MOLECULAR IDENTIFICATION AND PATHOGENICITY OF *PHYTOPHTHORA* *Species CAUSING* PINEAPPLE HEART ROT DISEASE IN UGANDA

BY

OKELLO CHARLES

B.VOC. AGRIC/ EDUC. (HONS.), KYU

REG. NO: 14/U/14249/GMCS/PE

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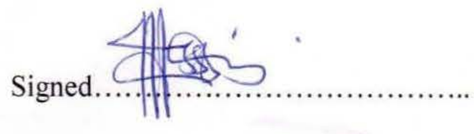
DECLARATION

I, Okello Charles hereby declare that this is my original work and has never been submitted either in whole or in part for any award in any university or institutions of higher learning.

Signed…………………………..

Date…11·12·2017…………………………

APPROVAL

This is to certify that this work was carried out under our supervision as university supervisors and is now ready for submission for examination.

Signed...

Associate Professor Bosco Bua

Department of Agriculture,

Kyambogo University

Date...11/12/17..............................

Signed...

Dr. Geoffrey Kawube

Department of Agriculture and Environment

Gulu University

Date...

DEDICATION

To my late parents, your love and passion for education and hard work for survival will always be cherished by those who benefited from your toils. Had the Creator granted you amnesty, you would have lived to see, witness and remember this moment! May your souls Rest In Peace. Amen

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ACRONYMS/ABBREVIATIONS

AMOVA	Analysis of molecular variance
BLAST	Basic local alignment search tool
DNA	Deoxyribonucleic acid
FAO	Food and agriculture organisation
IITA	International institute of tropical agriculture
ITS	Internally transcribed spacers
MEGA	Molecular evolutionary genetics analysis version 6
MUSCLE	Multiple Sequence Comparison by Log- Expectation
NaCRRI	National crops resources research institute
NARO	National agriculture research organisation
NGS	New generation sequencing.
PCR	Polymerase chain reaction
PHRD	Pineapple heart rots disease
rDNA	Ribosomal deoxyribonucleic acid
RFLP	Restricted fragment length polymorphism
SDW	Sterile distilled water
SLP	Single-locus probe
UNCTAD	United Nations conference on trade and development

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ABSTRACT.

Pineapple (*Ananas cosmosus* L.Merrill) has enormous potential for nutritional and health benefits, foreign exchange earnings, industrial growth and development in Uganda. The production of this crop is on the decline, because of outbreak of the pineapple heart rot disease. Therefore the objective of this study was to determine the molecular identity and pathogenicity of the *phtophthora* causal organism of pineapple heart rot disease. Four major pineapple growing districts in Central Uganda were surveyed for disease prevalence and samples collected for laboratory isolation and characterization between April-May 2016. Twenty one (21) samples of *phytophthora* heart rot pathogen were isolated from diseased pineapple tissues. Pathogenicity of the pineapple heart rot causal organisms was assessed on invitro pineapple leaves and green apple fruits in laboratory following koch's postulate. Internal transcribed spacer regions (ITSI, 5.8s ITSII rDNAs, from 11 isolates of *phytophthora* were analysed by polymerase chain reaction amplification and direct sequencing. The analysis of molecular variance (AMOVA) showed a significant genetic diversity within population (PhiPT= -0.068, P= 0.001). Intraspecific variability was detected. The genetic diversity of the population was measured by building phylogeneies based on these sequenced rDNA-ITS data using Muiltiple sequence alignment tool. The results of the pathogenicity test showed that 100% of the isolates were pathogenic apple fruits and healthy pineapple leaves with varying levels of virulence. Based on molecular identification, the sequence of amplified PCR products of DNA fragment of 850bp confirmed that the causal organism of pineapple heart rot disease was P.nicotianae. AMOVA indicated the variability within populations was (100%). These results also suggest that P.nicotianae has considerable evolutionary potential, which enable it to adapt to the new environment and overcome management strategies over time.

CHAPTER ONE: INTRODUCTION

1.1 Origin and distribution of pineapple

Pineapple (*Ananas comosus* L. Merrill) is native to southern Brazil and Paraguay especially the Parana-Paraguay river area where wild relatives occur. Pineapple was apparently domesticated by the Indians and carried through south and Central America long before the arrival of Europeans in the Indian sub continent (Purseglove, 1972; Bartholomew *et al.*, 2003). Pineapple was introduced to Philippines and later to Hawaii and Guam by the Spaniards early in the 16th Century (Morton, 1987). By 1820, American missionaries found pineapple growing wild and being cultivated in home gardens in Hawaii (Bartholomew *et al.*, 2012; Green and Scot, 2015). According to Purseglove (1972), pineapple was grown in China and South Africa in 1594 and 1655, respectively. Lutheran missionaries in Brisbane, Australia imported pineapple plants from India in 1838. The first plantings of pineapple in Israel were made in 1938 using materials from South Africa (Purseglove, 1972). Accordingly, pineapple is a crop of the Neotropical lowlands from Mesoamerica and the Antilles southward to Paraguay and the humid valleys along the Pacific coast of Peru (Clement *et al.*, 2010). However it is not clear when exactly when pineapple reached east Africa and Uganda in particular. Pineapple is a perennial herbaceous monocotyledonous crop of the family *Bromeliaceae* (UNCTAD, 2016). In fact, many members of Bromeliaceous are epiphytes living naturally on trees and rocks (Morton, 1987).

