Vinson *et al.*, 2011). Prolonged exposure to fungicides experienced by chemical applicants threaten the health of their unborn babies (Shim *et al.*, 2009). Therefore, it is necessary to understand chemical residues in the management of pests in relation to their efficacy (Duan *et al.*, 2016).

Ridomil® (metalaxyl 4% and mancozeb 64%) is one of the most widely used fungicide to manage late blight globally applied up to 10 -15 times per cropping season depending on weather conditions (Taylor *et al.*, 2013). Metalaxyl and mancozeb overuse on potato to manage late blight pose health risks arising from residues in food and drinking water (Atmaca *et al.*, 2018; Lopes *et al.*, 2009). Mancozeb is non-systemic preventive fungicide (Gullino *et al.*, 2010) usually broken down by plant to Carbon disulphide which accumulate in potato leaves and in pericardial layer of tubers if extensively applied (Alam *et al.*, 2012). The molecule is carcinogenic and antithyroid compound (ethylenethiourea) when exposed to light (Panganiban *et al.*, 2004). Prolonged exposure to mancozeb is associated with dermatitis and eye problems (Cole *et al.*, 1997) and therefore it is hazardous to occupational workers (Wesseling *et al.*, 2005). However, studies have shown Mancozeb being non-systemic do not dissipate into tuber tissues. Dissipation into the pericardial layer could be removed by peeling which is a usual preparation procedure for potato before cooking (Alam *et al.*, 2012; Ritu *et al.*, 2013). Recently Kim *et al.* (2017) reported that prolonged exposure to carbamates resulted in asthma and chronic problems including cancer. Metalaxyl is an acylanilide systemic fungicide with curative action against downy mildew and late blight. Multiple application especially during long rain season has been found to dissipate into crop products (Laurie *et al.*, 2015). Abass *et al.* (2007) reported that metalaxyl affects liver functionalities. Recently Zhang *et al.* (2017) found that metalaxyl influence breast cancer progression. Kinetics of metalaxyl have been studied in soil, grapes, cabbages and potatoes (Wang *et al.*, 2014; Yan *et al.*,

2015). Standards for metalaxyl have been set by European Food Safety Authority of European Commission that has set acceptable limits of 0.08 mg Kg⁻¹ body weight and Acute Reference Dose of 0.05 mg Kg⁻¹ body weight in daily consumption in directive (EC) 91/414/EEC. Therefore, there is need to determine effect of the frequency of fungicide application on chemical molecule residues in crop products.

There is need to embrace sustainable farming practices that focus on integrated disease management. This aligns with the need to enhance natural enemies and their integral role in crop pests management (Wang *et al.*, 2010). Choosing integrated pest and disease management supports to reduction in chemical dissipation into food system and maintaining environmental biodiversity (Erisman *et al.*, 2016). The practices should emphasize on increasing fungicide application interval to give the crop sufficient time to breakdown the molecules thereby lowering the chemical molecules accumulation in their sink.

The objective of this study was to determine the levels of metalaxyl dissipated into the tuber following Ridomil® application at different spray intervals.

6.2 Materials and Methods

6.2.1 Sample Collection

Tubers from the field trial (chapter 4) were harvested 20 days after Ridomil® application from unsprayed, 7, 14 and 21 day spray interval plots untreated with *T. asperellum*. Tubers were also randomly sampled from 10 buyers during market day from Limuru and Wakulima market Nairobi. The samples were washed with distilled water and air dried for 15 minutes. They were packed in polyethylene and placed in ice boxes before transporting the same day to the Chemistry Department, Pesticides Research Laboratories at University of Nairobi. The samples were dried further in the laboratory using anhydrous sodium sulphate.

6.2.2 Standards, Reagents and Instrumentation

Metalaxyl certified pure analytical standard grade (purity, > 99%) was obtained from Pest Control Products Board (PCPB) Headquarters, Nairobi. Ethanol, sodium hydroxide, distilled water, magnesium sulphate, dcm, and other analytical grade chemicals were purchased from local stockists in Nairobi. Metalaxyl was determined by capillary gas chromatography mass

spectrometer (GC – MS) using Mass Spectrometer model HP5771A and NIST library 2008. Mancozeb, a dithiocarbamate molecule residue is non systemic and therefore was not quantified.

6.2.3 Sample Processing

Potato tubers were washed with distilled water to remove soil particles. One tuber was randomly selected from the composite sample of each treatment and each tuber cut and homogenized using Hobart food processor.

6.2.4 Metalaxyl Extraction

Analysis of metalaxyl residues in the samples was performed by GC-MS model HP5771A. A sample of 10 g was picked using sterilized spatula and washed with 10 mL of dichloromethane: n-hexane (1:1, v/v). The wet sample was chemically dried using a polar extraction solvent of 3 g of anhydrous sodium sulphate for every gram of the sample before they were covered with aluminium foil and incubated overnight at 45 °C. The dried sample was placed in a thimble in the soxhlet set up. Hexane and acetone were put in 250 mL volumetric flask at a ratio of 1:1 and soxhlet extraction set for 16 hours in three replication (5 g each).

6.2.5 Purification of Samples of Extracts

Clean-up was done using silica. Briefly, chromatography clean-up column measuring 20 cm long by 2 cm in diameter was filled with 2 g, 1.8 g and 0.5 g of anhydrous sodium sulphate (Na_2SO_4), silica oxide (SiO_2) and activated charcoal respectively in that order. Exactly 2 g of Na_2SO_4 was added followed by tapping the column to settle the particles uniformly. Conditioning of the column was done with 15 mL of hexane-acetone mixture (1:1) and discarded. Soxhlet extracts were transferred in to the column and the tube rinsed with 1 mL of hexane-acetone mixture (1:1) five times and the filtrate immediately returned in to the column to avoid drying. Volumetric flask was used to collect the elute and then rotary-evaporated to 1.5 mL before transferring in to GC sample auto vial. Nitrogen stream was added to concentrate the extract to 1 mL. The extract was analyzed using GC – MS.

Recovery was conducted to confirm efficiency of method used in analyzing the molecules residue. Triplicates of 10 g of the tuber samples were injected with 50 μL of pure Metalaxyl standard solution of 1ppm which was extracted. The samples were chemically dried using 3 g of

activated anhydrous sodium sulphate and blended into homogenized powder using pestle and mortar. The analysis was conducted as described in section 6.2.5 above. Control samples (untreated tubers) and reagent blanks were also injected with 50 μL of pure Metalaxyl standard solution of 1ppm. Extraction, clean up and analysis procedures were conducted same way as described above to find out interferences due to substrate and reagents.

6.2.6 GC - MS Analysis and Quantification of Extract Samples

Metalaxyl residue levels were determined using GC – MS analysis where levels were read. Briefly, DB-5 fused silica capillary column measuring 30 m (long) by 0.25 μm (diameter) was coated with 5% phenyldimethylpolysiloxane. Helium gas at 99.9% purity was passed at a flow rate of 1.0 mL/min as a carrier gas. Operating temperatures were set as follows; oven temperature were initially set at 70 $^{\circ}\text{C}$ for 1 minute, increased at a rate of 15 $^{\circ}\text{C}$ per minute till it reaches 175 $^{\circ}\text{C}$, then at 2 $^{\circ}\text{C}$ per minute to 215 $^{\circ}\text{C}$, followed by 10 $^{\circ}\text{C}$ per minute to 265 $^{\circ}\text{C}$ and finally 20 $^{\circ}\text{C}$ per minute to 290 and held for 8 minutes. Injection volume (1 μL) was injected at temperature of 250 $^{\circ}\text{C}$ in split-less mode. Analysis of control samples from potted crop that included untreated potatoes were carried out alongside the treated samples. Field blanks consisted of distilled anhydrous sodium sulphate carried to track field contamination and then subjected to analytical procedures in three replicates.

6.3 Data Analysis

Standard errors were calculated to separate the values of the treatments.

6.4 Results

6.4.1 Assessment of Metalaxyl Residues

Metalaxyl percentage recovery was 98.8%. Mass spectrum of metalaxyl standard solution and ion chromatogram of metalaxyl was obtained after analysis using GC-MS. Similarly, the ion chromatograms for samples obtained from unsprayed, 7, 14 and 21 day spray intervals plots, Limuru market and Wakulima market were observed.

