MORPHOLOGICAL IDENTIFICATION AND *IN VITRO* EFFICACY OF FUNGICIDES IN CONTROL OF *PHYTOPHTHORA* CAUSING PINEAPPLE HEART ROT DISEASE IN UGANDA

BY

OCWA AKASAIRI
(BVoc. Agric/ Educ., KYU)
REG No. 14/U/14248/GMCS/PE

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DECLARATION

I, Ocwa Akasairi do hereby declare that this thesis is my original work and has never been submitted to any institution of higher learning for an academic award.

Signed.....

Date .....5/12/2016.....

APPROVAL

This is to certify that this work was carried out under our supervision as University supervisors.

Signed .....

Associate Professor Bosco Bua

Date .....01.12.18.....

Signed.....

Dr. Geoffrey Tusiime

Date .....

DEDICATION

I dedicate this work to Associate Professor Bosco Bua for the immeasurable mentorship and support right from my undergraduate studies to-date. I know without you, my graduate studies would have remained more of a dream than a reality! Additionally, to my late grandfather, you loved education. I wish you had stayed to witness the fruits of your investment.

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LIST OF ACRONYMS AND ABBREVIATIONS

ACMA	Amended Cornmeal Agar
EC_{50}	Effective concentration
CIAT	International Center for Tropical Agriculture
CMA	Cornmeal Agar
KYU	Kyambogo University
PDA	Potato Dextrose agar
PHRD	Pineapple Heart Rot Disease
NaCRRI	National Crops Resources Research Institute
NARL	National Agriculture Research Laboratories
UNDP	Uganda National Development Plan 2015

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ABSTRACT

Pineapple (*Ananas comosus* L. Merr) production in Uganda is being constrained by a diversity of factors including pineapple heart rot disease (PHRD). Information on PHRD causal pathogen is limited. Also, management options are clear. The objectives of this study were, therefore, to identify the casual pathogens causing PHRD and assess the *in vitro* reaction of the causal pathogens to fungicides. Pathogen isolation was done using amended corn meal agar. Macro and micro-morphological characteristics of the isolates were assessed. Pathogenicity of the isolates was tested using healthy pineapple plants based on Koch's postulate. *In vitro* reaction was determined using 10% V8 media amended with 0.1g/L Metalaxyl, 0.1 g/L Victory 72 powder, 0.01 g/L and 0.001 g/L Metalaxyl, 0.01 g/L and 0.001 g/L Fosetyl Al and un amended V8 media as a control. There was a significant difference ($P<0.05$) in the growth rates, hyphae diameter and asexual structure dimensions of the isolates. Isolates were characterized by a dense rosette and stoloniferous mycelial growth pattern in PDA media. Sporangia were non-caduceus, terminal, papillate and mostly ovoid, obpyriform and limoniform sporangia (32-56μm) with a mean length/breadth ratio of 1.32:1μm. Chlamydospores were spherical and thick-walled (25-42μm diameter). Sporangiophore branching was sympodial. All the isolates were sensitive to Metalaxyl and Victory 72 powder treatment. Based on the morphological characteristics of isolates, it was concluded that *Phytophthora nicotianae* is the species associated with PHRD in central Uganda. Additionally, Metalaxyl was the most effective chemical against the pathogen *in vitro*. Molecular characterization of the pathogen is recommended to confirm the identity of the species. It is also recommended that the efficacy of fungicides which have shown effectiveness be assessed further under field conditions.

CHAPTER ONE

INTRODUCTION

1.1 Production trend and importance of pineapple

Pineapple (*Ananas comosus* L. Merr) belongs to the *Bromeliaceae* family and is believed to have originated from South America (Clement *et al.*, 2010; Joy and Sindhu, 2012). The crop is ranked as the third important tropical fruit after banana and citrus (Hassan, *et al.*, 2011). Accordingly, pineapple accounts for over 20% of the total volume of global tropical fruit exports (Coveca, 2002; MUZARDI 2010; Olayinka, 2013). Globally, pineapple production was estimated at more than 19 million metric tonnes (MT) in 2011 (FAO, 2013), with about 50% production coming from Brazil, Thailand, Philippines and China. Prior to 2011, Philippines was the largest pineapple producer in the world. However, from 2013 to date, Thailand has become the largest global producer of pineapple (UNATAD, 2016) (Table 1). Currently, in East Africa, by 2008, Kenya (429065MT) was the leading producer country in the region followed by Tanzania (78000), Rwanda (18208) and lastly Uganda (1600) (MUZARDI, 2010).

Though being the last producer in East Africa, pineapple by far is the most developed and widely grown commodity in the fruit crop range and value chain in Uganda (UIA, 2001). The involvement of Non Government organizations (NGO's) like VEDCO strengthens the pineapple value chain by providing services in farmer extension and training services, market information and linking farmers to buyers (MUZARDI, 2010).

Table 1: Major producers of pineapple in the world

Rank	Country	Production (Metric tonnes)
1	Thailand	2 650 000
2	Costa Rica	2 484 729
3	Brazil	2 478 178
4	Philippines	2 397 628
5	Indonesia	1 780 889
6	India	1 456 000
7	Nigeria	1 420 000
8	China	1 000 000
9	Mexico	759 976
10	Colombia	551 133

Source: UNCTAD, 2016

Pineapple is a very important crop due to its numerous attributes. Pineapple is popularly known as a queen of fruits because of its excellent taste and flavor (Olanyinka, 2013). The fruit is eaten as fresh fruit, or can be canned and processed to produce juice concentrate. Pineapple possesses exceptional juiciness, vibrant tropical flavor and has immense health benefits. It is a rich source of calcium, potassium, dietary fiber and vitamin C. Pineapple also provides a good source of vitamin B1, B6 and copper which are cerebral toners that help to combat loss of memory, sadness and melancholy (Joy, 2010). Pineapple is a natural anti-inflammatory fruit rich in sulfur proteolytic enzyme bromelain used for tenderizing meat (Gap *et al.*, 2010; Sunantha and Saroat, 2011; Kujawska *et al.*, 2013). Among its other health attributes, pineapple has been used to treat rheumatoid arthritis as well as speed up tissue repair resulting from injuries, diabetic ulcers and general surgery (Joy 2010). Pineapple crowns and fine pineapple waste from factories can be fed to cattle, pigs and horses. Potted ethylated pineapple plants with fruits have been used as ornamentals for decoration. Besides, its health benefits, pineapple is financially relevant to tropical and subtropical areas of the world (Rodriguez *et al.*, 2015); with recent surveys in Uganda indicating that pineapple contributes to income of many farmers (Bua *et al.*, 2013).

1.2 Constraints to pineapple production

Despite, its importance, the global and local pineapple production is constrained by a number of abiotic, biotic and social economic factors (Bua *et al.,* 2013; Akhilome *et al.*, 2015; UNDP, 2015). Limited capital, poor transport, access to information, poor quality planting materials and low literacy levels are the key social economic factors affecting production globally (FAO, 2013; Olanyinka, 2013). The high perishability of pineapple also limits its Shelf life (Amao *et al.,* 2011), forcing farmers to sell produce at giveaway prices during peak seasons. This drastically reduces potential profits from pineapple enterprises. For example during a survey carried out in pineapple growing districts of central Uganda in 2015, farmers reported selling average size pineapples for as low as UGX 500 (about $0.15) (Bua, unpublished).

Abiotic factors such as drought severely reduce the yield of pineapple while excessive moisture provide a conducive environment for diseases to flourish (Bartholomew *et al.*, 2003; Aghighi *et al.,* 2015). Key biotic factors affecting pineapple production include weeds, pest and disease (Bua *et al.*, 2013).

In the Lake Victoria basin crescent in Uganda, pineapple heart rot disease among the major diseases causing economic losses to farmers (Bua, unpublished). The economic losses have been reported in other countries in the like China, Mexico and India (PHRD) (Shen *et al.*, 2013; Rodriguez *et al.*, 2015; Shreenivasa *et al.*, 2015).

1.3 Pineapple production areas and varieties in Uganda

Pineapple production in Uganda has no clearly documented history (MUZARDI, 2010). Pineapple is majorly grown in the Lake Victoria basin crescent (covering districts of Kayunga, Luwero, Mukono and Masaka), south of Lake Kyoga, western Uganda (covering districts of Ntugamo and Kabale) (FIT, 2007; MUZARDI, 2010; Bua *et al.*, 2013).

Varieties of pineapples grown currently in Uganda include: the small sized spiked (Sasirimu) variety and the large smooth Cayenne (smooth, spineless leaves) (MUZARDI, 2010). According to Bua *et al.* (2013), 70% of farmers in Mukono and Kayunga grow smooth cayenne. Preference for smooth cayenne is that it allows ratooning, is larger and juicier while Sasirimu gives one fruit in its life time and the farmer has to cut back or replant after each harvesting cycle (MUZARDI, 2010). A survey conducted in 2015 central Uganda revealed other varieties like Victoria and Red Spanish (*Kafaransa*) being grown (Bua, unpublished).

1.4 Statement of the problem

Pineapple heart rot disease is reported to be one of the most devastating diseases of pineapple in many parts of the world. For example, in China the incidence ranged from 25% - 30% (Shen *et al.*, 2013). Similarly, in India the incidence was upto 46% (Shreenivasa *et al.*, 2015). This implied serious economic losses (Rodriguez *et al.*, 2015; Shreenivasa *et al.*, 2015). The outbreak of PHRD in the Lake Victoria basin crescent threatens pineapple industry in Uganda (NARO, 2012). Currently, limited studies have been carried out in Uganda to determine the causal pathogen of the heart rot disease. Elsewhere, however, pineapple heart rot disease is reported to be caused by *Phytophthora cinnamomi* and *Phytophthora nicotianae* (Joy and Sindhu, 2012; Shen *et al.*, 2013; Rodriguez *et al.*, 2015). Whether these two *Phytophthora* species are also

involved in the Uganda PHRD epidemic is not known (NARO, 2012). Yet these pathogens are widely and spatially distributed (Hu, 2007; Joy and Sindhu, 2012) with associated crop destruction of upto 100% yield loss (Rohrbach and Schenk, 1985). Currently, farmers practice roguing to control PHRD but it's not effective (Bua, unpublished). This there're calls for inclusion fungicides as part of integrated disease management like In India, integrated pineapple heart rot disease management involving cultural practice and the use of 0.2% metalaxyl and 0.1% Fosetyl reduced the effect of PHRD on pineapple (Shreenivasa *et al.*, 2015). Consequently, as the first step in developing a disease management strategy, it was imperative to identify clearly the causal organisms associated with PHRD as well as make an *in vitro* assessment of the reaction of the PHRD causal pathogen to fungicides in Uganda.

1.5 Justification of the study

The outbreak of PHRD in the Lake Victoria basin crescent has threatened pineapple production and consequently the quantity exported (NARO, 2012; Bua unpublished). According to Rohrbach and Schenk (1985), under severe cases pineapple heart rot disease can cause 100% yield loss. Unless managed, the disease can result into considerable economic losses to communities that rely on pineapple as source of livelihood (Shreenivasa *et al.*, 2015). For example PHRD caused serious economic losses in Australia, Hawaii, Philippines, South Africa and Thailand (Coppens *et al.*, 1997; Rodriguez *et al.*, 2015). Pineapple heart rot disease has a potential of causing loss of susceptible pineapple varieties. Additionally, the offensive odour produced by rotting pineapple tissues pollutes environment. Accordingly, if left unabated, this would not only undermine food security but also interfere with general livelihoods of the local communities (Shen *et al.*, 2013).

1.6 General objective of the study

The general objective of this study was to establish the identity and reaction to fungicides of causal pathogens of pineapple heart rot so as to design a sustainable management package for the disease in Uganda.

1.7 Specific objectives of the study

The specific objectives were to:

i. Identify the causal organism(s) of PHRD in central Uganda.

ii. Determine the *in vitro* reaction of PHRD causal pathogen(s) to fungicides commonly used for the management of fungal diseases in central Uganda.

1.8 Hypotheses

i. Pineapple heart rot disease in Uganda in caused by *Phytophthora nicotianae*.

ii. There is no variation in reaction of pineapple heart rot disease causal pathogens population to fungicides Uganda.

1.9 Significance of the study

i. The results of this study when disseminated improve farmers' knowledge on PHRD recognition and causal pathogen.

ii. The findings of this research provide justification for the policy makers to allocate resources for management PHRD in Uganda.

iii. This information is helpful to the extension agents when guiding farmers on management of PHRD.

iv. This study provided a baseline on the basis of which future researchers can build on.

CHAPTER TWO

LITERATURE REVIEW

2.1 Symptoms of pineapple heart rot disease

Pineapple heart rot disease manifests as a syndrome where young leaves of infected plants fail to elongate and turn chlorotic; heart leaves wilt and turn brown; terminal whorl /heart leaves of infected plants can be pulled from the mother plant very easily; the base tissue of these leaves are water soaked and have a foul smell. Leaves may also turn red and yellow with necrotic leaf margins and tips; and the developing fruits change colour. As the disease progresses the plant collapses and dies (Joy and Sindhu, 2012; NARO, 2012; Shen *et al.*, 2013; Rodriguez *et al.*, 2015).

2.1 Etiology and epidemiology of pineapple heart rot disease

Pineapple heart rot disease is caused by a wide range of *Phytophthora* species (Drenth and Sendall, 2004) which belong to the class Oomycetes (Drenth and Sendall, 2001) in the Kingdom *Chromista* (Drenth and Sendall, 2001; Agrios, 2005; Kirk *et al.*, 2008). According to Sadeghy (2014), the genus *Phytophthora* contains very destructive plant pathogens that attack hundreds of plant species throughout the world. However, the most common species of *Phytophthora* consistently isolated from infected pineapple plants globally are *P. cinnamomi* and *P. nicotianae* (Rodriguez *et al.*, 2002; Drenth and Sendall, 2004; Zeng *et al.*, 2009; Joy and Sindhu, 2012; Shen *et al.*, 2013; Rodriguez *et al.*, 2015). *Phytophthora nicotianae* is reported to be a very destructive pathogen on numerous crops including coconut (*Cocoa nucifera*), macadamia (*Macadamia intergrifolia*), pineapple (*Ananas comosus*), taro (*Colocasia esculentum*), tobacco

(*Nicotianae tabacum*) among others (Drenth and Sendall, 2001) while *P. cinnamomi* is one of the species responsible for the majority of *Phytophthora* diseases in trees and shrubs (Hu, 2007; Schreier, 2013).

The pathogens' primary mode of reproduction is asexual. Fruiting bodies called sporangia develop from mycelium and produce zoospores (Gallup *et al.*, 2006). Less commonly, the mycelia of two different mating types can produce sexual structures called oogonia (female) and antheridia (male). When these structures combine, they produce sexual spores called oospores (Green and Scot, 2015). The pathogens spread through infected suckers, soil and water (Joy and Sindhu, 2012). During inoculation and penetration, zoospores make contact with the leaf or root surface, germinate and then enter the plant tissues. Later, during infection and pathogen development a fungus-like mycelium begins to grow in the basal white tissues of the crown within hours of penetration. As the mycelium develops, it spreads throughout the crown (Green and Scot, 2015).