1.2 Production of pineapple

Pineapple is a crop of tropical and subtropical regions grown in a number of countries. According ly, the top ten pineapple producing countries in the world are, Thailand, Costa Rica, Brazil, Philippines, Indonesia, India, Nigeria, China, Mexico and Colombia (FAOSTAT,2013, Adegbite and Adeoye (2015),. The major Asian Pacific producers of pineapple are Thailand, India, Philippines, China, Vietnam and Malaysia accounting for 47%

of the global production (FAO, 2011, Rodríguez *et al.*, 2015). Mean while in Latin America and Africa, the main producers are Brazil, Costa Rica, Nigeria, Ivory Coast and Ghana respectively (Ole, 2008; UNCTAD, 2013) (Figure1).

In Uganda pineapple is mainly grown south of Lake Kyoga and western Uganda (Bua *et al* 2013), Jumba and Freyer (2016) indicate that pineapple is grown in the central districts of Kayunga Luwero, Masaka.and Kayunga, is the leading pineapple growing area in the country because of its fertile soils and cheap labour (Muyanja andTuryagyenda, 2006).

According to Bolwig (2012), bimodal rainfall and an altitude of about 1200m above sea level, is suitable for pineapple cultivation, Total production of pineapple in Uganda is estimated at 3,265 tonnes perannum (FAOSTAT, 2009 and Zziwa et al2017).

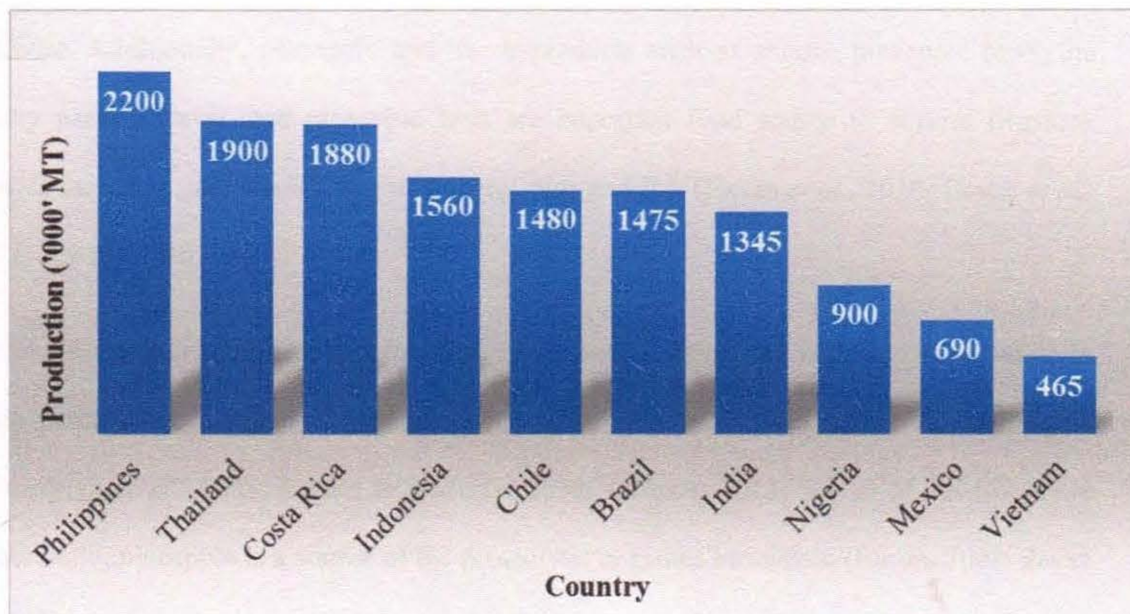

Figure 1: Major Pineapple producing countries in the world: Annual production in '000' metric tonnes.

Adopted from FAOSTAT (2013)

1.3 Importance of pineapple

Pineapple has emerged as one of the most lucrative commercial crops in central Uganda, where it contributes to increase in national revenue, household income, improved nutrition

and employment of the farming communities (Jumba and Freyer, 2016). Pineapple is an important horticultural tropical fruit grown in many countries in the tropics and sub-tropical regions. The fruit has a fine flavour and high nutritive value (Baruwa *et al.*, 2013; Joy *et al.*, 2016). Pineapple cultivation plays a vital role in the socio-economic development of many countries in the world. Pineapple production contributes greatly towards meeting food needs, employment opportunities and income of over 50 % of the farming communities especially the rural populace (Diao, 2010; WDI, 2011; Kongethavani *et al.*, 2015; Akhilomen *et al.*, 2015; Joy *et al.*, 2016).

According to Yapo *et al* (2011), pineapple provides an attractive flavour and refreshing sugar-acid balance contributing to its high demand as a dietary supplement to human nutrition. Additionally, pineapple and its byproducts such as shoots, pineapple baits, the thorny pandan leaves and pineapple bran are important food source to several livestock species including cattle, poultry, goats, sheep, pigs and fish (Hasan *et al.*, 2010; Heuze *et al.*, 2013; Joy *et al.*, 2016).

Pineapple fruits are used mainly for fresh home consumption and making juice, while in some parts of the world the fermented juice is used to make vinegar and alcoholic spirit (Pornsuriya *et al.*, 2008; Larsen and Marks, 2010; Baruwa, 2013; Joy *et al.*, 2016). More importantly, pineapple is a source of the proteolytic enzymes bromelain (Barun, 2008; Pavan *et al.*, 2012; Kanojiya *et al.*, 2013).

Green pineapple fruit is also used for making pickles (Joy *et al.*, 2016). Pineapple is also recommended as medical diet for certain diseased persons (Hasan *et al.*, 2010). Accordingly, pineapple has been identified as one of the horticultural crops with enormous potential for nutritional and health benefits, foreign exchange earnings, industrial growth and development (Fawole, 2008; Joy, 2010; Fakayode *et al.*, 2012; Adegbite and Adeoye, 2015)

1.4 Pineapple production

1.4.1 Ecological requirements

Pineapple is a tropical crop and grows in areas of average rainfall ranging from 1,200mm to 1,500mm per annum (Bartholomew, 2003). The crop thrives well in a temperature range of 25- 32^{0}C and a wide range of soil types, but well drained sandy loam with pH of 4.5-6 are preferred (Souza *et al.*, 1986; Py *et al.*, 1987;Malézieux and Bartholomew, 2003). According to Abbey (2005), the majority of pineapple plantations is rain fed and requires about 18 months to mature.