Spraying Ridomil[®] at 7 days spray interval had the highest levels followed by samples collected from Limuru market which were above acceptable limits of 0.08 mg Kg⁻¹ directed by European Food Safety Authority if consumed daily while 14 days spray interval was on the cut

line limit. Samples from plots sprayed at 21 days interval and Wakulima market indicated that they could be safer as they recorded undetectable limits below European Union limits (Table 12).

Table 10: Metalaxyl residues results

Treatment	Metalaxyl (mg Kg⁻¹)
7 day spray interval	1.69±0.02
14 day spray interval	0.08±0.00
21 day spray interval	<LOD
Limuru market sample	0.09±0.00
Wakulima market sample	<LOD
Unprotected	<LOD
LOD	0.05 mg/Kg

Key; LOD and < represents Limit of Detection and less than respectively

6.5 Discussion

Late blight remains a major challenge in ware and seed potato production especially when conducive conditions for *P. infestans* are prolonged during the cropping season requiring farmers to adopt short fungicides spray intervals. The discovery of metalaxyl (curative) co-formulation with mancozeb (Preventive) improved the management of late blight but recent emergence of more aggressive *P. infestans* has required farmers to apply fungicide with increased frequency especially during long rain season in Kenya. This could contribute to chemical dissipation into the tubers posing human and environmental hazard. Although metalaxyl with a maximum allowable limit of 0.03 mg/Kg of body weight per day has low mammalian toxicity, extensive application to manage late blight when conducive conditions are prolonged pose human health risks. The molecule is classified as EPA toxicity class III. Knowledge on the amount chemicals that flow into food products at point of consumption remains key in public health point of view.

Studies on possibility of Metalaxyl and Mancozeb dissipation into crop products and soil have been explored by scientists. Yan *et al.*, 2015 reported that Metalaxyl residue was observed but the levels were below 0.05 mg Kg⁻¹ following 7 day interval application in China. Further studies suggested that Metalaxyl residue levels were below detectable limits in potato tubers collected from either during short or long rain season (Kamau, 2017). Mancozeb deposition and

dissipation in vegetables has also been explored but found to be below detectable limits due to its non-systemic nature (Sharma *et al.*, 2006). The present study attempted to establish whether Ridomil® application to manage late blight on potato under Kenyan conditions contribute to Metalaxyl residue dissipation into tubers. The results of the study suggested that dependence on fungicide pose consumers' health. Spraying of Ridomil® at 7 days interval as recommended by the manufacturer resulted in a higher level of residue in tubers than set acceptable. The practice is common in all potato producing regions due to favourable weather conditions for late blight epidemics. Spraying at regimes of 14 days interval gave a level that was same as acceptable limits. This suggests that a longer spray intervals (probably 14-21 days) should be adopted and applied in combination with host resistance, biological control and cultural practices to manage late blight effectively. The results are inconsistent with Alam *et al.* (2012) results who reported metalaxyl persistence only in leaves in plots sprayed with Ridomil® at double dosage while mancozeb and metalaxyl were below detectable level following 7 days application regime in tubers. Mancozeb is non-systemic contact preventive fungicide and this reduced its detection at tuber tissues. Occurrence of metalaxyl residue in tuber tissues could be due to the inability of the potato crop to break down the molecule efficiently as result of increased fungicide application frequency.

The process of metalaxyl uptake by potato, break down and dissipation is influenced by a number of factors. Metalaxyl dissipation is affected by rapid degradation of the chemical as a result of dilution of the toxicant associated with crop growth coupled with conducive environmental conditions. The major metabolic pathways include hydrolysis and oxidation of the methyl ester which are dissipated by plant uptake, microbial degradation, photodecomposition or leached. In soil, metalaxyl acid is predominantly formed by soil microorganism which migrate to lower soil horizon resulting in ground water contamination (Sukul, 2006). Harvest period after the last fungicide application influence the amount of chemical residue dissipated into the tuber. Study conducted by Abd-Alrahman and Almaz (2012) indicated that harvesting tubers within 5 days after the last fungicide application resulted in residue concentration exceeding the maximum residue levels. Concentration of the fungicide during application affect the crop ability to break the chemical molecules into non-harmful compounds resulting in increased chemical dissipation into potato tubers. Use of higher dosage to manage late blight is prevalent especially when conducive weather conditions for blight development are prolonged. Abd-Alrahman and Almaz (2012) and Alam *et al.* (2012) in their study observed that increasing fungicide application rate resulted in

increased chemical residue. Environmental conditions including temperature, relative humidity and rainfall and plant physiology also influence chemical dissipation into crop products (Edward, 1975; Macharia *et al.*, 2009).

Residue analysis showed the levels of metalaxyl were above acceptable limits which indicates farm workers face double exposure as a result of increased fungicide application frequency that cause dermal or aerosol exposure even at low levels as reported by Damalas & Eleftherohorinos (2011) and Macfarlane *et al.* (2013) while mixing, applying the fungicides or working in treated field (Christensen *et al.*, 2015). Washing of the potato tubers after harvest to remove mud is rarely practiced. Studies have shown washing with tap water could decontaminate crop products by removing a percentage of pesticide residues especially from non-systemic fungicides including mancozeb (Cheverri *et al.*, 2004; Randhawa *et al.*, 2008). Some farmers leave the tubers in the field to harvest when market prices are conducive while others harvest and store for a shorter period which affect chemical residue in tuber tissues. This has not been explored to determine how long the residue molecules breakdown. Therefore chemical dissipation and deposition into crop products is a dynamic process which require scientists to evaluate the products continuously to provide informative advice to value chain actors

CHAPTER SEVEN

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

7.1 General Discussion

Dependence on the use of fungicides endangers human health and the natural ecosystem. Therefore, use of biological controls in the management of crop diseases could be eco-friendly and sustainable approach (Yao *et al.*, 2016). However, the use of biological controls has been reported to have a slower mode of action and greatly influenced by inoculum concentration. In the *in vitro* study, concentration of *T. asperellum* affected the growth of mycelial within a time frame which played key role in inhibiting mycelial growth and development of late blight. *Trichoderma asperellum* at 3.0×10^5 CFU/mL was unable to reduce development of both *P. infestans* and late blight development due to low concentration of the biocontrol. Similar results were observed by Fatima *et al.* (2015). This could be attributed to reduced competition ability for food and space against *P. infestans* and ineffective mycoparasitism ability (Keswani *et al.*, 2014) due to slow mycelial growth. Therefore, concentration, quick action and fast growth of biocontrol in relation to the target pathogen under all conditions are the most key important traits of biocontrol in enhancing their effectiveness. Ridomil® and mistress 72® did not allow leaf infection by *P. infestans* denoting that fungicides have a quicker action than biocontrols. Moreover, the fungicides did not inhibit mycelial growth of *T. asperellum*. Therefore, to minimize fungicides overuse, *T. asperellum* can be safely combined with fungicides to improve on late blight management.

Crops in the unsprayed plots were defoliated before complete tuberization while spraying with ridomil® at 21 days interval seemed ineffective that resulted in lower yield. On the other hand, seed pre-treated with *T. asperellum* had lower disease severity. This shows that *T. asperellum* constrained development of late blight early in the growth stages while fungicide application on foliage reduced *P. infestans* virulence. Rojas *et al.* (2013) reported that fungicide application offer both curative and preventive thereby reducing late blight severity. Chemical fungicides showed fast and effective control of late blight both *in vitro* and field experiments meaning they cannot be completely avoided, hence the need to combine them with other strategies to minimize their detrimental effects.

Effective disease management result in seed tubers of high quality in terms of size and quantity (Whisson *et al.*, 2015) which ultimately result in higher net farm income. Almaz (2012) observed that increasing fungicide application rate led to increase in chemical residue but did not

consider application interval. From cost benefit analysis, use of biocontrol in combination with fungicide had higher returns than when each treatment was applied singly. This shows that the technology could improve on net farm income.