After establishment, disease incidences and severities become high during wet seasons (Purwantara *et al.*, 2004), within a temperature range of 20-30°C and high relative humidity (Mounde *et al.*, 2012). For that reason the inoculum level of *Phytophthora* is usually very low during dry season but high during rainy season (Weste and Vithanage, 1979). This situation is worsened by presence of discarded infected plant materials that raises the inoculum levels (Dakwa, 1974). Saturated soils also exacerbate disease progress (Jung *et al.*, 2013). Recycling irrigation water can further exacerbate a *Phytophthora* disease problem; if inoculum gets into retention or irrigation ponds, use of infested water for irrigation can lead to infection of many

plants throughout the landscape (Schreier, 2013). Weather conditions therefore influence disease occurrence. Dakwa (1974) reported that the fungus may occur in the soil but not be readily available as primary inoculum if weather conditions are not favourable for disease development. During favorable conditions the chlamydospores germinate and hyphae infect young leaf or stem tissues. Because of the aggressiveness of the pathogen, the tissues that are infected produce disease symptoms (Drenth and Sendall, 2004).

2.2 Isolation of *Phytophthora* species associated with pineapple heart rot disease

Phytophthora grow slowly *in vitro* compared with saprophytic fungi and bacteria. Special techniques are required for their isolation (Drenth and Sendall, 2001; Jeffers, 2006). This is because they are liable to suppression by direct competition, antagonism and parasitism (Drenth and Sendall, 2001). The use of selective media usually overcomes these problems for example, *P. cinnamomi* shows medium-fast to fast growth on cornmeal agar (CMA) (Bernadovicova and Juhasova, 2005). However, antibiotics have to be added to the isolation media in order to suppress the growth of bacteria. Additionally, because *Phytophthora* species are out competed by many fungi, media which is low in terms of nutritional status is required for culturing. More importantly, plates of selective media used for isolations should not contain any free water or condensation on the lids as water encourages the growth and spread of bacterial contaminants. The commonly used media for *Phytophthora* isolation include CMA or V8 media amended with pimaricin, ampicillin, rifampicin, benomyl, pentachloronitrobenzene (PCNB) and hymexazol (Drenth and Sendall, 2001; Jeffers, 2006). V8 media facilitates easy detection by promoting sporulation of most *Phytophthora* species.

2.3 Morphological characterization of *Phytophthora* species associated with pineapple heart rot disease

Historically, *Phytophthora* species are delimited by their morphology (Appiah *et al.*, 2003). According to Schreier (2013), the genus *Phytophthora* has several attributes that are useful for phenotypic description hence the identification of more than fifty *Phytophthora* species based on morphological characteristics (Stamps *et al.*, 1990). Earlier, Waterhouse (1963) used mycelial growth patterns and sporangial characteristics like shape and sporangiophore branching to identify different *Phytophthora* species. Bernadovicova (2003), Bernadovicova and Juhasova (2005) also reported that mycelia growth, sporangia shape, size and shape of reproductive structures can be used in identification and specification of the biology of *Phytophthora* soil borne pathogens under specific conditions (Table 2). For example, the colony diameters from different *Phytophthora* species are reported to vary depending on the incubation temperatures (Mounde *et al.*, 2012). In fact, growth temperature serves as a distinguishing feature between *Phytophthora* species such as *P. citrophthora* (24-28°C), *P. syringae* (20°C) and *P. nicotianae* (> 30°C) (Erwin and Ribeiro, 1996; Mounde *et al.*, 2012). According to Mounde *et al.* (2012), colony characteristics and growth rates are also very useful in the identification of *Phytophthora* species by complementing sporangial characteristics in species differentiation.

Table 2: Selected morphological features of *Phytophthora* species causing pineapple heart rot disease

Morphological feature	*P. nicotianae*	*P. cinnamomi*
Sporangia papillation	Papillate	Non papillate
Sporangium shape	Varies from ellipsoid, ovoid, pyriform, obpyriform to sphe cal, with pron nent papilla	Includes ovoid, obpyriform, ellipsoid tapered at the base
Sporangium size	46.2x34.9 µm	60 x 36.0 µm
Sporangiophore branching	Sporangia irregularly or sympodially branched	Usually unbranched
Mycelium morphology	Hyphal swellings have been noted	coralloid, with abundant hyphal swellings and vesicles
Mycelium growth pattern	Dense rosette	Medium-dense, wooly mycelium, uniformly growing

Source: Drenth and Sendall, (2001).

Indeed, sporulation of *Phytophthora* cultures provides important clues for species identification (Drenth and Sendall, 2001). Additionally, variability and development of sporangium morphology provides a more complete understanding of the morphological plasticity of *Phytophthora* species that reveals previously uncharacterized morphotypes (Schreier, 2013). However, the most important characters to observe are sporangia morphology (shape, size), papillation of the sporangium (non- papillate, semi papillate, and papillate), sporangiophore morphology or branching (simple sympodium, compound sympodium, and umbellate sympodium) (Drenth and Sendall, 2001). Although, some species of *Phytophthora* produce

sporangia readily on the surface of the agar media, many species need to be cultured in dilute non-sterile soil extract under light conditions before they produce sporangia (Ferguson and Jeffers, 1999; Drenth and Sendall, 2001, .2004; Jeffers, 2006; Duncan *et al.*, 2008; Schreier, 2013). This is because inducing sporangium formation in the laboratory by *Phytophthora* is challenging (Chen and Zentmyer, 1970). Accordingly, mycelium growth habit and colony morphology are useful tools for describing species and subgroups within species (Aragaki *et al.*, 2001; Erwin and Ribe o, 1996). In fact, detailed recognition of features of mycelia, colony morphology and characteristics of sexual and asexual structures is very important for correct understanding of the whole infectious process and disease development (Bernadovicova and Juhasova, 2005). According to Erwin and Ribeiro (1996), *Phytophthora* exhibits varying cultural characteristics in V8 media, CMA and PDA media which aids in morphological analysis and identification.

Phytophthora cinnamomi on V8 media forms medium-dense, wooly hyphae that grow uniformly from the starting point. The hyphae grow in an aerial fashion above the surface of the agar filling the space between the medium and the petri dish lid (Hardham, 2005). Colony morphology of *P. cinnamomi* has also been evaluated using PDA media (Zentmyer, 1980). Nevertheless, mycelium growth habit is, at times, a challenge to characterize. Unlike colony diameter, it is largely subjective and exists on a gradient (Mbaka *et al.*, 2010). Distinguishing between the aerial and appressed growth habits depends on visual assessment of mycelium density in the agar and amount of aerial hyphae above the agar surface both of which vary on a continuum (Schreier, 2013). Additionally, *Phytophthora cinammomi* always exhibits wide variation in sporangia shapes and sizes both within and among its population with general shape of sporangia pre-

dominantly ovoid to ellipsoid with optimum growth temperature of 24°C (Mbaka *et al.*, 2010) and non papillate sporangia (Schreier, 2013). The colony morphology is always described as rosaceous, petaloid as it is always influenced by temperature. Because colony morphology is a non-stable character on certain media, this may necessitate adjustments of the species description (Mbaka *et al.*, 2010).

On the other hand, *Phytophthora nicotianae* forms a dense rosette stoloniferous colony (Palmucci *et al.*, 2013), produces non-caduceus, terminal, papillate and mostly obpyriform sporangia averaging 46.2 x 34.9 µm with the mean length/breadth ratio of 1:34 (Tao, *et al.*, 2011). *P. nicotianae* shows a distinctive dense rosette spreading aerial mycelium with coenocytic hyphae of up to 7-10 nm in diameter in V8 media (Mounde *et al.*, 2012). The sporangia are ovoid and terminal with prominent papilla that varies in size (18-61 µm x 14-39 µm) as well as produces intercalary sporangium. The colony diameter of *P. nicotianae* is 37.5mm (Mounde *et al.*, 2012). The diploid mycelium of *P. nicotianae* is hyaline, coenocytic and in culture it forms distinctive rosette pattern in the presence of V8 vegetable juice. However, the formation of sporangium in distilled water appears within 48 hours under perpetual white light (Erwin and Ribeiro, 1996; Hu, 2007).

According to Bernadovicova and Juhasova (2005), *P. cinnamomi* always shows a faster growth rate compared to other *Phytophthora* species. Growth rates ranges from 13.5-20.0 mm^{-1} (18.1±0.26) on V8 media and 5.0-14.0 mm^{-1} (10.5±0.2) was reported by Hurbuli (1995) on PDA media in seven days.

However, the limitation with the morphological method of identifying *Phytophthora* species is that morphological characteristics of the sporangia, mycelia and colonies used in the differentiation of fungal species are plastic, influenced by environment and often overlap between species (Mbaka *et al.*, 2010; Mounde, *et al.*, 2012).

2.4 Management of *Phytophthora species* associated with pineapple heart rot disease

Management practices available to manage *Phytophthora* causing pineapple heart rot disease include cultural practices, host resistance, chemical control and integrated disease management (IDM) (Drenth and Sendall, 2004). Cultural practices such as crop rotation, roguing, manuring and others are cheap and environmentally friendly (Drenth and Sendall, 2004). Also, the use of resistant pineapple cultivars presents an alternative to PHRD management. However, the challenge is that the survival structures of *Phytophthora* associated with PHRD stay for long in the soil hence may not easily be managed by crop rotation (Erwin and Ribeiro, 1996; Joy and Sindhu, 2012). Additionally, a report by Rodriguez *et al.* (2002) in Cuba indicated that there was no pineapple variety resistant to PHRD.

Fungicides with different modes of action for managing PHRD has been reported to be successful (Hu, 2007; 2008; Nina, 2014). For example in India, pineapple heart rot disease management involving the use of 0.2% Metalaxyl and 0.1% Fosetyl Al reduced the yield effect of PHRD on pineapple (Shreenivasa *et al.*, 2015). Similarly, in Hawaii, Metalaxyl and fosetyl Al when applied as preplant crown dips controlled pineapple heart rot caused by *Phytophthora parasitic*a and *Phytophthora cinnamomi* by reducing the mortality of pineapple plants. However, Fontema *et al.* (2005) reported that of overuse of chemicals like metalaxyl formulations for management of *Phytophthora* can result into chemical resistance and environment pollution.

2.5 Reaction of *Phytophthora* species associated with pineapple heart rot disease to fungicides

Management options for controlling and regulating common oomycete pathogens or diseases involves the use of growth-inhibiting fungicides (Nina, 2014). According to Hu (2007), the use of fungicides to control for *Phytophthora* diseases accounts for over 25% of the total annual global fungicide expenditure. Major fungicides with different modes of action include: mefenoxam/metalaxyl (Subdue MAXX); propamocarb hydrochloride (Banol; Previcure Flex); fosetyl-Al (Aliette); dimethomorph (Stauré DM) and etridiazole (Truban; Terrazole) (Hu, 2007; 2008; Nina, 2014). These fungicides are active against oomycetes (Erwin and Ribeiro, 1996; Hu, 2007). Fungicides can be used singly or in combinations for better results. Available information indicates that *Phytophthora* species have demonstrated a greater degree of sensitivity and reaction to some antifungals like metalaxyl, Fosetyl-Al and Phosphorous acid (Fenn and Coffey, 1983; Ganoo and Saumtally, 2001; Mukalazi *et al.*, 2001). The level of sensitivity depends on the concentration and the type of antifungal used. Rebollar-Alviter *et al.* (2010) reported that the combination of strong curative and protectant activity fungicides provides a sustainable curative disease program. Fosetyl-Al has a low activity against mycelial growth *in vitro* in low concentration (Fenn and Coffey, 1983; Boughalleb *et al.*, 2006). Metalaxyl based fungicides are routinely used world over for *Phytophthora* management because they show effective protective, curative and eradicative antifungal activity with most *Phytophthora* species (Gisi and Cohen, 1996; Narayana *et al.*, 2010). The use of metalaxyl formulations should, however, be carefully planned as high frequency of metalaxyl use may result into a possible emergence of metalaxyl-resistant biotypes (Fontema *et al.*, 2005). Reaction is normally scored as sensitive, intermediate and resistant (Ganoo and Saumtally, 2001; Mukalazi *et al.*, 2001; Kimberly *et al.*, 2010). In

China, 179 isolates of *P. parasitica* screened for sensitivity to metalaxyll yielded 41.9% which were of intermediate resistance and 58.1% was sensitive and none resistant. Additionally, in Virginia, 26 out of 96 isolates of *P. nicotianae* were highly resistant to mefenoxam with a mean EC_{50} value of 326.5 µg/ml while the remaining 70 were sensitive with an EC_{50} of <0.01 µg/ml. The resistant isolates exhibited higher infection rates and greater sporulation ability than sensitive ones. The intense selection pressure determines these sensitivity variations to mefenoxam fungicide (Wang *et al.*, 2013).

According to Ganoo and Saumtally (2001), these three sensitivity categories have a significant impact on disease management. In fact, resistance to chemical compounds may result into failure in disease control (Kimberly *et al.*, 2010). Therefore, *in vitro* reaction provides information about the potential for resistance to a chemical to develop in a pathogen and facilitates better examination of the mechanism of action. According to Elliott *et al.* (2015), resistance to chemical fungicides depends on a number of factors including mode of action of the chemical, biology of the target organism and patterns of usage in the nursery or field. For example, *P. cinnamomi* resistance development was as a result of prolonged chemical use (Dobrowolski *et al.*, 2008). Additionally, metalaxyl sensitivity of isolates based on sampling sites in two years 2001 and 2002 in Cameroon showed that metalaxyl sensitivity was significantly influenced by geographic origin of the isolates (Fontema *et al.*, 2005). In Tunisia, reports indicated that ridomil was less effective inhabiting mycelial growth *in vitro* at low concentration and at 100 - 1000µg/L ridomil/metalaxyl completely inhibited the development of *Phytophthora* while isolates in fosetyl grew by 6.5mm after 24hrs of incubation (Boughalleb *et al.*, 2006). Similarly, Wagner *et*

al. (2007), in California reported that *in vitro* inhibition of *Phytophthora* mycelial growth by metalaxyl can be achieved with concentration range of 1 - <1000 µg/L.

2.6 Pathogenicity of *Phytophthora* species associated with pineapple heart rot disease

Variability of pathogenicity has been widely investigated for many crop pathogens because selection for host resistance is dependent on the pathogenicity of the fungus (Huburli, 1995). Zentmyer (1980) outlined a number of studies which indicate there is variability in pathogenicity among isolates of *Phytophthora*. For example, in Australia and California, there were few pathogenic phenotypes (Huburli, 1995). However, Rodriguez *et al.* (2010) indicated that the majority of *P. nicotianae* isolates were pathogenic while others were not. In fact, the *P. nicotianae* caused disease incidence of 25% - 30% (Shen *et al.*, 2013; Bua, unpublished). In Kenya, *Phytophthora nicotianae* isolates produced progressive lesions of varying sizes (Mounde *et al.*, 2012).