1.4.2 Constraints to pineappleproduction

Pineapple production is hindered by a number of production constraints including abiotic and biotic. Abiotic factors affecting pineapple production globally are sunburn or sunscald which affect fruits prior to harvest from fields (Morton, 1987). However, Donkoh and Agboka (1997) highlighted weeds, soil fertility, harvesting and post-harvest handling methods, and environmental degradation as the key constraints in pineapple production. Other constraints identified were credit and price fluctuations, freighting challenges, plus several infrastructural problems which confined production to areas near major transport routes like seaports, Insufficient land, and little access to credit, poor roads and lack of transport is common (Kayitesi, 2011)). Among the diseases (biotic) are Pineapple mealy bug wilt disease (PMWD) and Pineapple heart rot disease (PHRD) (NARO, 2012; Bua *et al.*, 2013). Pineapple heart rot disease is a recent outbreak in Uganda with limited documentation on pathogen identity and pathogenicity as well as economic impact.Yet, reports from other parts of the world indicate that disease can cause 100% yield loss (NARO, 2012). Heart rot can be caused by *Phytophthora nicotianae B. de Haan var. parasitica Das* frequently called *Phytophthora parasitica Dast*. However heart-rot symptoms are the same, regardless of the *Phytophthora*

species causing them (Green and Scot, 2015). The most widely distributed species *are P. cinnamomi* and *P. nicotianae B. de Haan var. parasitica.*

1.5 Symptomatology of Pineapple heart rot disease

Pineapple heart rot manifest as a syndrome. The colour of the leaves changes to yellow or light coppery brown. Later, the leaves wilt causing the leaf edges to roll under, turn brown and eventually die. The heart leaves can easily be pulled off the mother plant and produce offensive odour (NARO, 2012; Joy and Sindhu, 2012; Shen *et al.*, 2013). In fact plants of all ages are attacked, but three to four month old plants are most susceptible. Fruiting plants or suckers on ratoon plants as well may be affected.

As the disease progresses sufficiently, the infection moves up through the fruit peduncle and rots the fruit (Shen *et al.*, 2013; Rodriguez *et al.*, 2015). The lesion diameter progresses slowly from the base of the leaf to the tip (Shen*et al.*, 2013; Green and Scot, 2015; Rodriquez *et al.*, 2015).

1.6 Statement of the problem

Pineapple is a tropical and subtropical fruit grown in many countries ofthe world. In Uganda it is mainly grown south of Lake Kyoga and western Uganda (Freyer and Jumba,2016). However, the prevalence of diseases such as pineapple heart rot disease caused by *Phytophthora* severely constrains the production of pineapple for both small and largescale farmers in Uganda (Bua *et al* 201; Shen *et al.*, 2013) . Yield losses of up to 100% have been reported from Hawai (Rohrbach and Schenck, 1985). Currently the disease is on the increase but there is no information on the identity and pathogenicity of the causal pathogen in Uganda.

In fact, the outbreak, of PHRD is a threat to the pineapple industry which demands an intervention especially the identification of the causal organisms and management of the

disease. However, in Uganda, information on the causal organism is scanty and limited as well and no attempt has been made to identify and assess pathogenecity of the causal pathogen (Bua *et al.*, 2013).This leaves a gap to be addressed. According to (NARO, 2012) proper identification and understanding of the pathogenicity and genetic diversity of the causal pathogens provides context specific entry point for development of appropriate management options. Therefore, as the first step to understanding and developing effective management strategy against pineapple heart rot disease, identifying causal pathogen, assessing the pathogenicity and genetic diversity of the PHRD causal organisms is imperative

1.7Justification for the study

Pineapple is a tradable crop and generates reasonable income to the households. It is used as a fruit as well as for producing juice for consumption.The increasing demand for pineapple in the world requests for an intensification of the cultivation which rely on the availability of planting material

Recent surveys conducted in Mukono, Masaka, Kayunga and Luwero districts show that pineapple heart rot disease is wide spread and devastating (Bua *et al,* 2013). However.the annual economic impacts of PHRD are impossible to estimate because of the species diversity, versatility of host range and ecological niche (Moralejo *et al.*, 2009). Accordingly, unless properly managed, the disease can cause 100% yield loss (Rohrbach and Schenck, 1985). Yet pineapple producing communities derive livelihood from the sales of pineapple. For instance, youths are employed in the pineapple production industry, pineapple fruits form essential components of the dietary needs of the people (vitamin C), feeds for livestock and the exported fruits generates foreign exchange for the economy of Uganda (Freyer and Jumba,2016). While the contribution of pineapple to the economy of Uganda and the welbeing of the general population is apparent, the alarming rate of spread of pineapple heart rot disease in central Uganda is a threat to food security, livelihoods and loss of pineapple

biodiversity (NARO,2012). Accordingly, there is need to identify, characterize and determine the pathogenicity of the causal pathogen of Pineapple heart rot disease through Morphological and Molecular means. However, morphological identification is unreliable, hectic, time consuming, plastic and sometimes causes misidentification (Hüberli *et al.* 2001; Eggers *et al.*, 2012). Accordingly, molecular characterisation remains the only viable option to identify and determine the genetic diversity of PHRD causal pathogen.