Application of fungicides at 7 days spray interval resulted in accumulation of metalaxyl residue as a result of insufficient time given to the crop to break the chemical into inactive compounds. The implication of this is consumers been exposed to health risks associated with metalaxyl. Therefore the government should put measures in regulating fungicides use and training farmers to adhere to post harvest techniques.

7.2 Conclusions

- i. From objective one, *T. asperellum* at 66% concentration inhibited the mycelial growth of *P. infestans*. *Trichoderma asperellum* mycelial growth was not inhibited by Ridomil® and Mistress 72® indicating possibility of combining the strategy in integrated disease management program to manage late blight
- ii. In the objective two, seed treatment with *T. asperellum* at 66% concentration in combination with Ridomil application at 7 and 14 days spray interval reduced late blight severity more than any other combination. Seed treatment by pericardial injection had lower disease score than dipping
- iii. Cost benefit analysis indicate that seed treatment with *T. asperellum* at 66% and 100% concentrations returns were affected by seasonality. Seed treatment with *T. asperellum* at 66% concentration in combination with Ridomil® application at 14 day spray interval had the highest marginal rate of return. Seed dipping had a higher net benefit than pericardial injection. Producing ware potato from rooted apical cuttings resulted to loss.
- iv. Spraying potato with Ridomil® at 7 days interval resulted into metalaxyl residue accumulation in freshly harvested tubers.
- v. Therefore the use of seed treatment with *T. asperellum* at 66% concentration in combination with Ridomil® application at 14 days interval could be effective in managing late blight, improving on yield and net farm income while minimizing chemical exposure to human and negative impacts on the environment. Reducing fungicide application will ultimately reduce cost of production, and safe guard human health (handling, exposure to

spray drifts and chemical residues in food systems) and environmental concerns as well as possibilities for emergence of fungicide resistant *P. infestans* strains.

7.3 Recommendations

Treatment of seed potato with *T. asperellum* at optimal concentration in combination with fungicide application could provide an effective and sustainable option in late blight management contributing to improved potato production, increased income among farmers and reduced exposure to chemical fungicide while spraying and consuming the crop products. Symptomless seed tubers are no guarantee of having healthy seed but have a potential of been source of primary *P. infestans* inoculum that accelerate late blight epidemics. Detection of the pathogen in this asymptomatic tuber is usually not easy. Therefore, seed treatment with *T. asperellum* that delay late blight infection could be a sustainable option while fungicide offer foliage protection against *P. infestans*.

Integrated disease management programme for late blight on rooted apical cuttings need to be established especially during long rain season in Kenya as 7 day spray interval was not sufficient in managing late blight severity. Future studies should determine mechanism applied by *T. asperellum* in promoting potato growth and late blight management. Although using rooted apical cuttings in potato production resulted in net loss, there is need to determine return on profit and complete budgeting associated with rooted apical cuttings against tuber if the harvested tubers are sold as seed in subsequent season. Use fungicide different with active ingredient in alternate with combination with seed treatment with *T. asperellum* could be explored in future studies to reduce fungicide application frequency. In addition, studies on metalaxyl dissipation in potato tubers following pre-harvest period need to be explored. Samples from Limuru market had higher residue limit than minimum residue level which was not the case in Ukulima market of Nairobi. Therefore is need for continuous monitoring of the potato tubers occasionally.

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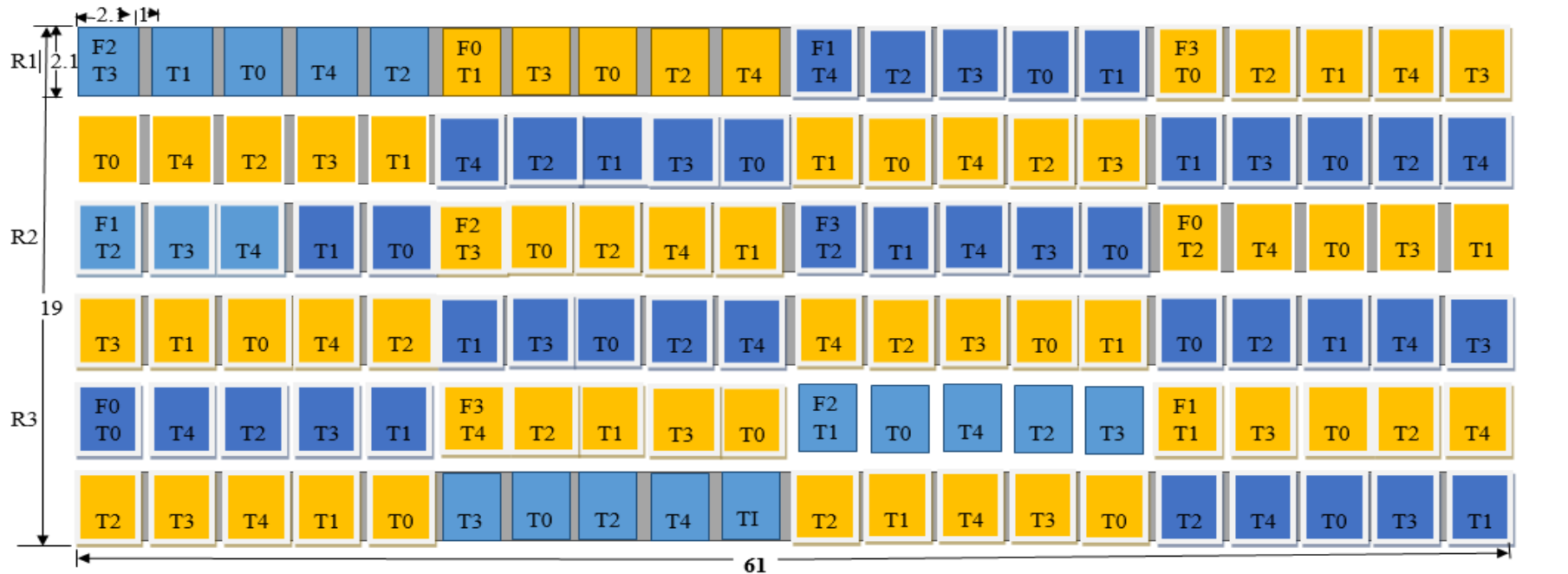
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APPENDICES

Appendix A: Randomized complete block design in split split arrangement for field experiment 1

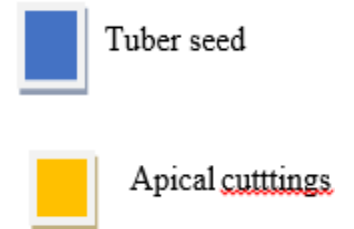


KEY

F0- unsprayed
 F1- 20 days interval
 F2- 15 days interval
 F3- 10 days interval

T0- untreated
 T1- 0 ml
 T2- 0.33 mL⁻¹
 T3- 0.66 mL⁻¹
 T4- Mistress 72[®]

R- Replication
 Unit; Metres



Appendix B: Randomized complete block design in split split arrangement for field experiment 2



Key; F0; unsprayed, F1 (7 days interval), F2 (14 days interval) and F3 (21 days interval)

T0; Untreated, T1; 100% *T. asperellum* concentration and T2; Mistress 72®

Seed dipping

Peridermal injection

Appendix C: Monthly average weather data

Month	Min Temp (°C)	Max Temp (°C)	Total Rainfall (mm)	Wet days	Relative Humidity (Rh%)
November 2018	10.8	24.8	471.8	9	66.4
December	11.2	23.6	624.1	13	69.0
January 2019	11.1	24.4	113.4	3	60.6
February	10.6	24.7	13.3	1	61.6
March	10.5	24.4	211.8	4	53.9
April	12.2	25.2	136.5	9	63.7
May	13.7	20.1	225.9	14	75.5
June	13.2	17.0	69.0	14	87.3
July	13.3	17.5	58.0	12	85.0

Appendix D: Mean squares for radial growth inhibition (mm) at 3, 5 and 7 days after inoculation