2.7 Literature review summary

Pineapple heart rot disease is caused by a wide range of *Phytophthora* species (Drenth and Sendall, 2004). The common species of *Phytophthora* consistently isolated from infected pineapple plants worldwide are *P. cinnamomi and P. nicotianae* (Joy and Sindhu, 2012; Shen *et al.*, 2013; Rodriguez *et al.*, 2015). Morphological characteristics of these pathogens in media such as mycelial growth patterns, sporangia characteristics, colony morphology and others aid their identification (Stamps *et al.*, 1990). This information is critical and fundamental to epidemiological studies and the development of control strategies (Mbaka *et al.*, 2010; Schreier, 2013; Akrofi, 2015). Besides, cultural practices have been reported to ineffective in managing *Phytophthora*. Additionally, no variety of pineapple has been reported to be resistant to PHRD

(Rodriguez *et al.*, 2002). According to Erwin and Ribeiro, (1996); Hu, (2007), fungicides remain a common management option for *Phytophthora* diseases worldwide However, over use of chemical formulations results into development of resistant biotypes (Fontema *et al.*, 2005) and environmental pollution. Therefore effective minimum fungicide concentrations must always be used. In Uganda, information on identity of PHRD causal pathogens and sensitivity of *Phytophthora* to fungicides is limited and scanty (NARO, 2012).

CHAPTER THREE

MORPHOLOGICAL CHARACTERIZATION OF *PHYTOPHTHORA* SPP ASSOCIATED WITH PINEAPPLE HEART ROT DISEASE IN UGANDA

3.1 Introduction

Pineapple production is among the important agricultural enterprises that many farmers are engaged in tropical and subtropical areas of the world (Rodriguez *et al.*, 2015). In Uganda, pineapple is majorly produced within the central districts including Kayunga, Mukono, Masaka and Luwero. The most cultivated pineapple variety in Uganda is smooth cayenne with traces of Victoria and Kafaransa (MUZAARDI 2010; Bua *et al.*, 2013). However, all these varieties are being threatened by PHRD (NARO, 2012). Infact, No sources of resistance to PHRD within the pineapple germplasm has been reported worldwide (Rodriguez *et al.*, 2002; 2015). This implies serious economic losses (Coppens *et al.*, 1997; Shreenivasa *et al.*, 2015).

Currently, In Uganda, farmers are employing several methods to manage PHRD including roguing and manuring. However, the methods used have proved not effective in managing PHRD (Bua, unpublished). To make matters worse, there is limited precision on identity of the causal organism (NARO, 2012) yet correct identification of the pathogen is critical to the development of appropriate disease control packages (Mbaka *et al.*, 2010; Schreier, 2013; Akrofi, 2015). Therefore, this study was conducted to identify the *Phytophthora* associated with PHRD in Uganda.

3.2 Materials and methods

3.2.1 Collection of symptomatic samples

One hundred twenty (120) symptomatic pineapple samples (leaves with symptoms of PHRD) were collected from the four districts of Masaka, Luwero, Mukono and Kayunga (Major pineapple producing districts) in the month of April 2016. The collected samples were packed in paper bags and taken to the laboratory for isolation of possible causal pathogens.

3.2.2. Isolates examination criteria

The collected samples were analyzed in the lab to isolate 37 isolates identified as *Phytophthora nicotianae* in trail I. Three isolates selected from each district based on district representation and growth rate were further assayed for morphological characteristics in trial II at NARL laboratories in May – August 2016 to confirm the results from the previous trial I. Both macro morphological and micro morphological characteristics were assessed. According to Hurberli (2001) and Mbaka *et al.* (2010), macro morphological features included colony type and growth rate while micro morphological features included sporangia shape, papillation, sporangiophore branching, sporangia length and breadth, chlamydospore and hyphae diameter.

3.2.3 Study location

The study were conducted in the laboratory at the National Crops Resource Research Institute (NaCRRI) Namulonge, Wakiso district (1200meters above sea level, 00° 31′ 30″ N, 32° 36′ 54″ E), National Agricultural Research laboratory (NARL) Kawanda (1190 meters above sea level, 0°25′05″ N, 32°31′54″ E) and in the screen house at the Department of Agriculture, Kyambogo University Kampala (1189meters above sea level, 00°20′54″N, 32°37′49″E).

3.2.4 Isolation of *Phytophthora* species

Isolation of PHRD causal organisms was done using cornmeal agar amended with 10mg pimaricin, 250mg ampicillin, 10mg rifampicin, 10mg benomyl, 25mg Pentachloronitrobenzene (PCNB) and 50mg hymexazol (PARBPH) as described by Drenth and Sendall (2001). The symptomatic pineapple leaves were washed under running water to eliminate soil. Five (5) mm pieces were cut off the disease lesions between healthy and diseased tissue. The cut tissue pieces were disinfested by immersion in a solution of 70 % ethanol (used because it was available) for 3 minutes, rinsed three times with sterile distilled water and dried with sterile paper towels. The dried leaf fragments were placed on cornmeal agar (CMA) amended as above (Drenth and Sendall, 2001; Mounde *et al.*, 2012; Rodriguez *et al.*, 2015). The petri-plates were incubated at 24°C in the dark for 2-3 days (Drenth and Sendall, 2001; Mbaka *et al.*, 2009; Mounde *et al.*, 2012). Pure cultures of *Phytopthora* species were obtained by sub-culturing hyphal tips onto freshly prepared corn meal agar (ACMA) as described above for 2-3 days.

3.2.5 Pathogenicity testing of *Phytophthora* isolates

The pathogenicity of *Phytophthora* isolates recovered from the infected plant tissues was confirmed by inoculating them on two month old healthy smooth cayenne pineapple plants (Shen *et al.*, 2013), grown out in the screen house to confirm that they were healthy (Palmucci *et al.*, 2013; Shen *et al.*, 2013). *Phytophthora* isolates used for the study were induced to sporulate following the protocol described by Jeffers (2006). Zoospore release was induced by incubating agar plugs with sporangia in non sterile soil extract solution (NS-SES) at 4°C for 30minutes to shock the sporangia. Isolates were later placed at room temperature for 10-20 minutes to burst the sporangia so as release zoospores (Saadoun and Allagui, 2008).

Isolate suspensions with zoospores at 10^8 ml^{-1} concentration were prepared and adjusted using a haemocytometer (Rodriguez *et al.*, 2002, 2015). The base of the heart leaves of pineapple plants were surface sterilized with 70% alcohol and blot dried. The disinfected basal heart leaves were inflicted with four wounds and inoculated with 10^8 zoospores ml^{-1} (Rodriguez *et al.*, 2015). Control pineapple leaves were wounded and inoculated with 4 ml of sterile distilled water. The inoculated pineapple plants were laid out in a complete randomized design with three replications. Pineapple plants were left under normal day and night cycle of illumination for three months in a screen house. Pineapple plants were monitored on a daily basis for one month (Rodriguez *et al.*, 2015) for PHRD symptom appearance. Once the symptoms appeared, information was recorded and plants left in the screen house.

3.2.6 Isolate characterization

3. 2.6.1 Radial growth rate and mycelium growth pattern

This was done using potato dextrose agar (PDA) as described by Drenth and Sendall (2001). Individual hyphal tips were cut from the edge of 37 actively growing colonies that caused PHRD symptoms in 3.2.4, placed on corn meal agar (CMA) and grown for 3 days following the procedure of Mbaka *et al.* (2010). After growth, 5mm agar discs were cut from the edge of actively growing colonies using a sterile cork borer and placed with the mycelia facing downwards in the centre of petri plates containing 10 ml of Potato Dextrose Agar (PDA). The plates with mycelia plugs were sealed with parafilm and incubated at 25°C in the dark for 7 days (Mounde *et al.*, 2012; Tsopmbeng *et al.*, 2014; Bevans *et al.*, 2015). The plates were arranged in a complete randomized design (CRD) in three replications for each isolate. Radial growth of the growing colonies was measured daily for one week along two lines intersecting at right angles at

the centre of the inoculum disc (Fenn and Coffey, 1983) (Appendix 2). Colony morphology of isolates was recorded after seven day as described by Erwin and Ribeiro (1996) and Drenth and Sendall (2001).

3.2.6.2 Sporangia production and morphology characterization

Test isolates were induced to sporulate by floating agar plugs of each isolates in non-sterile soils extract solution (NS-SES) (Jeffers, 2006). One kilogram of loam soil free from *Phytophthora* was collected from a field where trees were previously growing. Fifteen grams (15grams) of the soil was mixed with one litre of distilled water in a bottle and shaken for five hours using a rotary shaker. The suspension was allowed to settle overnight. The supernatant was decanted and centrifuged in falcon tubes of 50mls for 6 min at 6000rpm. Later the supernatant was filtered through Whatman filter paper to remove floating organic debris. The non-sterile soil extract soil solution (NS-SES) was stored for 3days in a glass bottle to allow it to age in the refrigerator at 4°C (Jeffers, 2006).

Each *Phytophthora* isolate was grown on 10% V8 juice agar at 25 °C in the dark for 3 days (Jeffers, 2006). The agar plugs (5mm) of young actively growing mycelia were cut, placed in 9 cm diameter petri dish floated covered with NS-SES (Drenth and Sendall, 2001; Jeffers, 2006). The flooded plates were incubated under continuous fluorescent light (18W, cool light) suspended 18cm above the cultures at room temperature (25° C) in order to induce production of sporangia. After 24hrs, individual plugs of each isolate were mounted on glass slides and observed under a light microscope (Zeiss, German) for the presence of sporangia. Sporangia associated features for characterization like sporangia shape, sporangia papillation and sporangiophore branching were examined at ×400 magnification and recorded following the

descriptors of Erwin and Ribeiro (1996) and Drenth and Sendall (2001). Chlamydospore and hyphae diameter was also examined (Palmucci *et al.,* 2013; Rodriguez *et al.*, 2015).

Sporangia length and width/breadth, chlamydospore and hyphae diameter were measured and recorded using the Zeiss camera (German) and software motic images (Rodriguez *et al.*, 2015). Thirty to fifty sporangia per isolate were selected and their sizes measured. Recorded features were compared with known characteristics in published identification keys (Waterhouse, 1963; Hall, 1993; Erwin and Ribeiro, 1996, Drenth and Sendall; 2001) as well as with the data from recently published papers describing *Phytophthora* (Mbaka *et al.,* 2010; Mounde *et al.*, 2012; Pao-Jen *et al.*, 2013; Palmucci *et al.*, 2013; Rodriguez *et al.*, 2015, Shand and Yamagata, 2016).

3.2.7 Data analyses

Data on radial growth rate, colony diameter and asexual structure dimensions were summarized and their means subjected to analysis of variance (ANOVA) using Genstat (15th edition) (Bekker *et al.*, 2005). Where there were significant differences, means were separated using Least Significant Difference (LSD) test at 5% probability level. Additionally, sporangia shape, papillation and sporangiophore branching frequencies were also generated using Genstat (15th edition).

3.3 Results

3.3.1 Isolation and pathogenicity tests of *Phytophthora* species associated with pineapple heart rot disease

The highest (42%) number of symptomatic isolates was recovered from Kayunga district followed by Luwero district (39.5%), Mukono (10.5%) and lastly Masaka (8%) (Table 3).

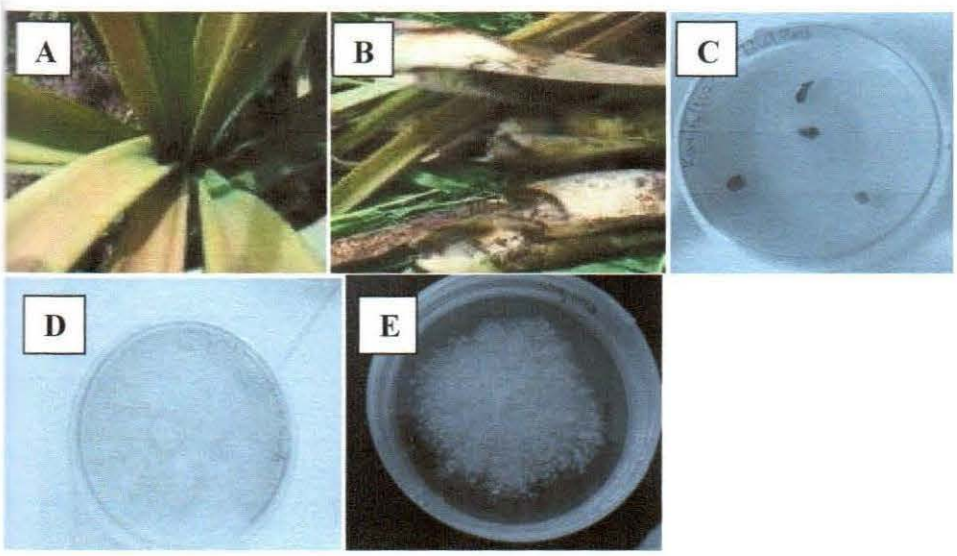

Figure 1: Isolation of PHRD causal organisms. **A**) Symptomatic pineapple plant in the field. **B**) Pineapple leaves extracted from PHRD infected plants in the field during survey. **C**) Infected pineapple leaf fragments plated on amended CMA. **D**) Pure culture of *Phytophthora* species growing in CMA. **E**) Isolate of *Phytophthora* species. growing in PDA media.

Symptoms characteristics of PHRD were observed within seven days after inoculation of the pineapple plants. The disease manifested as a pale green color and necrosis on the tips of the leaves followed by heart rot, browning of the base of the middle leaves coupled with foul smell (Figure 2). Of the 114 inoculated pineapple plants, 111 (97%) got diseased. Control plants did not develop disease (Figure 2). When re-isolated from the infected plants, the pathogens were able to re-infect the inoculated plants (new set of healthy pineapple plants) producing symptoms as above in accordance with Koch's postulates.

In general, all the symptoms observed on the inoculated pineapple plants were similar to those commonly observed in the field (Figure 2). Additionally, 97% (37/38) isolates inoculated in pineapple plants produced symptoms characteristic of PHRD (Figure 2).