1.8 Significance of the study

While no *Phytophthora* species has been conclusively proven a serious pathogen on the pineapple in Uganda, the findings would provide an understanding of the molecular identity and pathogenicity of the causal organisms in the country. It is also envisaged that the information and knowledge generated from this study will help the researchers to devise strategies to manage and reduce the loss of revenue and income inflicted by pineapple heart rot disease in Uganda.

The information generated from this study will also serve as a baseline reference to the scientists, academicians, extension workers and farmers. The findings will also provide justified reasons for intervention through proper resource allocation by policy makers.

1.9 General objective of the study

Identify causal pathogen of Pinneaple heart rot disease in Uganda.

1.10 Specific objectives

i. .To identify the causal organisms of PHRD using molecular means.

ii To ascertain the pathogenecity of *phytophthora,* the causal pathogen of PHRD in Uganda

1.11 Hypotheses

i Fungal isolates obtained from leaves showing PHRD disease symptoms are able to cause PHRD *in vitro*

ii. There exists genetic diversity among *Phytophthora* spp. causing PHRD in Uganda.

CHAPTER TWO: LITERATURE REVIEW

2.1Impacts of pineapple heart rot disease

Available literature indicates that PHRD is caused by *Phytophthora* species. Accordingly, *Phytophthora* infection reduces both the biological diversity of plant species, availability of food and protective cover for birds and small mammals (Cahill *et al.*, 2008). The most significant crop losses are caused by *Phytophthora* species (Ploetz., 2013; Nagel *et al.*, 2013) Globally, evidences indicate that the annual economic impacts of *Phytophthora* are impossible to estimate because of the species diversity, versatility of host range and ecological niche (Moralejo *et al.*, 2009). According to Nagel *et al.* (2013) and Panabieres (2016), the real economic impacts of PHRD is the integrated productivity loss, the costs of disease management and the eventual penalties for growing alternate crops of less value during rotation periods among others. In fact, *Phytophthora* diseases cause significant economic losses to agricultural systems on an annual basis amounting10–16% of the global harvest (Chakraborty and Newton, 2011). Accordingly, pineapple heart rot disease has immense impact on pineapple production emanating from a reduction in plant densities due to plant mortality (Marrero *et al.*, 2010). The disease lowers production, increases the risk of crop failure, threatens food security and reduces the profitability of pineapple production (Drenth and Guest, 2004).

Accordingly, *Phytophthora* causes severe diseases of many economically important crops including pineapple worldwide (Cooke *et al.*, 2000; Schena and Cook 2006; Zeng *et al.*, 2009; Spring and Thines, 2010, Das *et al.*, 2011; Wetzel, 2011). In fact, all *Phytophthora* species have the potential to cause destruction of natural ecosystems, particularly when different species are supported by conducive conditions for disease development (Jung *et al.*, 2005, Scott *et al.*, 2013, Dunstan *et al.*, 2016)

2.2 Aetiology and epidemiology of PHRD

Pineapple heart rot disease is reportedly caused by several species of the plant pathogenic water molds belonging to the class Oomycetes (Aghighi *et al*., 2012; Schreier, 2013; Green and Scot; 2015; Dunstan *et al*., 2015). Dick (2001) stated that *Phytophthora* genus in the kingdom Straminipila represents an important group of plant pathogens affecting many important crops around the world. According to Green and Scot (2015), pineapple heart rot disease affects the basal leaf tissues and can cause fruit rot as well. Reports from elsewhere showed the causal pathogens of PHRD as *P. nicotianae and P. cinnamomi.* However, *cinnamomi* is prevalent in the cool, wet soil at upper elevations whereas *P. nicotianae* is found in a wider range of soil conditions (Rohrbach and Apt 1986; Jung *et al.,* 2013). According to Green and Scot (2015), the infection process of these pathogens depends on variables such as topography, drainage, rainfall, and soil pH. The hyphae of germinating Chlamydospores infect roots or young leaves and stem tissues. Besides, the fruiting bodies called sporangia develop from the mycelium and produce zoospores. The zoospores penetrate the plant through the root and the stem where it causes decoloration of the leaves (Li *et al*., 2012). Exudates increase rate of movement towards the roots of the pineapple plant (Green and Scot, 2015). Accordingly, during overwintering, zoospores and oospores can be dispersed by surface water, irrigation water and air currents. However, *Phytophthora* species produces a large number of chlamydospores, which can persist in symptomless plants, debris and soil for a number of years (Zentmyer and Mircetich, 1966; Meng and Wang, 2008).

2.3 Classification of *Phytophthora* Species

According to Hynniewta *et al*., (2014), *Phytophthora* classification is ambiguous and highly controversial. Different taxonomists have recognized 60 to 120 species in the genus *Phytophthora* (Erwin and Ribeiro, 1996; Martin and Trooley, 2003). In fact most of the

confusion is due to free hybridization of different species and occurrences of intermediate forms.

Historically, a number of morphological and physiological criteria including sporangial structure, antheridial form, host specificity and breeding system (homothallic or heterothallic) has been used to classify members of *Phytophthora* genus (Martin and Trooley, 2003,2004 ; Martin *et al.*, 2014). However, there is growing evidence that molecular and ecological studies have become apparent tool for taxonomic groupings of disease causal organisms. According to van de Peer *et al.* (1996), the congruence between taxonomic inference derived from molecular data and that based on classical morphological taxonomy is a topic of interest in current studies.

Phytophthora resembles fungi in its morphology, since it has thread like mycelium (Stewart, 2011). However, *Phytophthora* and all other Oomycetes differ from the true fungi in many physiological traits that separate them into different kingdoms. As a group, Oomycetes are associated with aquatic habitat; their cell wall is composed of glucan and cellulose instead of chitin in fungi. Their mycelium is coenocytic, having no-septa or divisions as in true fungi. Also, their vegetative stage is diploid as opposed to haploid in true fungi (Drenth and Sendall, 2001).