Source of variation	df	Radial growth inhibition (mm) at days after inoculation		
		Three	Five	Seven
Model	7	2414.12*	2241.98*	2358.10
Rep	2	12.17*	20.39*	20.22*
<i>Trichoderma</i>	5	3374.90*	3130.62*	3223.26*
Error	10	15.37	6.52	7.89
CV%		6.65	4.23	4.85
Mean		59.17	60.22	58.28

* means significant ($P \leq 0.05$)

Appendix E: Mean squares for lesion size at 3, 5, 7, 9 and 11 days after inoculation

Source of variation	df	Lesion size (cm ²) at days after inoculation				
		Three	Five	Seven	Nine	Eleven
Model	5	0.04*	0.18*	0.57*	0.86*	1.58*
Rep	2	0.03*	0.01*	0.01*	0.01ns	0.01ns
<i>Trichoderma</i>	3	0.05*	0.30*	0.95*	1.43*	1.43*
Error	6	0.01	0.01	0.01	0.01	0.01
CV%		4.64	5.56	3.25	4.22	2.34
Mean		1.91	1.59	2.24	2.40	2.62

* and ns means significant and not significant at $P \leq 0.05$

Appendix F: Analysis of variance testing influence of season, fungicide regime, seed type, seed treatment concentrations and their interactions across on AUPDC and yield

Source of variation	AUDPC			Yield (t ha ⁻¹)	
	df	Mean squares	F values	Mean squares	F values
Model	67	2793536.9	161.55*	230.64	61.16*
Season	1	137618071.5	1828.43*	1647.46	221.80*
Rep	2	176503.5	2.34*	6.23	0.839*
Regime	3	8592630.9	114.16*	3916.46	527.27
Season x Regime	3	994430.1	13.21*	340.75	45.87
Main plot error	6	75265.7		7.42	
Seed	1	1021162.60	40.11*	0.03	0.01ns
Regime x Seed	3	789.927	0.03ns	0.18	0.07ns
Sub plot error	6	25458.6		2.53	
Seed treatment	4	4411735.6	255.13*	214.52	56.89
Regime x Seed treatment	12	3036.0	0.18ns	5.135	1.36
Seed x Seed treatment	4	789.9	0.05ns	0.18	0.05ns
Regime x Seed x Seed treatment	12	4100.9	0.24ns	0.67	0.18ns
Sub-sub plot error	172	17292.3		3.77	
CV%			7.24		12.06

* and ns means significant and not significant at $P \leq 0.05$

Appendix G: Analysis of Variance testing influence of season, fungicide regime, seed type, seed treatment concentrations and their interactions on AUPDC and yield in the short and long rain season

Source of variation	df	Short rain season				Long rain season			
		AUDPC		Yield (t ha ⁻¹)		AUDPC		Yield (t ha ⁻¹)	
		Mean squares	F values	Mean squares	F values	Mean squares	F values	Mean squares	F values
Model	55	366001.21	73.14*	148.23	89.72*	582297.40	94.69*	112.23	429.23*
Rep	2	12286.16	0.92*	7.43	1.31*	369042.93	2.50*	7.14	2.31*
Regime	3	275650.74	169.75*	2260.28	397.31*	7545051.80	51.02*	1997.85	646.76*
Main plot error	6	13405.79		5.69		147873.22		3.09	
Seed	1	5031.08	0.54*	32.02	31.2	1725600.83	34.18*	35.34	7.46*
Regime x Seed	3	3694.08	0.40ns	0.78	0.76	64237.26	1.27*	2.83	0.60ns
Sub plot error	6	9240.15		1.03		50482.95		4.74	
Seed treatment	4	3227422.5	644.94*	290.12	175.60*	1319103.18	214.51*	13.48	51.56*
Regime x Seed treatment	12	9578.73	1.91*	8.71	5.28*	8662.86	1.41*	0.47	1.80*
Seed x Seed treatment	4	6014.80	1.20*	1.19	0.72*	7889.98	1.28*	0.43	1.66*
Regime x Seed x Seed treatment	12	4948.22	0.99*	0.88	0.54ns	2647.76	0.43ns	0.26	1.00*
Sub-sub plot error	64	5004.19		1.65		6149.37		0.261	
CV%			6.87		6.86		3.05		3.79

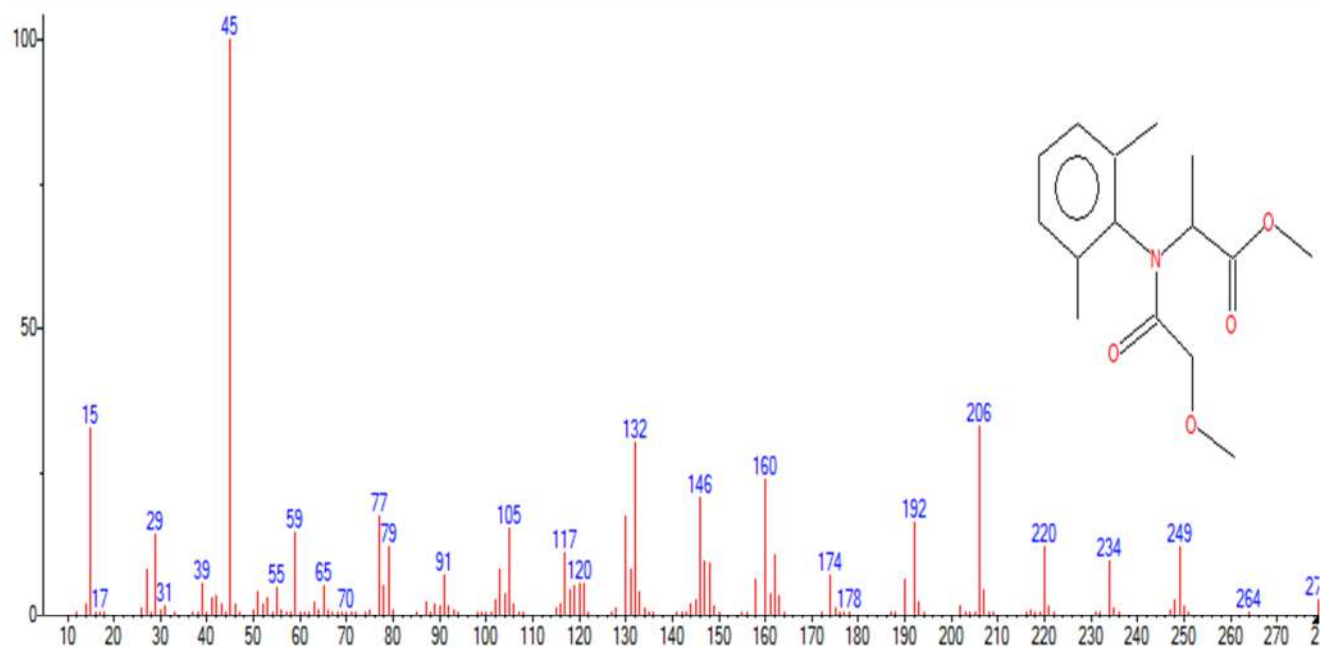
* and ns means significant and not significant at $P \leq 0.05$

Appendix H: Mean squares for AUDPC and yield on spray regime, seed treatment method and seed treatment rate

Source of variation	df	AUDPC		Yield (t ha ⁻¹)	
		Mean squares	F values	Mean squares	F values
Model	45	2341083.51	134.38*	173.38	27.03*
Season	1	78439068.34	1461.51*	129.52	53.02*
Rep	2	15557.84	0.28*	6.78	2.78*
Regime	3	5628321.78	104.87*	2075.24	849.44*
Season x regime	3	583697.10	10.88*	302.88	124.13*
Main plot error	6	53670.03		2.44	
Method	1	613741.67	9.60*	55.12	25.73*
Regime x method	3	5321.47	0.08ns	8.63	4.03*
Sub plot error	6	63945.42		2.14	
Seed treatment rate	2	3180975.05	182.59	186.990	29.15*
Regime x Seed treatment rate	6	20690.33	1.19*	1.281	0.20ns
Method x seed treatment rate	2	10887.51	0.62*	7.395	1.15*
Regime x method x seed treatment rate	6	18898.47	1.08*	2.217	0.35ns
Sub-sub plot error	48	17421.6		6.41	
CV%			8.11		16.24

* and ns means significant and not significant at $P \leq 0.05$

Appendix I: Chemical structure and ionic mass spectra for Metalaxyl standard



(mainlib) DL-Alanine, N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-, methyl ester

Name: DL-Alanine, N-(2,6-dimethylphenyl)-N-(methoxyacetyl)-, methyl ester

Formula: C₁₅H₂₁NO₄

MW: 279 CAS#: 57837-19-1 NIST#: 119023 ID#: 16121 DB: mainlib

Other DBs: RTECS, EINECS, IRDB

Contributor: NIST Mass Spectrometry Data Center, 1990.