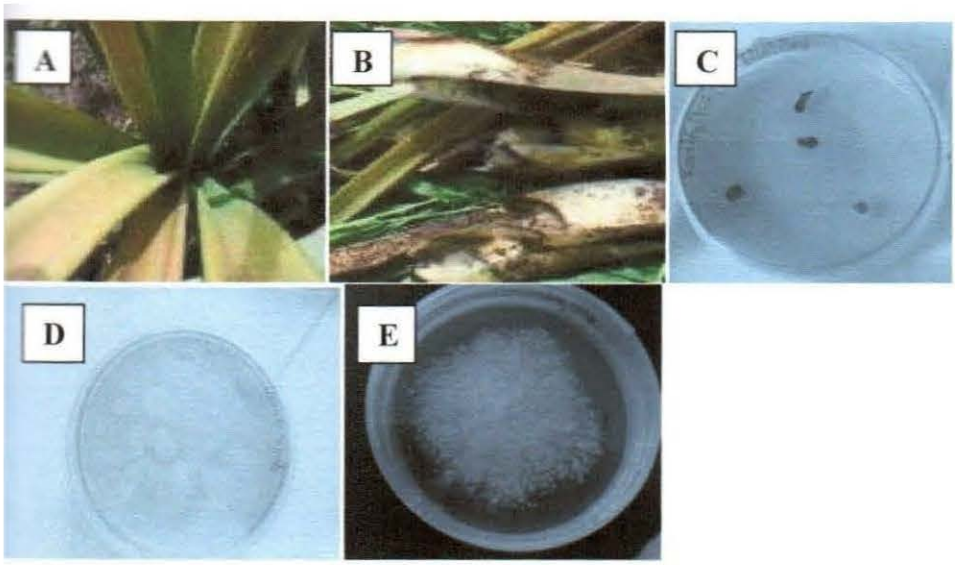

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Table 3: Origin and pathogenicity of *Phytophthora* isolates at Kyambogo University screen house, 2016

District	Isolate	Pathogenicity
Kayunga	KAY 01	++
Kayunga	KAY 02	++
Kayunga	KAY 03	++
Kayunga	KAY 04	++
Kayunga	KAY 05	++
Kayunga	KAY 06	++
Kayunga	KAY 07	++
Kayunga	KAY 08	++
Kayunga	KAY 09	++
Kayunga	KAY 10	++
Kayunga	KAY 11	++
Kayunga	KAY 12	++
Kayunga	KAY 13	++
Kayunga	KAY 14	++
Kayunga	KAY 15	++
Kayunga	KAY 16	++
Luwero	LUW 01	++
Luwero	LUW 02	++
Luwero	LUW 03	++
Luwero	LUW 04	++
Luwero	LUW 05	++
Luwero	LUW 06	++
Luwero	LUW 07	++
Luwero	LUW 08	++
Luwero	LUW 09	++
Luwero	LUW 10	++
Luwero	LUW 11	++
Luwero	LUW 12	++
Luwero	LUW 13	++
Luwero	LUW 14	++
Luwero	LUW 15	++
Masaka	MAS 01	++
Masaka	MAS 02	++
Masaka	MAS 03	++
Mukono	MUK 01	++
Mukono	MUK 02	++
Mukono	MUK 03	++
Mukono	MUK 04	--

++ Pathogenic, -- Non pathogenic, MAS: Masaka, KAY: Kayunga, MUK: Mukono, LUW: Luwero

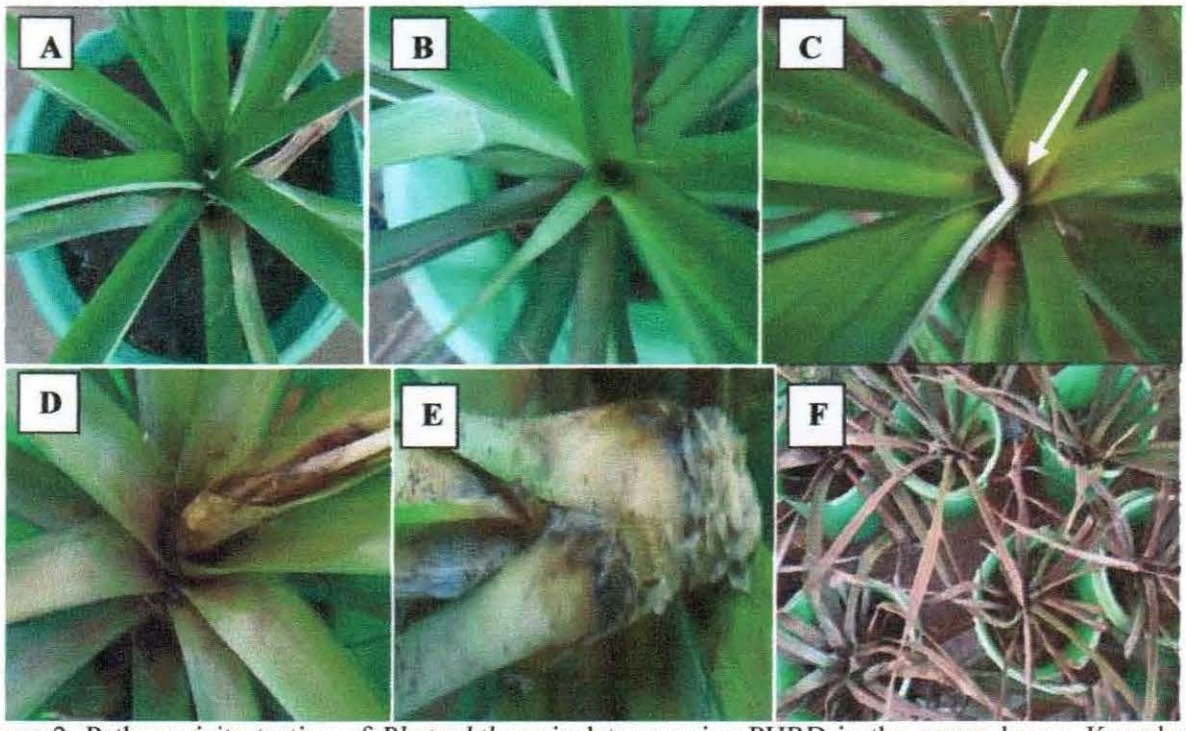
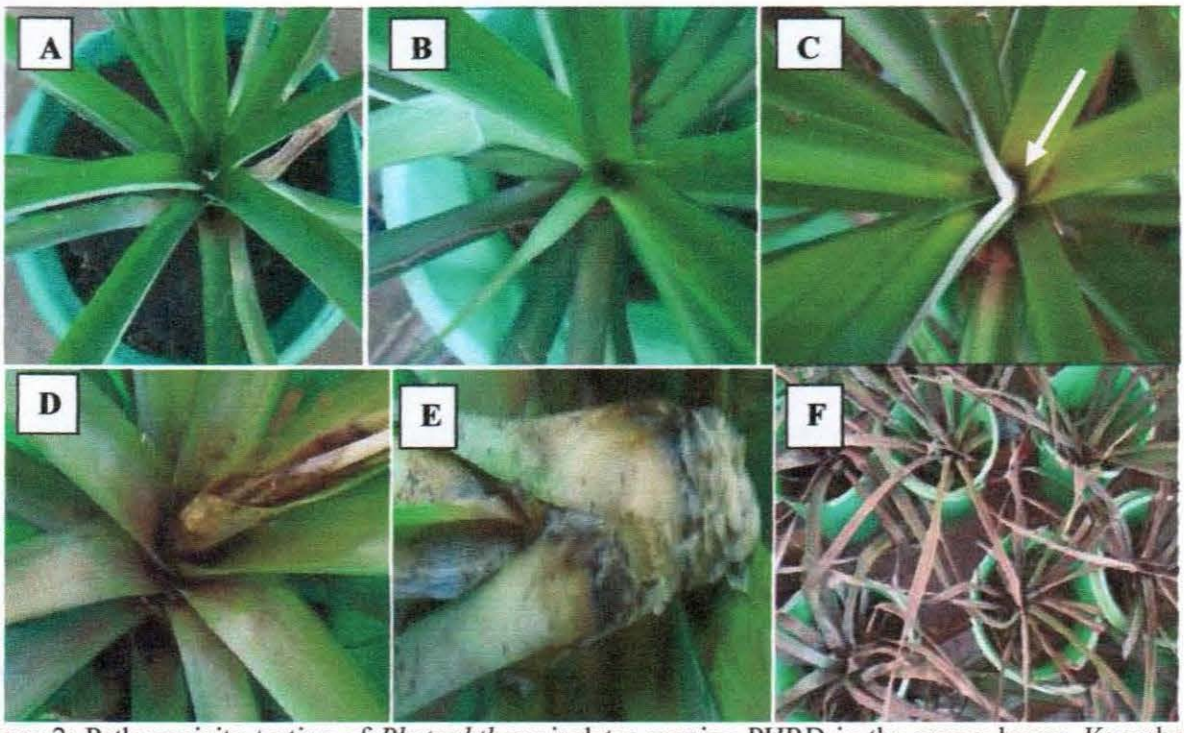


Figure 2: Pathogenicity testing of *Phytophthora* isolates causing PHRD in the screen house, Kyambogo University, 2016. **A**) Asymptomatic control pineapple plant month after inoculation. **B**) Asymptomatic pineapple plant close to three month after inoculation (Non pathogenic). **C**) Pineapple plants showing pale green colour and heart rot disease signs seven days after inoculation **D**) Pineapple plant exhibiting symptoms of PHRD 21 days after inoculation. **E**) Water soaked pineapple plant heart leaves exhibiting symptoms of PHRD 21 days after inoculation. **F**) Completely infected pineapple plants close to three months after inoculation.

3.3. 2 Identification of *Phytophthora* isolates causing pineapple heart rot disease

3.3. 2.1 Growth pattern, rate and colony morphology of *Phytophthora* isolates

All PHRD isolates formed a dense rosette growth pattern with white stoloniferous colonies on PDA (Figure 3). Overall, in trial I and II the mycelia were dense in growth habit (Figure 3).



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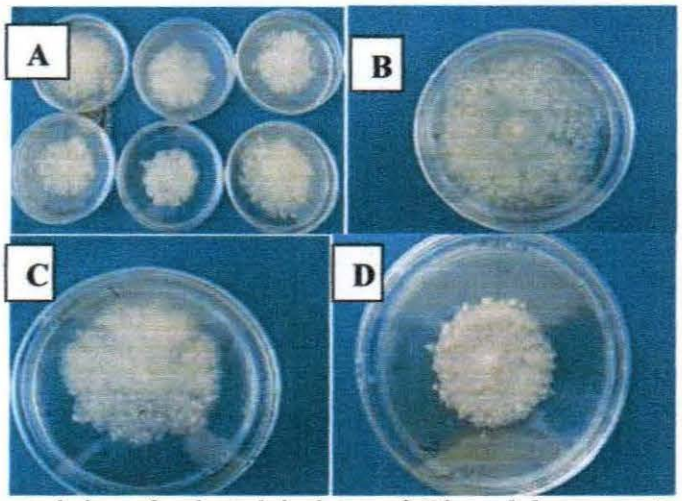

Figure 3: Colony characteristics of selected isolates of *Phytophthora species* in PDA. A) Isolates showing dense growth pattern in PDA in trial I. B-D) Isolates showing dense growth pattern in PDA in trail II.

There were significant differences ($P<0.05$) in growth rates amongst the isolates on PDA media (Table 4). On trial I in the first day, the highest and lowest colony diameters of 4.0 mm (KAY 10) and 1.2mm (LUW 11) respectively. On the 7th day, the highest and lowest colony diameters of 42mm (KAY13) and 20.17mm (LUW 12) respectively (Table 5). Results for the 2nd and 6th days are also presented in Table 4. Overall, the average colony diameters were 2.65mm and 25.83mm on the first and 7th days, respectively. A similar trend was observed when the experiment was repeated in Trial II (Table 5).

Table 4: Summary of ANOVA table for growth of *Phytophthora* isolates on PDA over seven day period at NARL, 2016

S. of variation	D.F	Mean squares						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
			Trial I					
Rep	2		0.62	0.37	2.5	1.39	0.99	1.49
Isolate	36	2.03***	13.45***	13.25***	45.09***	64.82***	87.09***	91.19***
Residual	62	0.45	0.72	0.99	2.03	2.32	2.16	4.28
			Trial II					
Rep	2	0.11	1.26	0.15	0.43	0.22	0.96	0.44
Isolate	11	2.88***	22.73***	41.97***	52.30***	95.87***	126.47***	128.41***
Residual	22	0.78	0.14	0.47	0.38	0.25	0.76	0.36

*** means significance at <0.001

Table 5: Colony diameter of *Phytophthora* isolates grown on PDA for a period of seven days at NARL, Trial I 2016

isolate	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
KAY 01	2.17	4.00	7.67	12.00	16.67	22.67	29.00
KAY 02	1.83	4.83	7.50	12.00	14.33	18.00	21.33
KAY 03	2.33	4.83	6.50	10.33	13.50	16.83	21.17
KAY 04	2.67	5.17	8.00	12.33	15.83	21.83	23.83
KAY 05	2.33	6.00	8.67	12.50	19.17	33.00	20.50
KAY 06	1.83	4.33	7.33	12.33	17.50	17.50	24.17
KAY 07	2.67	5.33	7.50	13.70	16.37	18.33	25.00
KAY 08	1.17	4.50	6.33	8.33	11.33	22.00	19.17
KAY 09	2.17	5.33	7.00	13.50	17.17	23.50	29.00
KAY 10	4.00	8.00	17.00	22.00	27.17	35.00	39.00
KAY 11	2.63	4.50	6.67	13.67	14.50	17.33	22.33
KAY 12	3.33	11.4	16.20	20.33	25.2	28.83	31.83
KAY 13	4.50	13.2	18.30	15.50	31.00	36.83	42.00
KAY 14	1.65	4.51	6.50	13.01	15.17	18.33	22.17
KAY 15	3.18	5.50	8.50	12.33	15.33	18.67	21.17
KAY 16	2.17	4.83	7.00	11.17	13.17	18.17	22.50
LUW 01	3.67	6.00	8.00	11.00	14.00	18.17	21.33
LUW 02	1.83	4.67	6.33	9.83	13.33	16.50	20.33
LUW 03	2.17	4.83	7.67	10.83	17.00	17.33	26.80
LUW 04	1.83	6.67	8.88	13.67	16.00	21.33	26.50
LUW 05	1.67	4.50	6.50	13.00	15.17	18.33	22.17
LUW 06	2.00	5.33	8.17	16.00	18.00	22.00	25.67
LUW 07	2.00	5.5	7.00	12.67	16.83	21.33	25.50
LUW 08	3.83	8.33	10.2	16.83	20.50	24.83	27.67
LUW 09	1.87	5.50	7.83	15.17	18.17	22.90	27.00
LUW 10	1.17	5.50	7.67	12.67	17.17	18.33	22.17
LUW 11	1.20	7.83	13.0	21.85	26.67	32.67	34.83
LUW 12	2.00	4.67	6.67	11.83	14.83	16.67	20.17
LUW 13	1.67	4.17	7.50	13.17	17.2	18.33	22.83
LUW 14	2.50	6.00	8.83	15.17	18.33	21.5	24.67
LUW 15	8.00	13.00	15.00	19.00	22.00	24.00	28.00
MAS 01	5.00	10.00	14.00	18.00	22.00	24.00	25.00
MAS 02	5.00	11.00	15.00	19.50	23.00	26.50	28.00
MAS 03	2.13	5.17	9.00	15.33	18.50	21.00	25.50
MUK 01	3.00	10.1	15.2	20.83	26.26	31.50	35.27
MUK 02	2.67	5.17	6.83	13.50	15.00	17.50	20.50
MUK 03	1.33	7.67	10.00	13.17	22.67	26.23	27.83
Mean	**2.65**	**6.48**	**9.43**	**14.31**	**18.36**	**22.48**	**25.83**
LSD $_{(5\%)}$	**1.05**	**1.38**	**1.62**	**2.33**	**2.48**	**2.39**	**3.38**
CV(%)	**10.1**	**4.8**	**6.5**	**3.6**	**2.5**	**3.6**	**2.2**