Currently, the *Phytophthora* genus is assigned to the Order Peronosporales and Phylum Oomycota within the group of heterokont, biflagellate organisms that comprise the kingdom Chromista (Cook *et al.*, 2000; Niklaus, 2012). *Phytophthora* is a diverse fungal species causing disease on a broad range of both temperate and tropical crops (Farhana *et al.*, 2013). According to Martin and Tooley (2004), *Phytophthora* is a complex genus within the Oomycetes containing approximately 67 described species that occupy a variety of terrestrial ecological habitats. Similarly, Panabieres *et al* (2016) noted that the genus *Phytophthora* was

considered to comprise approximately 60 species until the end of the last century. This number has doubled in the last 15 years, reaching approximately 120 described species (Martin *et al.*, 2014). Hansen *et al.* (2012) associated this blossom to changes in species concepts, the refinement of identification tools and subsequent resolution of species complexes, development of largescale surveys of natural ecosystems and new habitats, reports on new diseases and identification of new species arising from interspecific hybridization events.

2.4 Characterization of *Phytophthora* Species. causing PHRD

2.4.1 Molecular identification of *Phytophthora* pathogen causing heart rot in Uganda

2.4.1.1 Molecular characterising techniques

A higher number of new species and taxa of *phytophthora* were described during the last 15 years (around 56 species by 1999 (Erwin and Ribeiro, 1996; Jung *et al.*, 1999). Over 120 species described by 2014 (Brassier. 2009; Kroon *et al.*, 2012; Milenkovic*et al.*, 2014). However the characteristics of some of the newly described species are not included into previously listed keys and are not following the natural classification of the species within this genus (Cooke *et al*, 2000). Accordingly, molecular studies seem to offer very important criteria in final identification of the species in natural phylogeny (Cooke *et al.*, 2000; Kroon *et al.*, 2004).

Phytophthora belong to the Oomycete plant pathogens with several unique and identifying characteristics. Besides, the morphological approaches, other methods have been used to simplify and improve the accuracy of identification of isolates to a species level including use of protein patterns (Bielenin *et al.*, 1988), isozymes (Oudemans, 1994), serology (Oudemans and Coffey, 1991), restriction fragment length polymorphism (RFLP) (Liu *et al.*, 1997), analysis of nuclear and mitochondrial DNA (Moslemi and Darin, 2007) single strand conformational polymorphism (SSCP) (Bush *et al.*, 2006) and analysis of the internal

transcribed spacer (ITS) region of the ribosomal DNA (rDNA)(Salati *et al.*, 2012;Schoch *et al.*, 2012). Relatedly, the improved efficiency and accuracy of the diagnostic methods used to distinguish different *Phytophthora* species, serological methods and a range of molecular techniques have been developed (Liu *et al.*, 1997; Causin *et al.*, 2005; Panabières *et al.*, 2016). In fact, among these techniques, PCR based diagnostics have assumed a dominant role for some species of *Phytophthora* in recent years (Bonants *et al.*, 1997; Dobrowolski, 1998; Causin *et al.*, 2005; Das *et al.*, 2011). Accordingly, the use of PCR, nested PCR and DNA sequence analysis of internal transcribed spacer (ITS) regions has become routine for the detection, identification, classification and phylogenetic analysis of many fungi at the species level (Taylor *et al.*, 2000; Bowers *et al.*, 2007, Farhana *et al.*, 2013).

Kong *et al.*, (2003) cited that several PCR protocols have been reported for detecting *Phytophthora* species. According to Ippolito *et al.*, (2002), the majority of these protocols use primers derived from the internal transcribed spacers (ITS). Nevertheless, technical limitations related to post amplification procedures (gel electrophoresis and ethidium bromide staining) and cross contamination still limit large scale applications of PCR for plant pathogen diagnosis (Ippolito *et al.*, 2004).

However, species-specific probes and primers have proved useful in *Phytophthora* disease diagnosis because of the high levels of sensitivity and specificity for accurate identification (Kong *et al.*, 2003). Accordingly, some DNA-based methods are advantageous because pathogen isolation is not required and polymerase chain reaction (PCR) amplification can be performed directly from DNA extracted from infected tissue (Luck and Gillings, 1995; Paplomatas, 2006). According to (Meng and Wang (2000), DNA-based techniques have become an effective means of identifying plant pathogens by assessing levels of genetic diversity in targeted *Phytophthora* species. Additionally, rDNA sequence information from representative strains of each of the species are used to reveal evolutionary relationships

among the different biological species of *Phytophthora* (Drenth *et al.*, 2006; Meng and Wang, 2009). DNA sequence data obtained in phylogenetic studies have also been used to differentiate *Phytophthora* species. According to Cooke *et al.*, (2000), specific regions that have been examined include the large and small subunits of the ribosomal RNA (rRNA) and the ITS regions of the rDNA. In fact, the population genetic studies based increasingly on molecular methods have improved ability to identify fungal species and determine their natural relationships. With recent methodological advances, molecular markers are increasingly used for semi-quantitative analyses of fungal communities (Ihrmark *et al.*, 2012).