10 largest peaks:

45 999 | 206 326 | 15 324 | 132 299 | 160 234 |
146 203 | 77 173 | 130 173 | 192 162 | 105 151 |

Synonyms:

1. Ridomil
2. Apron
3. CGA-48988
4. Metaxanin
5. N-(2,6-Dimethylphenyl)-N-(methoxyacetyl)-dl-alanine methyl ester
6. Subdue
7. Methyl 2-[(methoxyacetyl)-2,6-dimethylanilino]propanoate #
8. Metalaxyl-m
9. Metalaxyl

Estimated non-polar retention index (n-alkane scale):

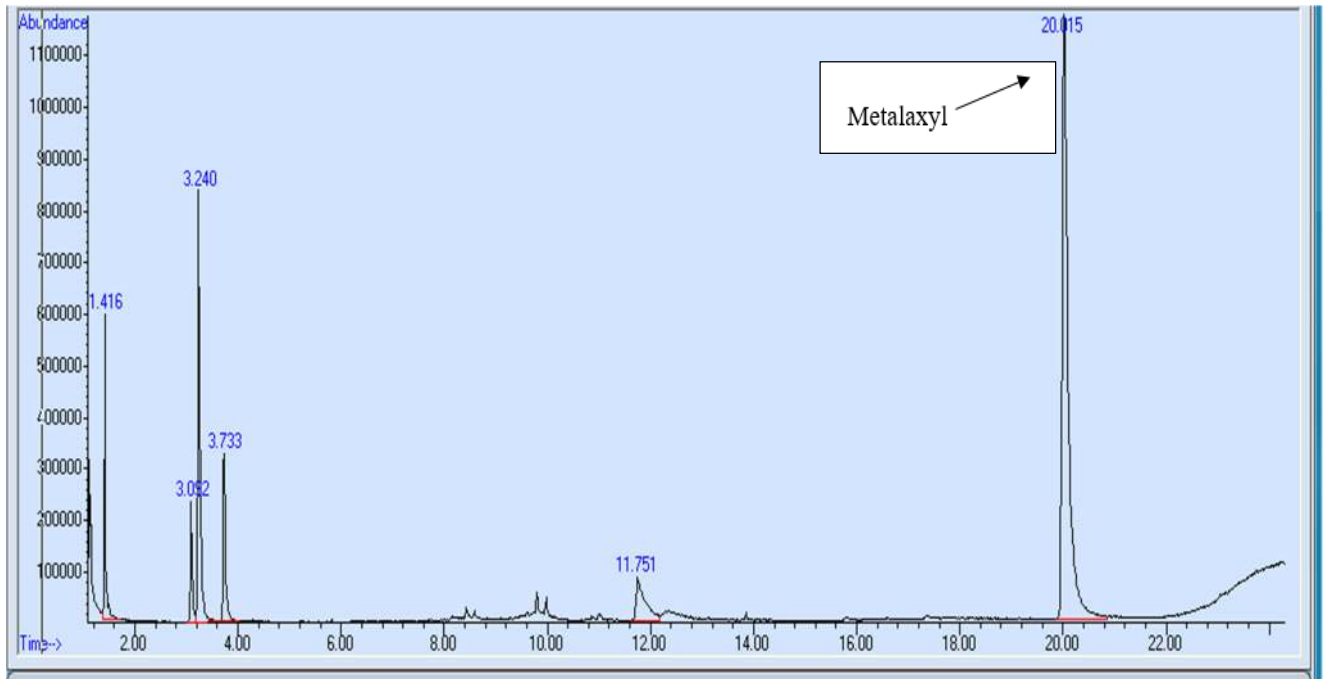
Value: 1996 iu

Confidence interval (Diverse functional groups): 89(50%) 382(95%) iu

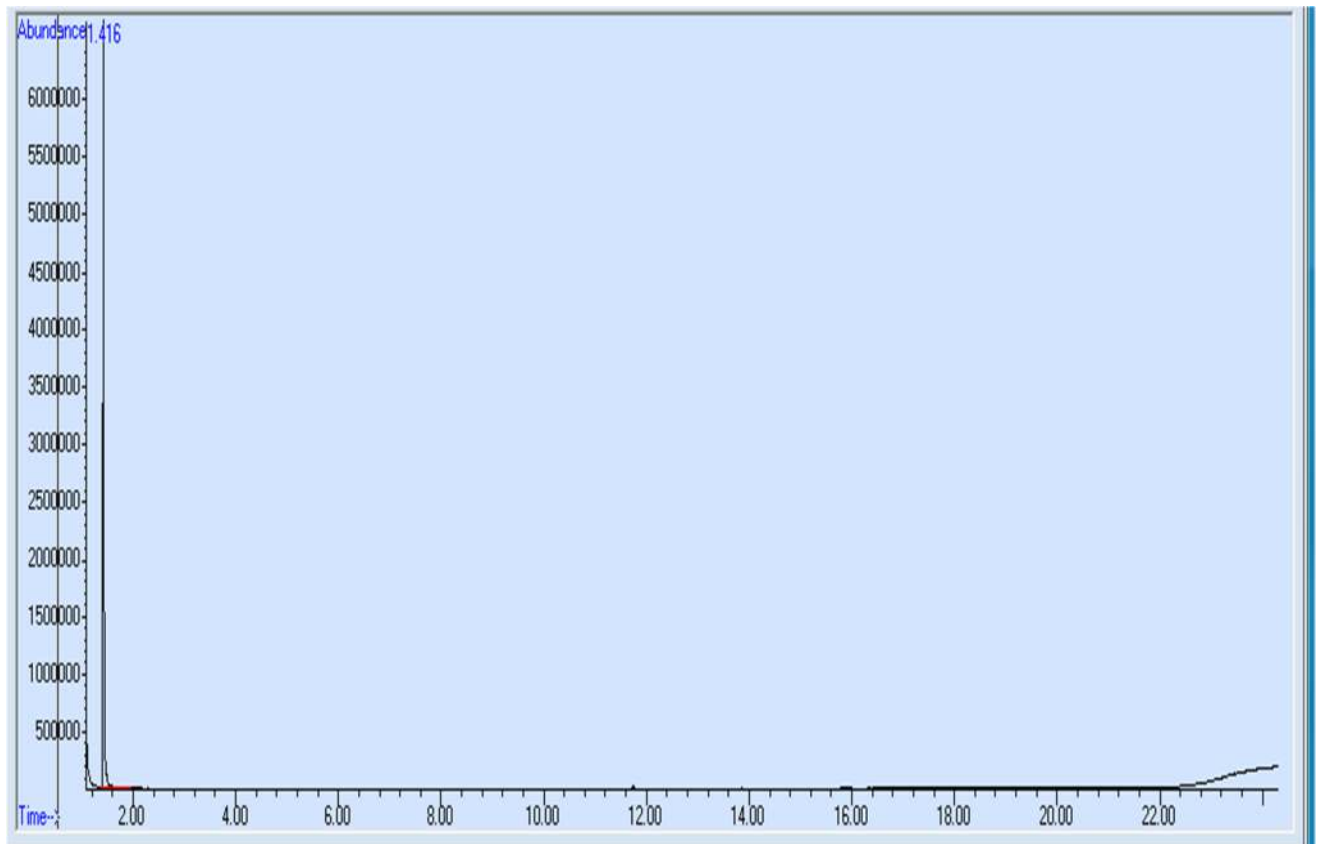
Retention index:

1. Value: 1882.9 iu
- Column Type: Capillary
- Column Class: Standard non-polar
- Active Phase: HP-1
- Column Length: 12 m
- Carrier Gas: He
- Column Diameter: 0.2 mm
- Phase Thickness: 0.33 um

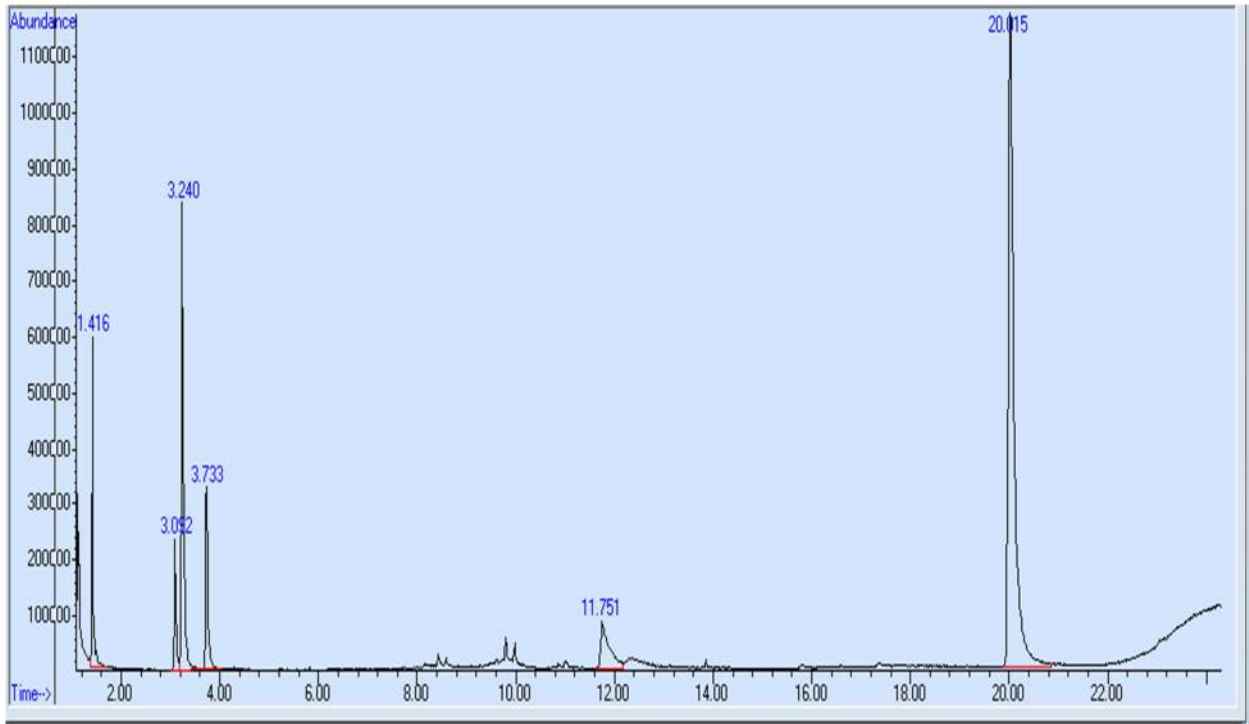
Appendix J: GC-MS ion chromatogram for Metalaxyl standard



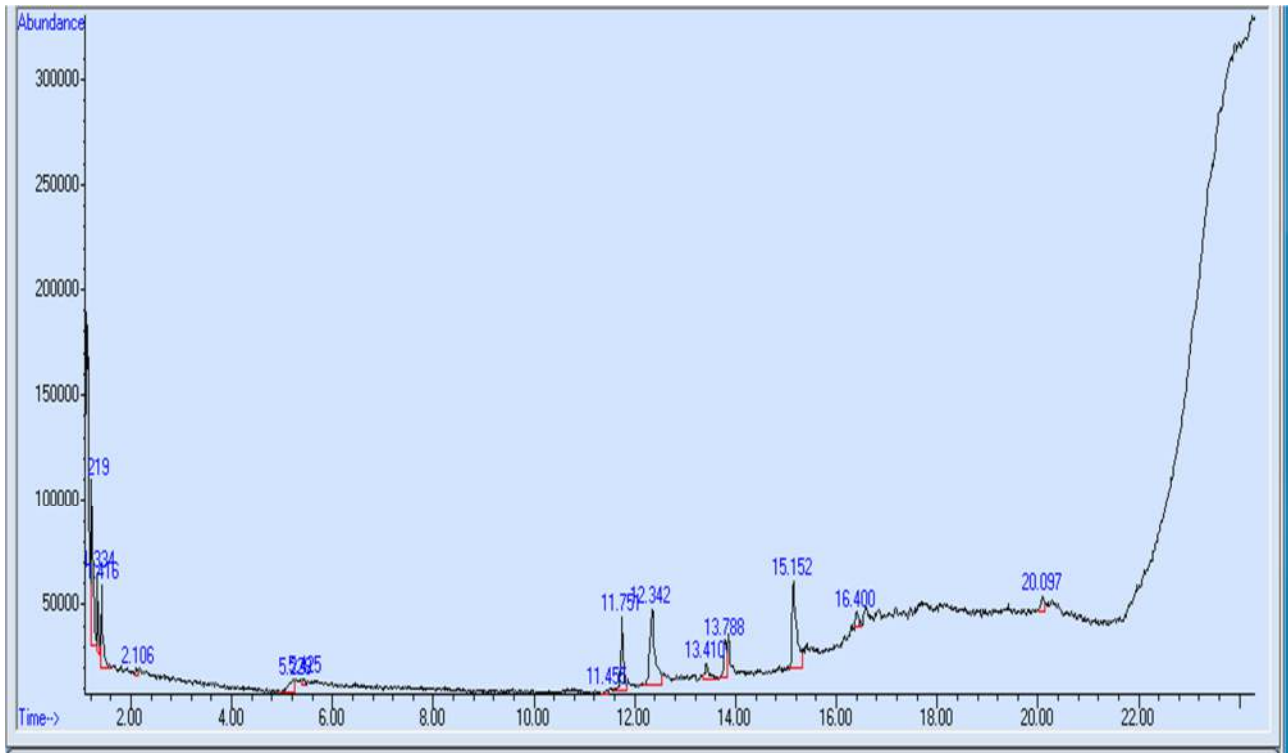
Appendix K: GC-MS ion chromatogram for unsprayed tuber samples



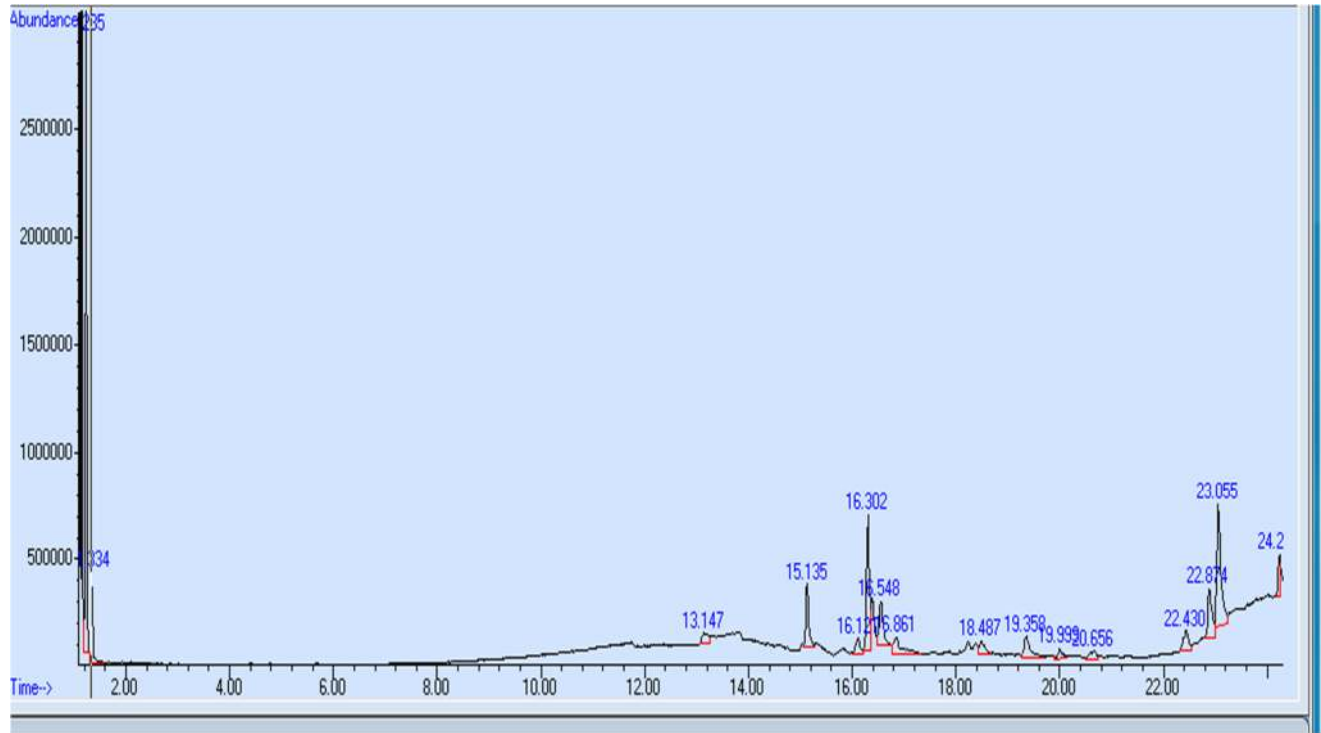
Appendix L: GC-MS ion chromatogram for tubers sprayed at 7 days interval