Table 6: Colony diameter of *Phytophthora* isolates grown on PDA for a period of seven days at NARL, Trial II, 2016

Isolates	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
KAY 03	2.95	6.15	10.43	14.12	17.42	20.85	23.75
KAY 08	2.22	4.96	6.86	8.54	11.87	15.55	19.17
KAY 10	5.85	9.21	16.65	21.26	28.54	34.37	38.15
LUW 03	3.94	6.15	9.50	12.50	15.24	19.32	22.44
LUW 05	2.42	5.00	8.25	11.00	17.00	23.00	27.22
LUW 14	2.55	5.54	8.67	12.64	18.92	21.64	24.62
MAS 01	6.25	12.32	17.50	20.55	25.74	28.56	31.72
MAS 02	3.50	7.50	13.55	17.95	21.32	26.73	29.23
MAS 03	4.21	8.32	13.46	18.25	22.11	25.86	28.55
MUK 01	3.25	6.55	10.55	14.45	19.13	22.54	26.56
MUK 02	3.65	7.42	14.95	20.22	25.35	29.95	34.75
MUK 03	3.00	6.25	9.85	12.95	15.95	18.24	21.64
Mean	**3.65**	**7.11**	**11.69**	**15.35**	**19.88**	**23.88**	**27.32**
LSD $_{(5\%)}$	**1.35**	**1.78**	**2.10**	**2.77**	**2.95**	**2.75**	**4.26**
CV (%)	**7.3**	**6.0**	**5.9**	**3.4**	**3.1**	**3.0**	**2.8**

3.3.2.2 Micro morphological characteristics of *Phytophthora* isolates

All (100%) of the isolates were able to sporulate when flooded with NS – SES. Accordingly, varying temperature from 25^{0}C to 4^{0}C resulted in successful release of zoospore by all the isolates (Figure 4).

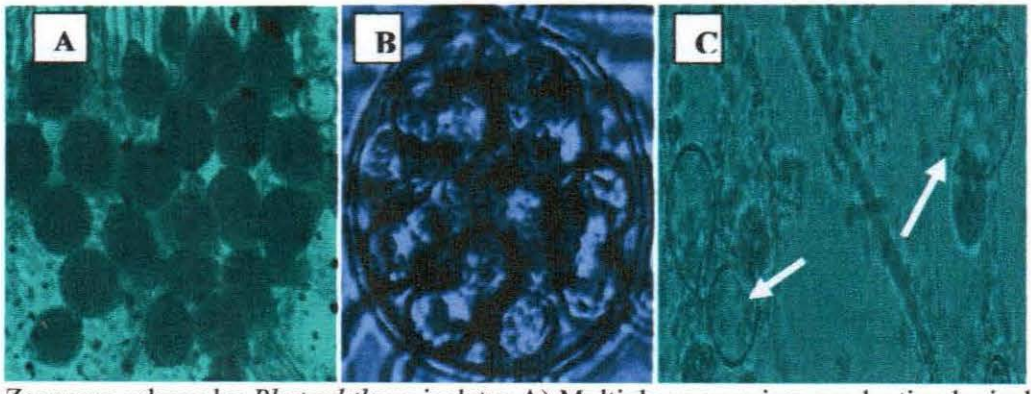

Figure 4: Zoospore release by *Phytophthora* isolates A) Multiple sporangium production by isolates after being flooded with NS - SES. B) Differentiation of the sporangium cytoplasm to release zoospores. C) Empty sporangia that released zoospores

The sporangia dimensions (length and width) as well as the chlamydospore and hyphal diameters were significantly different (P<0.001) amongst all the isolates from the four districts (Table 6). The widest and narrowest hyphae diameters were recorded from isolates MUK 04 (8.6 µm) and LUW 01 (3.0µm). Overall, the average hyphae diameter was 5.6µm (Table 7).

The isolates produced coralloid aseptate hyphae (Figure 5). Sporangia shape of all isolates varied although sporangia were mostly ovoid. However, there were also limoniform and obpyriform sporangia. Additionally, sporangia were all non-caducous and papillate, with prominent pedicels. Sporangiophore branching was compound sympodium for all the isolates (Figure 5; Appendix 1).

Table 7: Summary of ANOVA table for micro morphological characteristics of *Phytophthora* isolates at NARL, 2016

		Trial I			
		Mean squares			
Source of variation	**D.F**	**sporangia length**	**sporangia breadth**	**Chlamydospores diameter**	**Hyphae diameter**
Rep	2	14.09	0.78	10.73	0.40
Isolate	36	65.92***	32.98***	80.51***	5.18***
Residual	72	17.34	0.21	13.11	0.19
		Trial II			
Rep	2	0.08	0.01	0.06	0.11
Isolate	11	52.11***	29.33***	45.95***	8.81***
Residual	22	0.16	0.11	0.19.	0.07

*** means significant at <0.001

Table 8: Micro morphological characteristics of 37 *Phytophthora* isolates isolated from PHRD infested pineapple leaves at NARL, Trial I 2016

Isolate	Sporangia length (µm)	Sporangia breadth (µm)	Sporangia Length/breadth ratio	Chlamydospore diameter (µm)	Hyphae diameter (µm)
KAY 01	51.1	37.6	1.35 : 1	32.0	6.0
KAY 02	51.7	40.4	1.27 : 1	27.0	7.5
KAY 03	46.1	34.0	1.36 : 1	37.5	5.0
KAY 04	56.0	40.0	1.40 : 1	38.0	6.0
KAY 05	55. 0	41.5	1.33 : 1	42.0	6.0
KAY 06	45.7	37.5	1.22 : 1	41.0	6.7
KAY 07	52. 0	37.5	1.38 : 1	32.0	6.0
KAY 08	33.2	30.85	1.11 : 1	41.0	6.5
KAY 09	52. 0	37.5	1.39 : 1	32.0	6.0
KAY 10	42. 0	30.3	1.39 : 1	40.0	3.0
KAY 11	54. 0	42.0	1.29 : 1	No	4.0
KAY 12	47.3	37.2	1.27 : 1	37.5	5.7
KAY 13	46.7	33.0	1.42 : 1	24.7	6.0
KAY 14	54.7	42.0	1.30 : 1	30.7	6.0
KAY 15	50.3	39.0	1.28 : 1	40.0	3.5
KAY 16	50.3	39.0	1.28 : 1	40.0	3.5
LUW 01	47.6	34.0	1.40 : 1	25.0	6.0
LUW 02	50. 0	38.5	1.29 : 1	28.0	5.5
LUW 03	51.8	35.5	1.46 : 1	30.0	4.5
LUW 04	51.8	35.5	1.46 : 1	30.0	4.5
LUW 05	56. 0	40.0	1.40 : 1	38.0	6.7
LUW 06	57.5	39.0	1.47 : 1	40.0	3.3
LUW 07	52.3	42.0	1.25 : 1	40.0	6.7
LUW 08	51.8	35.5	1.46 : 1	40.0	4.5
LUW 09	51.3	39.5	1.29 : 1	38.5	4.0
LUW 10	52. 0	41.0	1.27 : 1	39.5	7.5
LUW 11	45.7	36.0	1.27 : 1	41.0	6.5
LUW 12	45.7	35.7	1.28 : 1	39.0	6.2
LUW 13	51.7	40.7	1.27 : 1	39.0	7.0
LUW 14	51.3	40.3	1.27 : 1	33.7	6.0
LUW 15	47.5	37.0	1.28 : 1	33.5	5.0
MAS 01	46. 0	39.0	1.17 : 1	37.0	5.0
MAS 02	47. 0	39.5	1.21 : 1	37.0	4.5
MAS 03	52. 0	38.5	1.35 : 1	32.0	6.0
MUK 01	42. 0	30.0	1.40 : 1	38.0	5.0
MUK 02	52.3	41.0	1.29 : 1	38.6	6.0
MUK 03	52. 0	44.5	1.17 : 1	41.0	8.6
Means	**49.8**	**38.1**	**1.32:1**	**37.6**	**5.6**
LSD $_{(5\%)}$	**6.79**	**0.75**		**5.91**	**0.72**
CV (%)	**8.30**	**1.22**		**10.17**	**7.75**

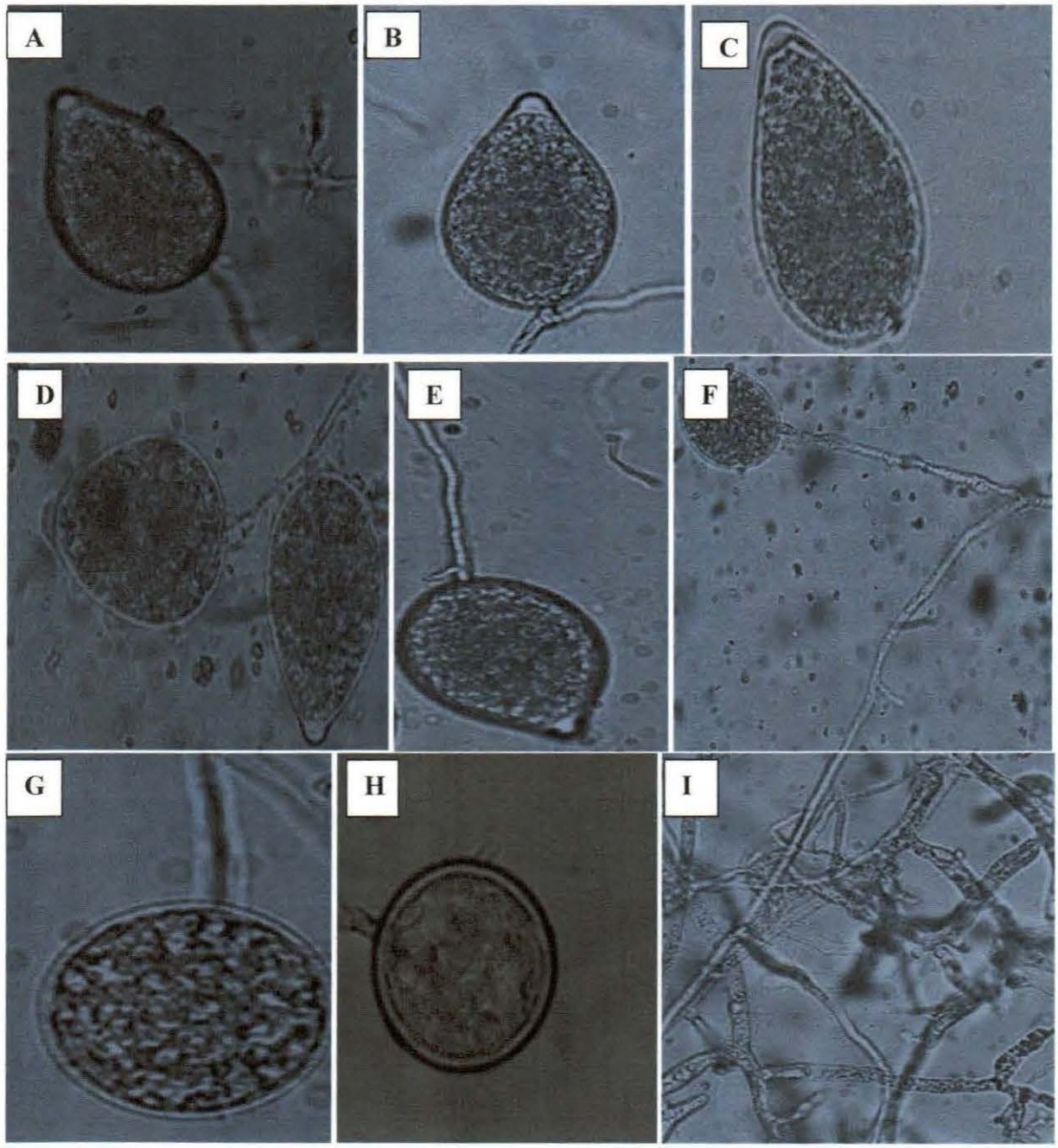

Figure 5: Micro morphological features of *Phytophthora* spp *isolates*. **A** and **B**) Limoniform Sporangia. **C**) Ovoid sporangium. **D** and **E**) Papillate sporangia. **F**) Sympodium sporangiophore branching. **G and H**) Terminal chlamydospores. **I)** Aseptate hyphae.

The longest and shortest isolate sporangia lengths were 57.5µm (LUW 06) and 33.2µm (KAY 08) respectively. The average sporangia length was 49.8µm. In contrast, the widest and narrowest sporangia breadths were 44.5µm (MUK 03) and 30µm (MUK 01 respectively. The average sporangia breadth was 38.1µm; accordingly, the length to breadth ratio was 1.32:1. The highest proportion (97%) of the isolates produced chlamydospores with diameter ranging from 25-42µm in contrast to 3% that did not produce chlamydospores (Table 6). When the experiment was repeated in trial II, all Isolates produced chlamydospores with similar diameter as in Trial I. For other parameters, a similar trend was observed (Figure 6).