2.4.1.2 Genomic DNA extraction and sequencing of Phytophthora isolates

Scientists such as Moslem *et al.* (2010) maintain that there is rapid and efficient method of molecular identification involving DNA extraction, PCR amplification and sequencing. According to Pitkäranta *et al.* (2008), good-quality DNA is required to perform conventional polymerase chain reaction (PCR) for phylogenic analyses with specific primers. However, fungal polyphenols can decrease the yield and purity of extracted DNA making DNA viscous, glue-like and non-amplifiable in PCR by inhibiting Taq polymerase enzyme activity as well as interfering with accurate DNA digestion (Alatar *et al.*, 2012). In fact, a good isolation protocol should be simple, rapid and efficient, yielding appreciable amounts of high-quality DNA suitable for molecular analysis (Križman *et al.*, 2006).

Different methods for DNA extraction have effectively been applied for many plant species. In addition, there are several commercial DNA isolation kits, but their high cost per sample makes it economically difficult for large scale genomic applications (Ahmed *et al.*, 2009). DNA is treated with DNasefree ribonuclease A, since large amounts of RNA in the sample

can chelate Mg2+ and reduce yield in PCR. It also involves successive longterm RNAse treatment with all steps carried out at room temperature.

RNAse treatment degrades RNA into small ribonucleotides that do not contaminate the DNA preparation and yields RNAfree pure DNA (Jena *et al*., 2010). Moreso, RNA contamination is removed by dissolving nucleic acids in the presence of RNase (Matasyoh *et al*., 2008; Muge *et al*., 2009). In light of new discoveries, analysis of microbial communities based on amplification and sequencing of genetic markers has revolutionized fungal ecology (Hibbett *et al*., 2009). Molecular identification through DNA barcoding of fungi has become an integrated and essential part of fungal ecology research and has provided new insights into the diversity and ecology of many different groups of fungi (Bellemain *et al*., 2010).

Additionally, in mycology study, sequences from the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA are commonly used for the identification of fungi (Naumann *et al*., 2007; Nilsson *et al*., 2008). According to Begerow *et al*. (2010) and Ihrmark *et al*. (2012), the ITS regions have high evolutionary rates and are flanked by highly conserved regions with suitable target sites for universal primers. The ITS2 region contains less genetic information than the entire ITS. Accordingly (Ihrmark *et al*., (2012) and El-Elimat *et al*.,(2013), observed that shorter amplicons lead to higher PCR efficiencies, meaning that fewer cycles are needed to obtain the desired product concentration and ensure successful amplifications is not hampered by scarcity of template or high inhibitor concentrations. Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Conrad *et al*., 2012). Interestingly internal transcribed spacer (ITS) regions located between the 18S and 28S rRNA genes have emerged as the most common target for molecular based identification (Crane, and Burgess,2013). Although the sequences from the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA are commonly used for the identification of

fungi, the ITS sequences in *Phytophthora* species may not be the best source for development of species-specific PCR primers because the interspecific differences in this part of the sequence are too small (Ryeberg *et al.*, 2008) . However, the same authors suggested that the use of RAPD-derived fragments could be a valid alternative. Surprisingly, it was found that attempts to overcome the problems of specificity caused by small differences in the ITS regions, did not give satisfactory results (Gazis *et al.*, 2011; Blaalid *et al.*, 2013)

Nonetheless, there are many sequences from environmental samples whose species affiliation remains unknown because they cannot be satisfactorily matched to a sequence of known taxonomic identity (Bastias *et al.*, 2006). Moreover, several molecular approaches now provide powerful adjuncts to the culturedependent techniques. These approaches in particular are PCR and rRNA based phylogeny, the identification of uncultured organism's techniques dependent on DNA melting behaviour or singlestrand rDNA conformation (Bush *et al.*, 2006).DNAbased molecular diagnostics now offers a valuable tool to assist in the identification of a number of plant pathogens including *Phytophthora* species (Li *et al.*, 2012). In addition, DNAbased molecular diagnostics help to trace genetic linkages between species and isolates from different geographical origins. This diagnostic method uses a fragment of ribosomal DNA, termed the internal transcribed spacer (ITS) region (Cook, 2000a). These primers amplify the entire ITS region (Kendall and Paul, 2005).

2.4.1.3 Markers for identifying *Phytophthora* species

DNA markers have now become a popular means for the identification of plant, animal and microbial species. This technology has been widely used in the identification of germplasm resources and determination of genetic diversity in populations of *Phytophthora* pathogens (Zhang *et al.*, 2007; Li *et al.*, 2012).

In the last decade, molecular markers have been widely used to study the genetic diversity of different *Phytophthora* species (Ochwo *et al*., 2002; Yang *et al*., 2008; Li *et al*., 2012). Sequence polymorphisms include insertions and deletions of base pairs or sections of DNA, as well as substitutions in nucleotide sequences such as single nucleotide polymorphisms SNPs (Paplomatas, 2006). Accordingly molecular markers should be selectively neutral, that is they should not be under selective pressures so that they do not reflect different local adaptations to natural or human selection (Ihrmark, *et al*. 2012).

Fungi can therefore be identified at the species level by primers designed on selected conserved sequences like the rRNA gene cluster followed by further characterization of the amplified fragments (Paplomatas, 2006). . RNA genes also occur in multiple copies with up to 200 copies per haploid genome arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes (Horton and Brans, 2001).