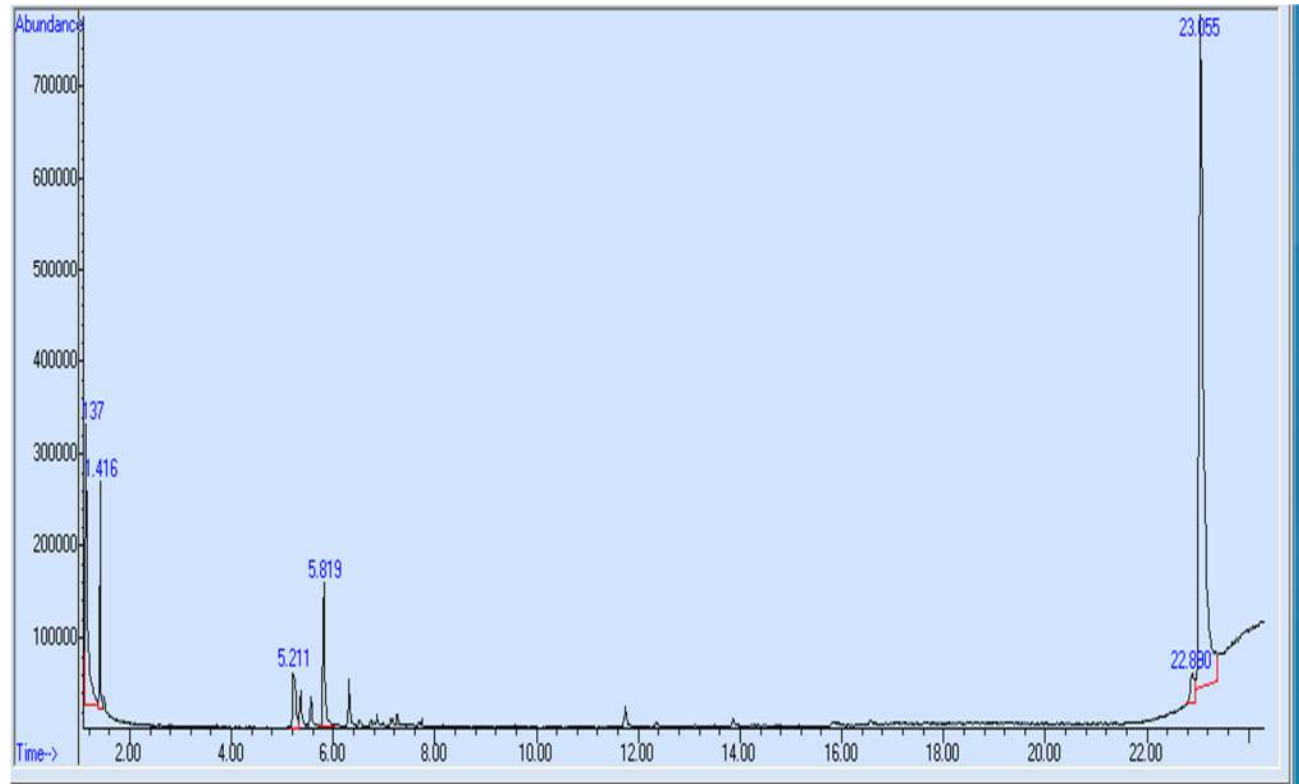
Appendix M: GC-MS ion chromatogram for tubers sprayed at 14 days interval



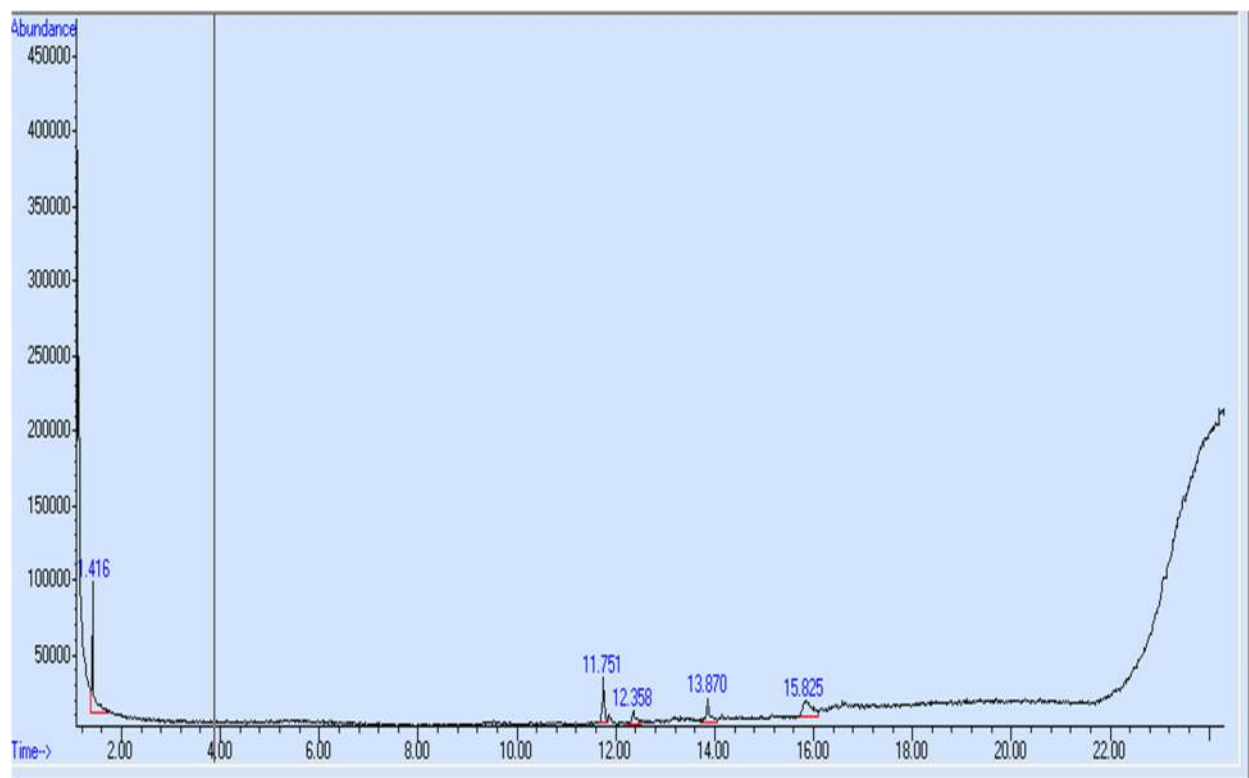
Appendix N: GC-MS ion chromatogram for tuber samples sprayed at 21 days interval



Appendix O: GC-MS ion chromatogram for Limuru market samples



Appendix P: GC-MS ion chromatogram for Wakulima market tuber samples



Appendix Q: List of publication

Vol. 14(5), pp. 148-157, May, 2020
DOI: 10.5897/AJMR2019.9195
Article Number: E2EA6E663641
ISSN: 1996-0808
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African Journal of Microbiology Research

Full Length Research Paper

In vitro* efficacy of *Trichoderma asperellum* and detached leaflet assay on late blight pathogen: *Phytophthora infestans

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Received 20 August, 2019; Accepted 24 October, 2019.