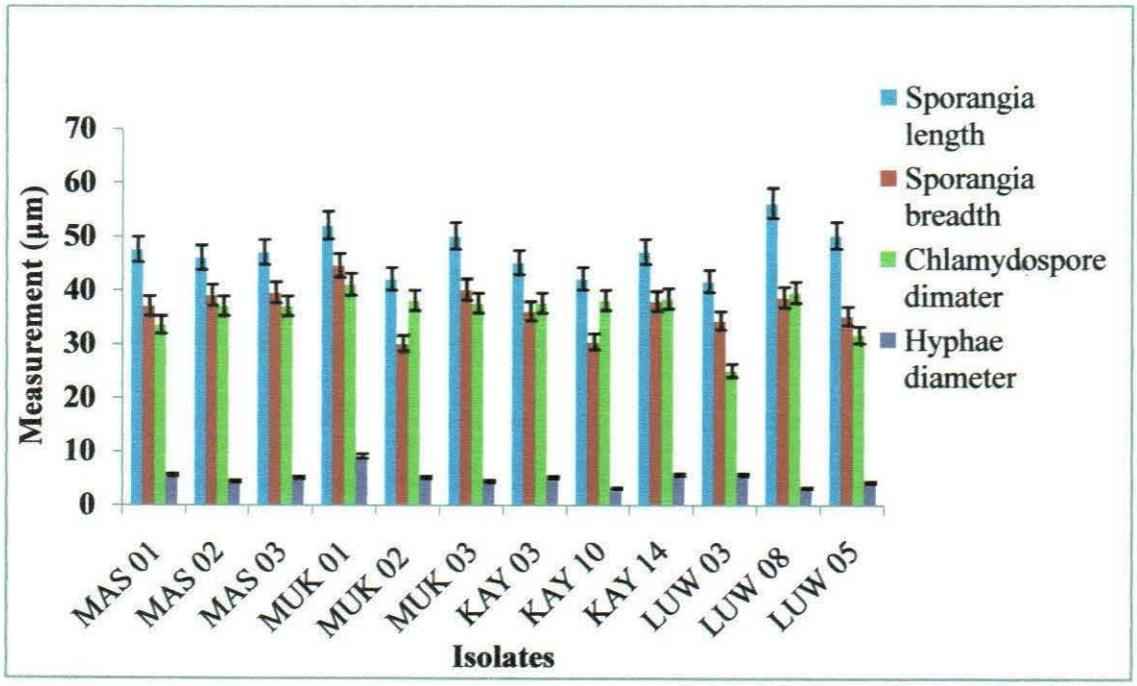

Figure 6: Micro morphological characteristics of 12 selected *Phytophthora* isolates at NARL, trial II 2016.

3.4 Discussion

The objective of this study was to identify the causal organism(s) of PHRD in central Uganda using morphological characteristics.

3.4.1 Isolation of *Phytophthora* species associated with pineapple heart rot disease

Pineapple heart rot disease causal organisms were isolated using amended Corn meal agar (CMA). This could probably be because CMA agar was deficient in nutrients hence prevented rapid growth of other organisms. Besides, *Phytophthora* being a slow growing organism, was given time to get established as a result of CMA media being low in nutrient content. Additionally, amendments of the media improved efficiency of isolation by directly suppressing contaminants. These results therefore corroborated the earlier reports of Drenth and Sendall (2001) that *Phytophthora* grows slowly *in vitro* and therefore requires media that is "weak" in nutritional terms to allow it to establish. For that reason, growth of bacterial and fungal contaminants is reduced, allowing colonies of *Phytophthora* to become established. To improve effectiveness of *Phytophthora* isolation from infected materials, addition of antibiotics like ampicillin rifampicin (to restrict the growth of gram positive and gram negative bacterial) and antifungals like pimaricin, benomyl, pentachloronitrobenzene (PCNB), and hymexazol (to restrict non *Phytophthora* fungus) is recommended (Drenth and Sendall, 2001; Jeffers, 2006).

3.4.2 Sporulation of *Phytophthora* species isolates

In this study 100% of the isolates were able to sporulate when flooded with NS – SES; in fact, isolates sporulated irrespective of the origin. Available literature indicates no relationship between sporulation of *Phytophthora* isolates with geographical origin. Sporulation by NS – SES could be because NS – SES provided nutrients that induced sporulation of *Phytophthora.* This

result is consistent with Jeffers (2006) and Zentmyer and Chen (1969) that *Phytophthora* sporulates easily when isolates are flooded with sufficient NS – SES According to Zentmyer and Chen, (1969) NS-SES contains bacteria in the genera *Chromobacterium* and *Pseudomonas* that supply some substance that is necessary for the fungus to produce sporangia, a substance which *Phytophthora* cannot form by itself in culture (Zentmyer and Chen, 1969). Additionally, bacteria cause a sudden reduction in food supply through competition, which forces *Phytopthora* into the sporulation stage instead of the vegetative stage (Zentmyer and Marshall 1959; Zentmyer and Chen, 1969).

3.4.3 Pathogenicity of *Phytophthora* species isolates recovered

Pathogenicity testing is normally used to identify and confirm unknown pathogens. In this study, 97% (37/38) of the isolates were pathogenic to pineapple plants. In fact, 97% of the inoculated pineapple plants in trial I produced symptoms characteristic of PHRD. These was due to high aggressiveness by *Phytophthora* isolates resulting into infection shown by appearance PHRD symptoms. This in agreement with a report by Rodriguez *et al.* (2015) that the majority of *P. nicotianae* isolates were pathogenic producing 95% infection on plants upon inoculated. The symptoms produced following inoculation of pineapple plants were characteristics of PHRD observed in other parts of the world. The symptoms started with water soaked lesions at the base of the leaves and the heart of the pineapple plant, the leaves turned a lighter green, the tips become necrotic and develop a characteristic foul smell. This is probably a result of physical hyphae penetration that allows entry of other secondary organisms (fungi and bacteria) which cause oxidation hence accumulation of cellular degradation residues (Agrios, 2005; Arévalo-Galarza, 2012; Rodriguez *et al.*, 2015). Accordingly, *Phytophthora* zoospores are attracted to invade elongation and differentiation zones producing progressive symptoms (Galiana *et al.*,

2005; Attard *et al.*, 2010). A report by Duniway (1983) and Shreenivasa *et al.* (2015) indicated that most cases of heart rot in pineapples are common during rainy season with temperatures oscillating between 24-26 °C and relative humidity of 90-100 %. Wet conditions are known to lead to high inoculum densities of the pathogen in the soil thereby increasing infection of susceptible hosts. Additionally, pathogenicity of the pathogen isolates relates to ability to produce high sporangia numbers. According to Shea *et al.* (1978), sporangial size and number have a relationship with pathogenicity. Thus, the ability of an isolate to produce greater numbers of sporangia may provide it with the potential to release more zoospores making it more virulent (Mbaka *et al.*, 2010). This coincides with the results of this study where all isolates produced considerable number of sporangia. The sporangia released zoospores that caused rapid infection of pineapple plants resulting into appearance of PHRD symptom within seven days. The high pathogenicity of *Phytophthora* spp isolates suggests that this pathogen is a serious threat to pineapple production in Uganda.

3.4.4 Morphological characterization of *Phytophthora* species associated with pineapple heart rot disease

In this study, all the isolates were identified as *P. nicotianae*. *P. nicotianae* was mostly characterized by mostly ovoid papillate non caduceus sporangium (32.4-56×30-41.5µm, length/breadth ratio of 1.32:1). Hyphae were coralloid and sporangiophores were sympodially branched. Also, the largest proportion of isolates (97%) produced terminal chlamydospores (25 to 42 µm in diameter) with exception of one isolate. Earlier, reports indicated the presence of intercalary and terminal chlamydospores in *P. nicotianae* (Hall, 1993). Similarly, Palmucci *et al.* (2013) reported that *P. nicotianae* produced persistent, mono- and sometimes bipapillate,

spherical to ovoid, ellipsoid, obpyriform sporangia (28–54×42–46 µm; length/breadth ratio of 1.3:1). However, terminal and intercalary chlamydospores (25 to 48 µm in diameter) and sexual structures were not observed in Argentina. Similarly, a report from Taiwan indicated that *P. nicotianae* produced sporangia with length 40 (48.2) - 55µm breadth of 30 (36.6) – 45µm and length to breadth ration of 1.06 – 1.43µm. The chlamydospores diameter was between 30-45 µm (Pao-Jen *et al.*, 2010). Gallup *et al.* (2006) in North Carolina indicated that hyphae of *P. nicotianae* were irregular with the width of 3-11µm with few numerous hyphal swellings. Sporangia were ovoid, pear-shaped, or spherical, very conspicuous papillae (Sizes of sporangia vary (18-70 x 14-39 µm) with isolate and the growth medium and chlamydospores ranges from 13 to 60 µm in diameter which coincides with the findings of this study. In China, Ho and He (2011) reported that *P. nicotianae* produced non-caduceus, terminal, papillate and mostly obpyriform sporangia, av. 46.2x34.9 µm with the mean length/breadth ratio as 1.34:1 Chlamydospores were spherical, thick-walled (av. 30.2 µm diameter. All this is in agreement with the findings of this study.

Phytophthora nicotianae isolates exhibited both macro-morphological (growth rate) and micro-morphological (sporangial morphology) variations. The differences observed could be due to variation in temperature, soil conditions, and altitude from where samples were picked. Earlier, Mbaka *et al.* (2010) reported phenotypic variations (radial growth rate, colony morphology) and sporangial dimensions within isolates of *Phytophthora cinnamomi* in Kenya. Similarly, in another study, Bernadovicova and Juhasova (2005) reported statistical significant differences in growth rates and sporangia dimensions within *Phytophthora* species isolates in Slovakia. Accordingly, Mounde *et al.* (2012) from Kenya reported that *P. nicotianae* colony diameter

varied between 5mm – 40mm/day between day one and day seven after incubation at 24^0C. In this study growth rate was slightly lower with colony diameters of isolates ranging between 3 - 25mm between day one and day seven after incubation at 24°C -26°C.

Accordingly, better understanding of phenotypic variation within the causal pathogen population is vital for development of management strategies for a disease (Mbaka *et al.*, 2010).

The fact that isolates tested induced characteristic heart rot disease symptoms on inoculated pineapples confirms that *Phytophthora* is a principal causative agent of PHRD in central Uganda. Accordingly, this study has confirmed previous studies from other parts of the world that PHRD is caused by *Phytopthora* species (Mbaka *et al.*, 2010; Shen *et al.*, 2013; Rodriguez *et al.*, 2015). These findings therefore have demonstrated that pineapple heart rot disease in Masaka, Mukono, Luwero and Kayunga districts in Uganda was caused by *P. nicotianae*. Similarly, in China and Mexico, *P. nicotianae* was confirmed as the causal organisms of PHRD (Shen *et al.*, 2013; Rodriguez *et al.*, 2015). Additionally, among the major constraints for pineapple growers in India was heart rot caused by *Phytopthora parasitica* that causes serious economic damage (Shreenivasa *et al.*, 2015).

The identification of all isolates from the four districts of Uganda as *Phytophthora nicotianae* has confirmed that the pathogen is wide spread in central Uganda. The high prevalence of *P. nicotianae* could be because the four districts are located at high elevations, with high rainfall that favors the survival and movement of *Phytophthora* zoospores. Favourable temperatures and poorly drained soils in this area could have also favoured the spread of PHRD through swimming of zoospores. These findings confirm reports that *P. nicotianae* is most prevalent at

high temperatures (Ricci *et al.*, 1990; Erwin and Rebeiro, 1996). Additionally, Weste and Vithanage, (1979) reported that inoculum levels of *Phytophthora* is usually very low during the dry season but high during the rainy season. According to Mounde *et al.* (2012), *P. nicotianae* in Kenya was confined within coastal lowlands compared to other parts of the country. In contrast, a survey of *Phytophthora* species attacking crops in Hainam province in China revealed that *P. nicotianae* was the predominant species (Zeng *et al.*, 2009). The fact that *P. nicotianae* is reported as one of the most destructive pathogens that cover a wide ecological habit, calls for an immediate intervention because the tropical regions where Uganda falls are among the major pineapple production areas. According to many authors, *Phytopthora* pathogens are very destructive to crops and their wide spatial distribution makes management difficult (Hu, 2007; Joy and Sihdhu, 2012).

CHAPTER FOUR

IN VITRO REACTION OF *Phytophthora nicotianae* ISOLATES TO FUNGICIDES

4.1 Introduction

The study reported in Chapter Three (this thesis) identified *Phytophthora nicotianae* as the causal organism of PHRD in central Uganda. *Phytophthora nicotianae* (syn: *Phytophthora parasitica*) is reported to be destructive on numerous herbaceous annuals and perennial plant species (Erwin and Ribeiro, 1996; Benson and von Broembsen, 2001). *Phytophthora* is disseminated to neighboring plants by splashes of sporangia (Benson and von Broembsen, 2001). Thus management of diseases caused by *Phytopthora* pathogens can be difficult.

Chemical control remains a primary approach for *Phytophthora diseases* (Hu *et al.*, 2008). Metalaxyl and Fosetyl Al are some of the major compounds used for management of *Phytophthora* diseases in plants (Jeffers and Miller, 2001). Due to the high effectiveness of Metalaxyl, farmers are motivated to repeatedly use this product. As a result, Metalaxyl resistance has been increasingly developing in pathogens including *Phytophthora* spp (Jeffers *et al.*, 2004). In Uganda victory powder which is an alternative fungicide to Metalaxyl is used by farmers for managing other fungal diseases. However, little is known about the sensitivity of *P. nicotianae* to Metalaxyl, Fosetyl Al and Victory powder in Uganda. Information on fungicide reaction is critical to developing management strategies (Hu *et al.*, 2008; Akrofi, 2015). The objective of this study therefore was to assess *in vitro* reaction of *P. nicotianae* isolates to Metalaxyl, Fosetyl Al and Victory powder in Uganda.

4.2 Methods and materials

4.2.1 Experimental location and fungicides

The study was conducted in the laboratory at National Agricultural Research Laboratories Kawanda (NARL) (1190 meters above sea level, 0°25′05″ N, 32°31′54″ E) in central Uganda. Experiments were carried out twice in 2016. Twenty isolates from study I (this thesis) were assessed for their sensitivity to the fungicides Metalaxyl [Methyl N-(methoxyacetyl)-N-(2, 6-xylyl)-DL-alaninate], Victory (Metalaxyl 8% + mancozeb 64%), and Fosetyl-Al [(Aluminum tris (O-ethyl phosphonate)].

Three fungicides were compared to assess their efficacy for management of PHRD in Uganda. Victory 72 powder efficacy was tested because it's local availability in Uganda (can act as an alternative fungicide for Metalaxyl). Fosetyl Al was included in the study because it has been used in other countries like India for management of *Phytophthora nicotianae* causing PHRD (Shreenivasa *et al.*, 2015). Later, concentrations 0.01 and 0.001 g/L Metalaxyl was introduced due to the fact that 0.01 g/L Metalaxyl caused complete suppression of *Phytophthora nicotianae* growth *in vitro.*

4.2.2 Media preparation and amendment

V8 media (10%) was prepared following the description of Jeffers (2006). Appropriate quantities of the fungicide solution were then poured in autoclaved media after cooling to about 55-60 °C (Hu *et al.*, 2008). In trial I of this study, 20 isolates randomly selected from experiment I. Each of the 20 isolates was grown on 10% V8 agar in petri plates for 7days at 25°C (Kimberly *et al.*, 2010). Thereafter, a 5 mm diameter mycelial disc was taken from fungal culture and transferred to the center of a petri plate containing 10 ml of clarified V8 agar amended with fungicides

Metalaxyl and Victory 72 at a concentration of 0.1 g/L Metalaxyl and Victory 72 powder. Control plates without fungicides were included in the study. Culture plates for each isolate were arranged in complete randomized design (CRD) and replicated three times. In trial II, eight isolates selected based on district representation were further assayed for fungicide sensitivity in the same manner described in trial I.