According to Erselius *et al*., (1998), *Phytophthora* isolates are identified by genotypic markers using restricted fragment length polymorphism (RFLP) fingerprints for moderately repetitive probe. . However, the choice of a technique to use usually depends on the research question being addressed (Pereira *et al*., 2008). For instance, when the interest is detecting the presence or absence of a specific organism in a sample, conventional polymerase chain reaction PCR is used with specific oligonucleotide probes (primers) to detect the organism in question (White *et al*., 1990). Infact, the PCR based identification have also been widely used in the identification of other phytopathogens (Aboshosha *et al*., 2007; Mwang'ombe *et al*., 2008; Zitnick-Anderson and Nelson, 2015; Lakshman *et al*., 2016). Sanger sequencing is another common molecular method that is usually employed when a deeper analysis of DNA is required (Pereira *et al*., 2008). Accordingly, Sanger sequencing has been an important tool

in the study of pathogen populations and prevalence as well as the advancement of the molecular species concept (Sutton *et al.*, 2006; Bardgett, 2010; Jana *et al.*, 2005).Sanger sequencing of DNA enables analysis of DNA for several projects such as identification of homologous genes across species or identification of mutations or merely identification of individual species for determination of relationships among organisms. Identification of species in Sanger sequencing is achieved by performing a basic local alignment tool (BLAST) search of the sequence in NCBI database (Jamal *et al.* 2007). However, for fungi, universal primers such as those targeting the ITS region of rRNA are used (White *et al.*, 1990).

2.4.1.4 Genetic diversity of pineapple heart rot causal pathogens

Contemporary analytical tools for the analysis of population genetic variation are powerful resources to infer the evolutionary history of populations of pathogens (Grünwald and Goss, 2011; Grünwald, 2012). Accordingly, understanding of the evolutionary history of pathogens can, in turn inform management of emerging epidemics (Grünwald, 2012). According to Liu *et al.* (1997), species richness (number of species within a community) and species evenness (the sizes of species populations within a community) are the two essential parameters for defining community structure and diversity. However, interest in sequenced-based analysis of environmental samples ('environmental barcoding') has increased in the past decade as it allows to study abundance and species richness of fungi at a high rate and more reliably than conventional biotic surveys (Bellemain *et al.*, 2010).

According to Ryberg *et al.*, (2008), the possibilities of using molecular methods have therefore facilitated a deeper understanding of fungal biology. The internal transcribed spacer (ITS) of nuclear DNA (rDNA) is the preferred DNA barcoding marker for the identification of single taxa and mixed environmental templates ('environmental DNA barcoding) (Bellemain *et al.*, 2010). Accordingly, Choi *et al.* (2006) observed that internal transcribed

spacers (ITS) sequence analysis of ribosomal DNA (rDNA) and mitochondrial encoded cytochrome c oxidase 2 (COX-II) genes have increasing importance in evaluating the taxonomic and phylogenetic relationships of species with different degrees of intra-specific diversity. The genetics of *Phytophthora* species historically have been understudied and yet genome size among members of the genus varies greatly. The genetic diversity of this population is measured by sequencing DNA for several loci and building phylogenies based on these data (Schreier, 2013). Naturally, because of this great volume of data, scientists would like to establish DNA homologies by applying one or more of the highly innovative alignment methods available today in an automated high-throughput fashion (Covert *et al.*, 2004).

The advent of the next-generation sequencing (NGS) technologies has helped in understanding of comparative evolution and simple relationship between taxa at varying levels of classification (Dodsworth *et al.*, 2014). Whereas, Bybee *et al.*, (2011), say approaches of NGS include amplicon sequencing using barcoded primer, other authors say NGS approach bases on phylogenomics of the full complement of protein-coding genes (Zhou *et al.*, 2012). According to Das *et al.*, (2011), the rRNA genes are highly conserved and are suitable for determining relationships among distantly related organisms. However, understanding the trends in genetic diversity of *Phytophthora* is used as hypothetical mechanisms of evolution and speciation. Genotyping techniques are allowed to track the dissemination of clonal lineages from source points (Hulvey, 2010).In fact, a pathogen with high genetic variation has a high evolutionary potential and is more likely to quickly adapt to new conditions such as a resistant host or changing environment. However, the rise of new races seems to be too rapid to be accounted for by chance mutation, gene flow, recombination and subsequent natural reselection (Stewart, 2011).

Several primers have been developed for species of *Phytophthora* that amplify both nuclear and mitochondrial genes. The determining loci are the internal transcribed spacer (ITS) region. This locus encodes non-structural RNA that is degraded during ribosome assembly (Ristaino *et al.,* 1998). Consequently, there is relatively little evolutionary pressure acting on this locus (Baldwin, 1992). Low evolutionary pressure yields higher rates of mutation and more inter- and intraspecific variation, making the ITS region a phylogenetic determinant (Suh *et al*., 1993).The locus is used to derive phylogenetic relationships in the genus *Phytophthora* and as a determinant for species identification (Cooke *et al*., 2000; Kroon *et al*., 2004;Blair *et al*., 2008). The ITS sequence can be used to measure intraspecific variability (Maeder *et al*., 2010; Pandey and Ali, 2012).

2.4.2 Pathogenicity of *Phytophthora Species*

In the recent decades, a large body of evidence has been gathered on the increased variability in reaction and pathogenicity of *Phytophthora* species backed by emergence of new populations in a number of geographical areas owing presumably to pathogen migration (Fry 2008; Michalska *et al.,* 2016). It has also been suggested that there are numerous physiological races for each phase of the disease and each growing region seems to have its unique population of physiological races (Adipala and Wandera, 2001). An important aspect of the noted variation is the variability in pathogenicity toward the host. Quantitative assessments of the fitness parameters of pathogens have been conducted mostly with detached-leaf or leaflet assays (Goodwin *et al.* 1994; Świeżyński *et al*., 2000).

According to Drenth and Sendal (2001), almost all species within the genus *Phytophthora* are formidable plant pathogens. A number of studies which indicate variability in pathogenicity among isolates of *Phytophthora* have been conducted (Zentmyer, 1980, Kogeethavani *et al*., 2015). According to Rodriguez *et al.* (2010), the majority of *P. nicotianae* isolates are pathogenic to pineapple plants.