Late blight is highly variable adapting to new fungicides and overcoming host resistance. The objective of the study was to determine efficacy of *Trichoderma asperellum* against *Phytophthora infestans* and its compatibility with fungicides. *T. asperellum* at 33% (3×10^8), 66% (7×10^8) and 100% (1×10^7 CFU/mL; g/L), Ridomil® (Metalaxyl 4% + Mancozeb 64%) and Mistress 72® (Cynamoxil 4% + Mancozeb 64%) were plated alongside *P. infestans* *in vitro* and detached leaflets assay. Results indicated that Ridomil® and Mistress 72® completely inhibited mycelial growth of *P. infestans* *in vitro* and in detached leaves. The 33% *T. asperellum* concentration had the lowest inhibitory power (38.0%) while 66% (91.10%) and 100% (91.30%) *T. asperellum* concentrations were not significantly different ($P=0.05$). Lesion sizes were not significantly different in 66% *T. asperellum* (1.91 cm²) and 100% (1.89 cm²) concentration while 33% concentration (3.5 cm²) and untreated (3.55 cm²) did not differ significantly. Ridomil® and Mistress 72® had no significant effect on *T. asperellum* mycelial growth. The results suggest that *T. asperellum* at 66% was effective in managing late blight. Results further indicate that *T. asperellum* could be used in combination with fungicides for effective and economical option.

Key words: *Trichoderma asperellum*, *Phytophthora infestans*, detached leaf assay, *in vitro*.

Efficacy of *Trichoderma asperellum* Seed Treatment and Ridomil® Application in Managing Late Blight on Potato

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Received January 08, 2021; Revised February 09, 2021; Accepted February 23, 2021

Abstract Potato seed tubers latently infected with *Phytophthora infestans* initiate late blight that requires early fungicide application raising economic and human concerns. The objective of the study was to determine the efficacy of *Trichoderma asperellum* seed treatment and Ridomil® (Metalaxyl 4% and Mancozeb 64%) application to manage late blight. Ridomil® was applied at 21-, 14- and 7-day intervals on seed tuber and apical cuttings pre-treated with *T. asperellum* at 33 % (3×10^6), 66% (7×10^6) and 100% (1×10^7 CFU/mL) concentration by either dipping or injection. Results revealed that 7- and 14-day spray intervals were not significantly different ($P=0.05$) in terms of yield and late blight severity. Rooted apical cuttings had 7.4% higher disease severity resulting in 2.3% lower yield than crop from seed tubers. *T. asperellum* at 66% and 100% concentrations reduced disease severity by 26% and 27% respectively. Pericardial injection had 8.3% higher yield and conversely 7.8% higher disease severity than dipping. The combination of *T. asperellum* at 66% concentration with a 14-day spray interval provided better late blight management. The results suggest that seed treatment by dipping using 66% *T. asperellum* suspension could increase fungicide application interval by 7 days while improving on yield.

Keywords: *Trichoderma asperellum*, apical cuttings, Ridomil® (Metalaxyl 4% and Mancozeb 64%), *Phytophthora infestans*, dipping, injection

Cite This Article: Kilonzi Jackson Mutuku, Mafurah Juma Joseph, Nyongesa Moses Wabomba, and Kibe Antony Mwangi, "Efficacy of *Trichoderma asperellum* Seed Treatment and Ridomil® Application in Managing Late Blight on Potato." *World Journal of Agricultural Research*, vol. 9, no. 2 (2021): 42-52. doi: 10.12691/wjar-9-2-1.

Cost Benefit Analyses in Managing Late Blight Through *Trichoderma asperellum* Seed Treatment and Ridomil[®] Application on Potato

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Received: October 18, 2019

Accepted: January 10, 2020

Online Published: June 15, 2020

doi:10.5539/jas.v12n7p32

URL: <https://doi.org/10.5539/jas.v12n7p32>

Abstract

Fungicides overuse in management of late blight has led to increased cost of production and biodiversity issues. To better understand costs and benefits associated with seed treatment to reduce fungicide application, apical cutting and tuber seed crop were pre-treated by either peridermal injection or dipping using *T. asperellum* suspension at 33%, 66% and 100% concentration and then sprayed separately with Ridomil[®] (Metalaxyl 4% + Mancozeb 64%) at 21, 14 and 7 (Manufacturer's Recommended Regime) days interval. Results showed that apical cuttings had 7.5% higher disease severity and 0.2% lower yield than crop from seed tuber. Untreated and 33% *T. asperellum* were not significant different ($p = 0.05$) in disease and yield scores. *T. asperellum* concentration at 66% and 100% reduced disease severity by 26% and 27% resulting to 30% and 29% yield increment respectively. Spray interval of 14 days did not differ significantly from 7 day spray interval in terms of disease score and yield but the regime MRR% was double 7 day spray interval. Maximum yield loss was observed in unprotected plots followed by treatments sprayed at 21 days interval. Combination of 66% and 100% *T. asperellum* concentration with 14 days interval resulted to higher yield and marginal rate of return compared to any other combination. Seed dipping was four times profitable than injection. The findings suggest that seed treatment at 66% and 100% *T. asperellum* concentration by dipping in combination with Ridomil[®] application at 14 days interval significantly reduced late blight epidemics and improved on yield and consequently increased net farm income.

Keywords: seed treatment, apical cuttings, cost and benefit, *Trichoderma asperellum*, Ridomil[®] (Metalaxyl 4% + Mancozeb 64%)

Appendix R: Conference and seminar papers

1. Kilonzi, M.J., Mafurah, J.J. and Nyongesa, W.M. (2019). Effect of reduced Ridomil® application frequency on late blight in potato (Oral presentation); Soil Science Society of East Africa Conference, 19 – 22nd November, 2019
2. Kilonzi, M.J., Mafurah, J.J. and Nyongesa, W.M. (2019). Efficacy of *Trichoderma asperellum* seed treatment and Ridomil® application and their cost benefits on potato late blight. (Oral presentation). CESAAM summer school, 17 – 20th November, 2019
3. Kilonzi, J, Nyongesa, M., Mafurah, J., Oyoo, J. (2020). Effect of ridomil® application regime in late blight (*Phytophthora infestans*) management on yield and pesticide residue in potato tubers. THEMATIC AREA; Crop Protection and Plant Breeding. Oral presentation. HAK conference at Pwani university, 1st to 4th December 2020
4. Kilonzi, J.M, Mafurah, J.J, and Nyongesa, M.W. (2020). Efficacy of *Trichoderma asperellum* seed treatment and ridomil® application interval in managing late blight (*Phytophthora infestans*) on potato (*solanum tuberosum*). International Biennel conference at Egerton University, 24th to 28th Nov 2020

Appendix S: Research permit

THIS IS TO CERTIFY THAT: **MR. KILONZI MUTUKU JACKSON** of **EGERTON UNIVERSITY, 0-90100** **MASII**, has been permitted to conduct **research in Kiambu County** on the topic: **EFFICACY OF TRICHODERMA ASPERELLUM SEED TREATMENT AND RIDOMIL® APPLICATION TO MANAGE LATE BLIGHT (PHYTOPHTHORA INFESTANS) ON APICAL CUTTING AND TUBER SEED OF POTATO (SOLANUM TUBEROSUM)** for the period ending: **3rd May, 2020**

Permit No : NACOSTI/P/19/20573/30123
Date Of Issue : 6th May, 2019
Fee Received :Ksh 1000

Applicant's Signature  **Director General for Science, Technology & Innovation** 