Since all the isolates were sensitive to the fungicides at concentration 0.1g/L, eight isolates were selected basing on district representation for further fungicide assays at lower concentrations 0.01 and 0.001g/L metalaxyl and Fosetyl Al (replaced victory powder) to examine behavior of isolate growth at reduced concentrations of fungicides following the same manner described in the previous experiment of 0.1g/L metalaxyl and Victory 72 powder. The study was conducted in two trials (Trial I and II).

4.2.3 Data collection and analyses

Colony measurements were recorded on the third, fifth and sixth day after transfer. This was done by measuring the diameter of the colony and subtracting 5mm to correct for the plug length. Measurement did not go beyond the 6^{th} day since some isolates had already covered the petri plate (Al-Masri *et al.*, 2015). Isolates were scored as resistant or sensitive (Hu, 2007) depending on whether or not they grew in 0.1g/L metalaxyl. Percentage inhibition for each isolate was calculated as the difference between the mean colony diameter of fungicide amended plates and control plates expressed as a percentage (% ratio) (Bekker *et al.*, 2005), mathematically expressed as:

$$\text{Percentage inhibition} = \frac{(C - T) \times 100}{C}$$

Where:

C = colony diameter (mm) on the control plate

T = colony diameter (mm) on the test plate

Data on colony diameters was analyzed using analysis of variance in Genstat computer programme (15th edition) (Bekker *et al.*, 2005). Where there were significant differences between means were separated using the Least Significant Different (LSD) test at 5% probability level.

4.3 Results

4.3.1 Reaction of *Phytophthora nicotianae* isolates to Metalaxyl and Victory 72 powder

The growth on V8 media was highly significantly ($P<0.001$) isolates during the third and fifth days. However, there were no significant differences ($P>0.05$) on the sixth day. The effect of fungicides on the growth of isolates in the media was not significant ($P<0.05$). Similarly, a no significant ($P<0.05$) isolate × fungicide treatment interaction was observed for the colony diameters. In trial II, the results followed the same trend (Table 8).

In general, all the isolates tested from all the districts were sensitive to Metalaxyl and Victory 72 powders at 0.1 g/L. In fact, there was almost no fungal growth observed on V8 media treated with Metalaxyl and Victory 72 powder as opposed to the control in all the seven days (Figure 7).

Table 9: Summary of ANOVA for the effect of Metalaxyl and Victory 72 powder on growth of 20 *Phytophthora nicotianae* isolates at NARL, 2016.

		Trial I		
Source of variation	D.F	Day three	Day five	Day six
Isolate	19	6.51***	2744.21***	28.09 NS
Treatment	2	22066.94 NS	64271.34 NS	100488.94 NS
Isolate × Treatment	38	6.51 NS	7.44 NS	2865.09 NS
		Trial II		
Isolate	19	7.21***	2649.89***	27.55 NS
Treatment	2	22011.71 NS	65211.45 NS	104337.74 NS
Isolate × Treatment	38	7.57 NS	9.25 NS	2732.23 NS

*** means significance at <0.001

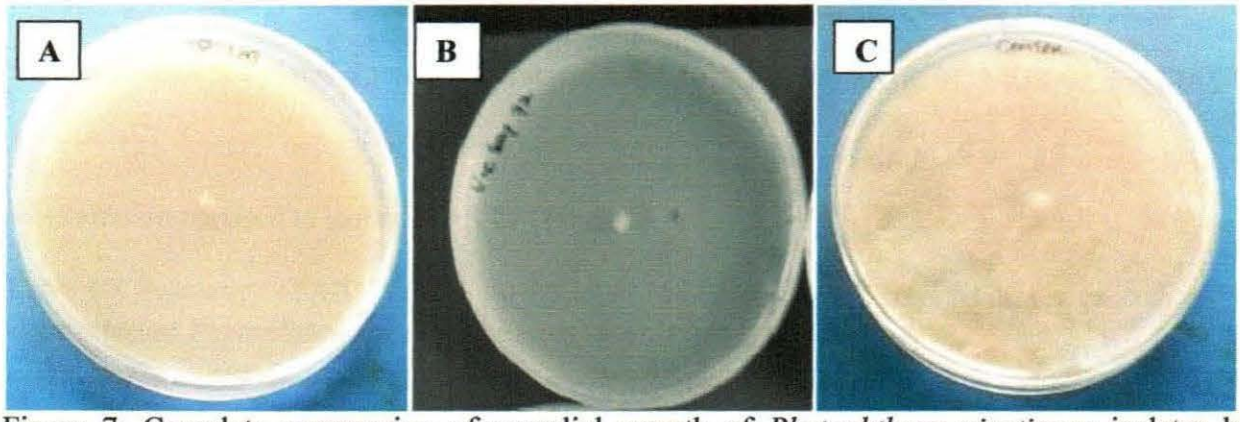

Figure 7: Complete suppression of mycelial growth of *Phytophthora nicotianae* isolates by Metalaxyl and Victory 72 powder at NARL, 2016 **A)** Complete suppression at 0.1g/L Metalaxyl on the seventh day of incubation. **B**) Complete suppression at 0.1g/L Victory 72 powder on the seventh day of incubation. **C**) Control plates with *Phytophthora nicotianae* isolate growth.

4.3.2 Reaction of *Phytophthora nicotianae* isolates to Metalaxyl and Fosetyl Al

The growth of isolates slightly varied in V8 media ($P<0.05$) in both trial I and II on the third and fifth days. However, the growth was highly significantly different by the sixth day ($P = 0.001$). Additionally, different fungicides significantly affected the growth of isolates in media. Accordingly, different fungicide concentrations greatly affected the growth of *Phytophthora*

nicotianae isolates (P = 0.001). However, isolate × treatment influenced the colony diameters of isolates (P<0.05) only on the sixth day (Table 9).

The highest and lowest colony diameters were recorded in Fosetyl Al and Metalaxyl fungicides, respectively (Figure 8). The highest and lowest colony diameters in 0.001 g/L Fosetyl Al amended plates were 71.67 mm (MAS 01) and 13.67mm (LUW 14) compared to 71.33mm (MAS 01) and 7.50mm (LUW 14) in metalaxyl amended plates respectively. Accordingly, at 0.01g/L Fosetyl Al, the highest and lowest colony diameters were 71.89 mm (MAS 01) and 17.61 mm (MUK 01) compared to 77.44mm (MAS 01) and 0.00mm (MUK 01; MAS 02) in Metalaxyl amended plates. In general, isolates KAY 01, KAY 14, MUK 01, MUK 02 and MAS 02 had the lowest colony diameters with complete suppression at 0.01 g/L Metalaxyl concentration opposed to isolate MAS 01 (Figure 8).

Table 10: Summary of ANOVA for the effect of Metalaxyl and Fosetyl Al on the growth of eight selected *Phytophthora nicotianae* isolates at NARL, 2016

		Trial I		
Source of Variation	D.F	Day three	Day five	Day six
Isolate	7	1132.2^{*}	956.8^{*}	967.9^{***}
Treatment	2	1533.8^{*}	2959.6^{***}	4037.5^{***}
Concentration	1	2658.5^{*}	5115.1^{***}	6048.9^{***}
Isolate × Treatment	14	100.3^{NS}	69.2^{NS}	378.8^{*}
		Trial II		
Isolate	7	1163.4^{*}	982.1^{*}	997.5^{***}
Treatment	2	1499.5^{*}	3031.8^{***}	4133.5^{***}
Concentration	1	2742.2^{*}	5178.6^{***}	6132.3^{***}
Isolate × Treatment	14	111.4^{NS}	80.3^{NS}	290.3^{*}

*** Means significant at 0.001 * significant at 0.05 NS Not significant

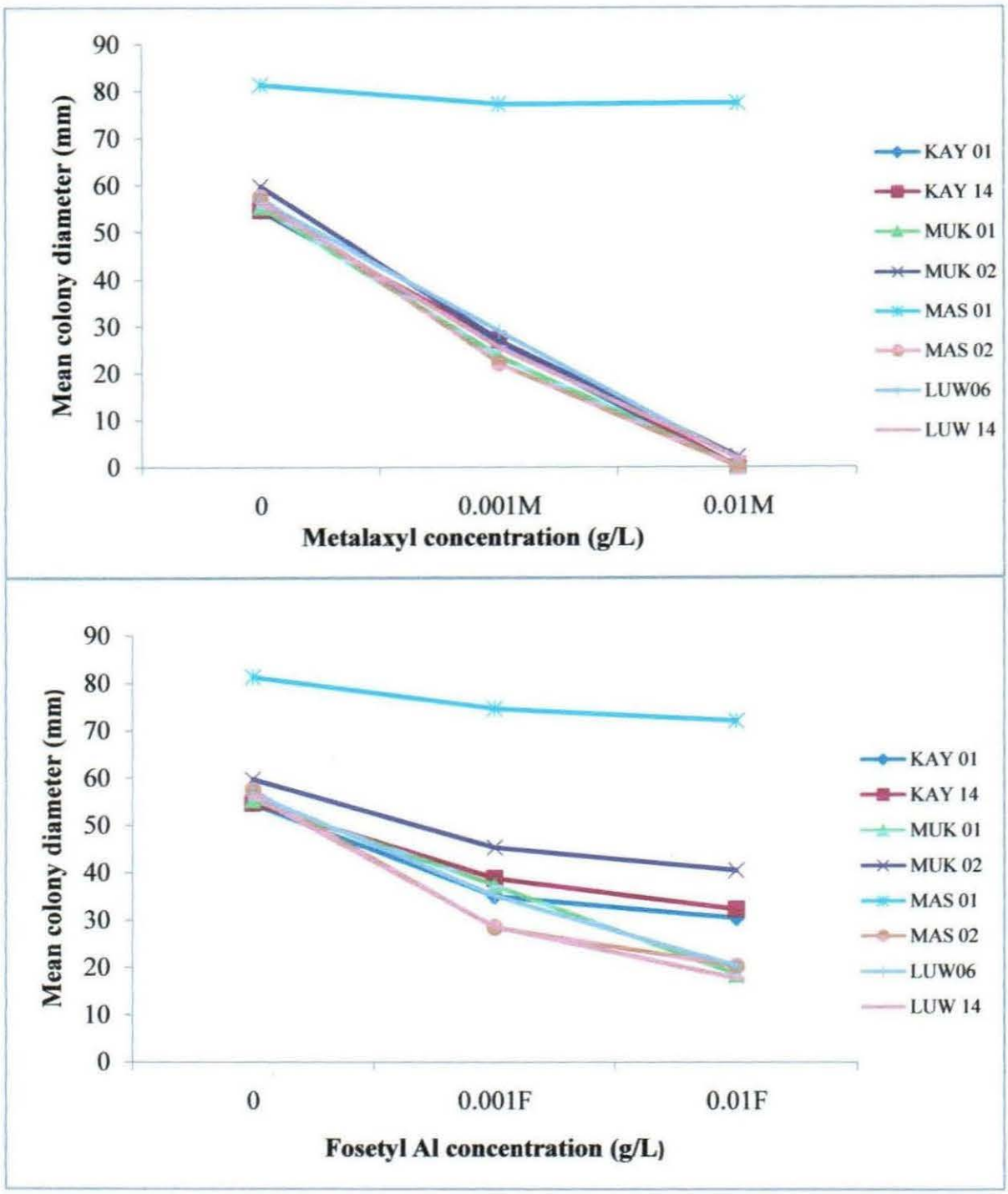

Figure 8: Mean colony diameter of eight *Phytophthora nicotianae* isolates in different Metalaxyl and Fosetyl Al concentrations at NARL, 2016.

In terms of inhibition, Metalaxyl at concentration 0.01g/L produced highest mycelial growth inhibition of isolates as opposed to Fosetyl Al. In fact all the isolates used were highly inhibited with exception of MAS 01 that had the lowest inhibition at concentration 0.01g/L metalaxyl. At concentration 0.001g/L, Metalaxyl, the highest inhibition was 61.51% (isolate MAS 02) while the lowest inhibition was 5% (isolate MAS 01) respectively compared to 53% (LUW 14) and 8.59% (MAS 01) in Fosetyl Al respectively (Figure 9). Accordingly, at concentration 0.01g/L, Metalaxyl, the highest inhibition was 100% (MUK 01) and the lowest was 11% (MAS 01) compared to 72% (LUW 14) and 8.59% (MAS 01) (Figure 9).

In general, low mycelial growth was observed in all Metalaxyl amended plates even at reduced concentrations compared to plates amended with Fosetyl Al (Figure 10). The results did not differ from the first trial in the second trial (Figure 9).

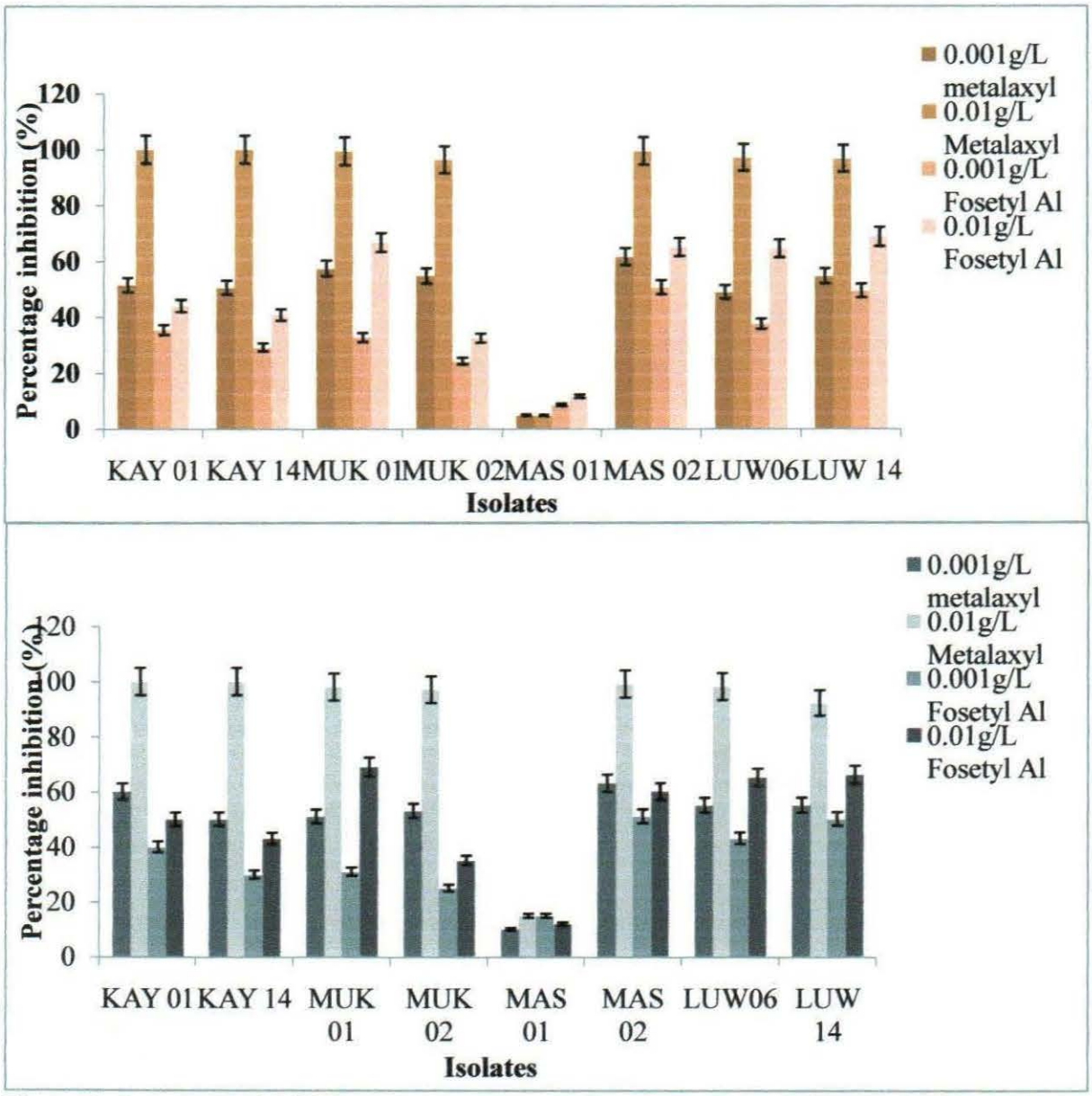

Figure 9: Percentage inhibition of eight *Phytophthora nicotianae* isolates at different Metalaxyl and Fosetyl Al concentrations at NARL trial I and II, 2016.

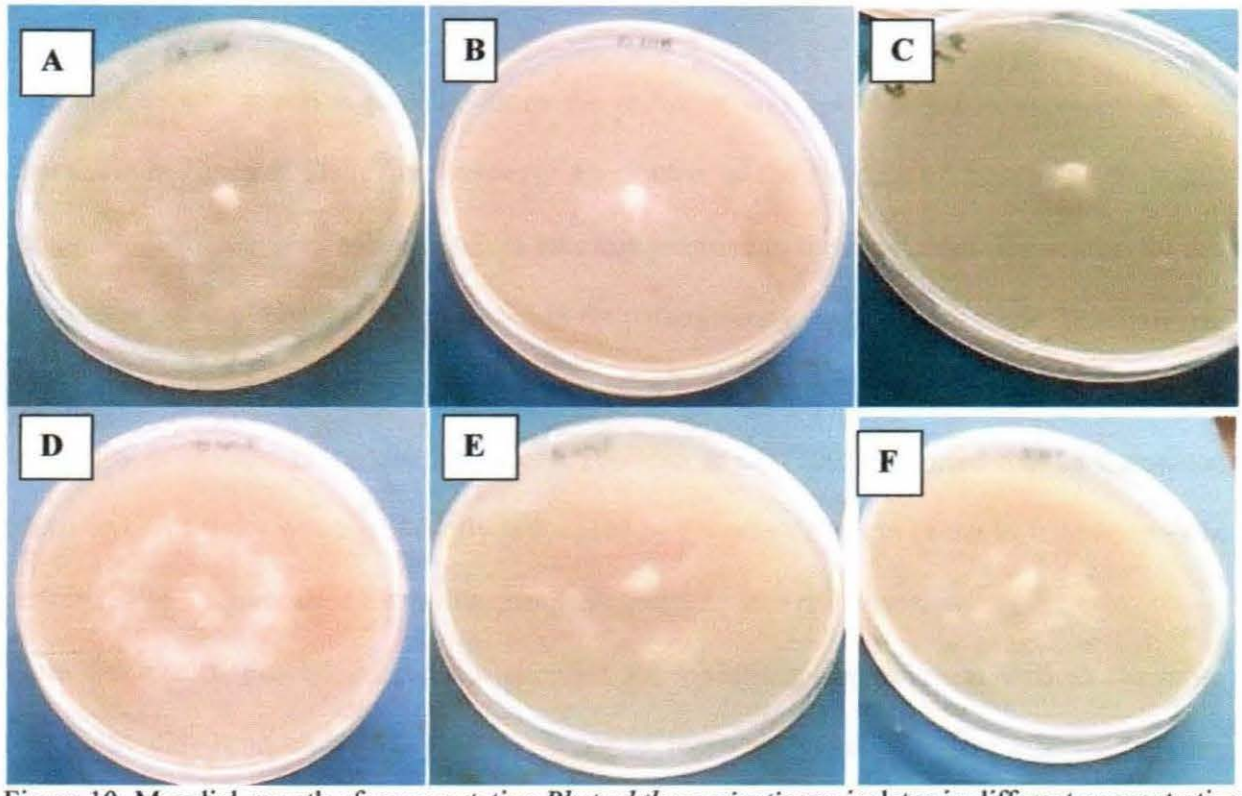

Figure 10: Mycelial growth of representative *Phytophthora nicotianae* isolates in different concentrations of Metalaxyl and Fosetyl Al. **A)** Control plate. **B and C)** Mycelial growth in petri plates amended with 0.01 g/L **Metalaxyl concentration. D) Mycelial growth petri plate amended with 0.001g/L** Metalaxyl. **E)** Mycelial growth in the petri plates amended with in 0.01 g/L Fosetyl Al. **F) Mycelial growth** in the petri plates amended with 0.001g/L Fosetyl Al.

4.4 Discussion

The objective of this study was to determine the *in vitro* reaction of PHRD causal pathogen(s) to Metalaxyl, Fosetyl Al and Victory powder for the management of fungal diseases in central Uganda. The results of this study have shown that all the isolates of *Phytophthora nicotianae* were sensitive to Metalaxyl and Victory powder at 0.1g/L and a varying response to Metalaxyl and Fosetyl at concentrations lower than 0.1g/L. This shows that Victory 72 powder can be used for managing PHRD disease in absence of Metalaxyl which is normally scarce. These results suggest that these fungicides can suppress *Phytophthora nicotianae* and hence can be used in the management of PHRD in Uganda. Sensitivity of *Phytophthora nicotianae* isolates was probably

attributed to low selection pressure since the pathogen was not exposed to excessive use of chemicals in Uganda. There was however a variation in the reaction of isolates to the tested fungicides. For example, isolate MAS 01 was less sensitive to the fungicides. The reason for this is not clear but could be due to a mutation in the pathogenicity gene of this isolate. This however needs to be proved in other studies. Some studies have pointed to geographical influence on sensitivity of isolates to certain fungicides. Fontema *et al.* (2005) reported that in Cameroon Metalaxyl sensitivity was significantly influenced by geographic origin of the isolates because in certain regions, farmer used excessive chemicals resulting into resistance.

From the results, Fosetyl Al did not suppress *Phytophthora nicotianae* as well as the other fungicides did. Fenn and Coffey (1983) reported that Fosetyl-A1 was less effective against *Phytophthora* and some other diseases caused by Peronosporales. Similarly, Boughalleb *et al.* (2006) in Tunisia reported that Metalaxyl and Fosetyl Al were only effective in inhibiting mycelial growth *in vitro* at high concentrations. Additionally, Wagner *et al.* (2007), in California reported that *in vitro* inhibition of *Phytophthora* mycelial growth by Metalaxyl can be achieved with a concentration range of 0.001-< 1 g/L. In India, a study by Padmaja *et al.* (2015) revealed that Metalaxyl (2.5 g/L) reduced *in vitro* growth of *Phytophthora nicotianae* by 92.9 %. However, this study has shown that at low concentration (0.1g/L), *Phytophthora nicotianae* growth was totally inhibited. This could probably be a result of limited or non-exposure of this fungal population to these fungicides.

Low inhibition of mycelia by Fosetyl Al at low concentration in this study is in agreement with Rohrbach and Schenke (1985) who reported that high *in vitro* inhibition of mycelial growth of *P. nicotianae* by Fosetyl Al can be achieved with concentrations between 0.3-1g/L. Similarly, Fenn

and Coffey (1984) in California reported that complete mycelial growth inhibition of *Phytophthora* species using Fosetyl Al requires the use of high concentrations.

In general, the result of this study has an implication on the management of PHRD in Uganda. Infact, this result shows that fungicides can be incorporated in integrated management of PHRD in Uganda.

CHAPTER FIVE

GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 General discussion

The identification of *Phytophthora nicotianae* as a principle cause of PHRD in Uganda has an implication towards the development of PHRD management strategies. Infact, diversity of pathogen populations has been reflected in the variation of macro and micro morphological features examined in this study. Several authors have reported that development of successful management strategies for a disease relies on the identity of the pathogen and extent of phenotypic variation within the causal *Phytophthora* pathogen (Mbaka *et al.*, 2010; Akrofi 2015) being well understood.

This study has also demonstrated that Metalaxyl is a promising chemical for the control of PHRD in Uganda. However, this needs to be incorporated into integrated management due to devastating effects of over using chemicals to the environment. For example, in India, integrated disease management of pineapple heart rot disease involving selection of healthy planting materials and use of 0.2% Metalaxyl and 0.1% Fosetyl Al reduced the effect of PHRD on pineapple (Shreenivasa *et al.*, 2015).

5.2 Conclusions

The study has shown that PHRD in Uganda is caused by *Phytophthora nicotianae*. The pathogen exhibited a great diversity indicated by variation in macro and micro morphological features.

The study has also shown that all the *Phytopthora nicotianae* isolates were sensitive to Metalaxyl and Victory 72 powder *in vitro* at 0.1g/L.

Metalaxyl was more effective at suppressing the growth of *Phytophthora nicotianae* isolates compared to Fosetyl Al *in vitro*.

5.3 Recommendations

Arising from objectives and conclusions of this study, the following are recommended.

i. Molecular characterization of isolates in this study is needed to confirm the true genetic identity of *Phytophthora nicotianae* isolates in order to validate these results.

ii. Studies on fungicide concentration need to conducted so as to determine the minimum effective concentration of Metalaxyl, victory 72 and Fosetyl Al for management of PHRD in Uganda.

iii. Field studies are required to determine the efficacy of metalaxyl and Victory 72 powder fungicides for the management of PHRD in Uganda.

iv. The results of this study be adopted and used in creating awareness on PHRD causal pathogen in Uganda.

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APPENDICES

Appendix 1: Frequency of Sporangia shapes and sporangia papillation for different isolates at NARL, 2016

	Sporangia shape presence and frequency (%)				
Isolate	**Ovoid**	**Limoniform**	**Obpyriform**	**Frequency**	**Sporangia papillation**
KAY 01	✓	✓	✓	100	Papillate
KAY 02	✓	✓	✓	100	Papillate
KAY 03	✓	✓	✓	100	Papillate
KAY 04	✓	✓	✓	100	Papillate
KAY 05	✓	✓	✓	100	Papillate
KAY 06	✓	✓	✓	100	Papillate
KAY 07	✓	✓	✓	100	Papillate
KAY 08	✓	✓	✓	100	Papillate
KAY 09	✓	✓	✓	100	Papillate
KAY 10	✓	✓	✓	100	Papillate
KAY 11	✓	✓	✓	100	Papillate
KAY 12	✓	✓	✓	100	Papillate
KAY 13	✓	✓	✓	100	Papillate
KAY 14	✓	✓	✓	100	Papillate
KAY 15	✓	✓	✓	100	Papillate
KAY 16	✓	✓	✓	100	Papillate
LUW 01	✓	✓	✓	100	Papillate
LUW 02	✓	✓	✓	100	Papillate
LUW 03	✓	✓	✓	100	Papillate
LUW 04	✓	✓	✓	100	Papillate
LUW 05	✓	✓	✓	100	Papillate
LUW 06	✓	✓	✓	100	Papillate
LUW 07	✓	✓	✓	100	Papillate
LUW 08	✓	✓	✓	100	Papillate
LUW 09	✓	✓	✓	100	Papillate
LUW 10	✓	✓	✓	100	Papillate
LUW 11	✓	✓	✓	100	Papillate
LUW 12	✓	✓	✓	100	Papillate
LUW 13	✓	✓	✓	100	Papillate
LUW 14	✓	✓	✓	100	Papillate
LUW 15	✓	✓	✓	100	Papillate
MAS 01	✓	✓	✓	100	Papillate
MAS 02	✓	✓	✓	100	Papillate
MAS 03	✓	✓	✓	100	Papillate
MUK 01	✓	✓	✓	100	Papillate
MUK 02	✓	✓	✓	100	Papillate
MUK 03	✓	✓	✓	100	Papillate
MUK 04	✓	✓	✓	100	Papillate

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KAY 03	✓	✓	✓	100	Papillate
KAY 04	✓	✓	✓	100	Papillate
KAY 05	✓	✓	✓	100	Papillate
KAY 06	✓	✓	✓	100	Papillate
KAY 07	✓	✓	✓	100	Papillate
KAY 08	✓	✓	✓	100	Papillate
KAY 09	✓	✓	✓	100	Papillate
KAY 10	✓	✓	✓	100	Papillate
KAY 11	✓	✓	✓	100	Papillate
KAY 12	✓	✓	✓	100	Papillate
KAY 13	✓	✓	✓	100	Papillate
KAY 14	✓	✓	✓	100	Papillate
KAY 15	✓	✓	✓	100	Papillate
KAY 16	✓	✓	✓	100	Papillate
LUW 01	✓	✓	✓	100	Papillate
LUW 02	✓	✓	✓	100	Papillate
LUW 03	✓	✓	✓	100	Papillate
LUW 04	✓	✓	✓	100	Papillate
LUW 05	✓	✓	✓	100	Papillate
LUW 06	✓	✓	✓	100	Papillate
LUW 07	✓	✓	✓	100	Papillate
LUW 08	✓	✓	✓	100	Papillate
LUW 09	✓	✓	✓	100	Papillate
LUW 10	✓	✓	✓	100	Papillate
LUW 11	✓	✓	✓	100	Papillate
LUW 12	✓	✓	✓	100	Papillate
LUW 13	✓	✓	✓	100	Papillate
LUW 14	✓	✓	✓	100	Papillate
LUW 15	✓	✓	✓	100	Papillate
MAS 01	✓	✓	✓	100	Papillate
MAS 02	✓	✓	✓	100	Papillate
MAS 03	✓	✓	✓	100	Papillate
MUK 01	✓	✓	✓	100	Papillate
MUK 02	✓	✓	✓	100	Papillate
MUK 03	✓	✓	✓	100	Papillate
MUK 04	✓	✓	✓	100	Papillate

Appendix 2: Summary of study activities. **A**) Collection of samples. **B**) Autoclaving of media. **C**) Isolation of PHRD pathogen. **D**) Incubation of cultures. **E**) Sorting of pure isolates. **F**) PHRD pure isolates. **G**) Pathogenic test. **H**) Examination of PHRD symptoms in screen house. **I**) Data collection.