When pathogenic species of PHRD is tested against a healthy pineapple plant, symptoms developed include wilting, leaf browning, easily pulled heart leaves and offensive smell (Stewart and Carrison1992). Plant pathologists have devoted considerable attention to the evolution of pathogenicity, while the evolution of virulence has generally been the subject of animal pathologists and evolutionary biologists. Virulence is fundamental to understanding the life history of pathogens (Lenski and May, 1994). Evolution of virulence in plant pathogens is still poorly understood but the knowledge is important for the effective use on plant resistance and sustainable disease management (Jiao *et al*., 2016)

2.4.2.1 Virulence of *Phytophthora spp.*

The amount of disease induced by a pathogenic strain on a susceptible host is a key component of pathogen fitness (Cook *et al*., 2012). Such adaptive traits (pathogen fitness) contribute to the epidemiological success of this pathogen and closely correlate with spore production and infection frequency. Lesion growth rate and the period from inoculation to sporulation (incubation/latent period) are important components of aggressiveness Cooke *et al.* (2012). Aggressiveness is a key adaptive trait expressed by the ability of sporangia or zoospores to infect and colonise host tissue. When combined with efficient dissemination, survival from season to season (fitness), aggressiveness determines the success of particular pathogens (Liu *et al*., 2016).

2.5 Literature summary

Pineapple heart rot disease is reportedly caused by genus P*hytophthora* belonging to the class Oomycetes (Green and Scot; 2015; Dunstan *et al*., 2015), kingdom Straminipila, Dick (2001). However evidence elsewhere, indicates that the disease is caused by *Phytophthora* species including *P.nicotianae* and *P.cinnamomi* (Joy and Sindhu, 2012; Shen *et al*., 2013; Rodriguez *et al*., 2015). The infection process of these pathogens depends on variables such as topography, drainage, rainfall, and soil pH (Green and Scot; 2015). According to Joy and

Sindhu, (2012) the primary mechanisms of spread of *Phtophthora* are through infected suckers, soil and water. When pathogenic species of pineapple heart rot PHR) pathogen is tested against a healthy pineapple plant, symptoms developed include wilting, leaf browning, easily pulled heart leaves and offensive smell (Stewart and Carrison 1992). Accordingly,pineapple heart rot disease has immense impact on pineapple production emanating from a reduction in plant densities due to plant mortality (Marrero *et al.*, 2010).On a negative note *Phytophthora* heart rot infection reduces both the biological diversity of plant species, availability of food and protective cover for birds and small mammals (Cahill *et al.*, 2008). Although, reported in some parts of the world, the identity, genetic diversity and pathogenicity of pineapple heart rot disease causal pathogen is still not well understood in Uganda. Molecular technique targeting the internal transcribed spacers (ITS) region using ITS primers has been adopted for identification of pathogen. The virulence or amount of disease induced by a pathogenic strain on a susceptible host is a key component of pathogen fitness (Cook *et al.*, 2012). Such adaptive traits (pathogen fitness) contribute to the epidemiological success of this pathogen. Also lesion growth rate and the period from inoculation to sporulation (incubation/latent period) are important components of aggressiveness which is akey adaptive trait expressed by the ability of sporangia or zoospores to infect and colonise host tissue.Cooke *et al.* (2012). Aggressiveness determines the success of particular pathogens (Liu *et al.*, 2016).

CHAPTER THREE: MOLECULAR IDENTIFICATION OF *PHYTOPHTHORA* CAUSING PINEAPPLE HEART ROT DISEASE (PHRD) IN UGANDA

3.1 Introduction

Pineapple heart rot disease is one of the most common diseases of pineapple caused by the *Phytophthora* species (Erwin and Ribeiro, 1996; Drenth *et al*., 2006; Abad *et al*., 2011; Sadeghy *et al* 2014). Previously, the identification of many plant pathogens including *Phytophthora* species was based on morphologcal characteristics (Drenth *et al*., (2006). However, morphological identification may be inefficient and time wasting due to emergence of numerous species of *phytophthora* and variability in pathogen population (Grünwald and Hoheisel, 2006). Thus, molecular identification is the only effective tool that can classify *phytophthora* to species level with minimal error of misidentification caused by overlaps in pathogen characteristics. In addition, knowledge of variability in the pathogen population is also important for plant breeding and the resulting improvement programes for disease management (Grünwald and Hoheisel, 2006).

Therefore the objective of this study was to identify and determine the genetic diversity of the of pineapple heart rot disease causal pathogen.

3.2. Materials and methods

3.2.1 Collection of plant samples for isolation of *Phytophthora* Species

Both symptomatic and asymptomatic samples (184) were collected from 92 farmer fields in the districts of Mukono, Kayunga, Luwero and Masaka all in central Uganda. Twenty six (46) samples were picked per district. Samples were collected between April and May 2016. The distance between each fields was approximately 3Km. Three leaf samples were collected from symptomatic and asymptomatic pineapple plants in each farm by cutting with sterilized scissors. The scissors were sterilized with 20% Jik® (sodium hypochlorite) after each plant to avoid cross contamination between samples and disease transmission between plants and

fields (Ippolito,*et al.*, 2004). All collected samples were placed in sampling bags, labeled and transported in cooler boxes to the laboratory at Kyambogo University. On arrival, the samples were stored at 4°C awaiting further processing.

3.2.2 Media preparation

Different culture media were prepared for isolation of pineapple heart rot disease causal pathogens.

3.2.2.1 Corn meal agar (CMA)

Corn meal agar media was prepared by dissolving 17g of the CMA powder (Sigma–AldrichChemieGmbH. Steinheim, Germany) in one litre of distilled water and autoclaved at 121°C and 15 psi for 15minutes and cooled to about 60°C before amending with 10mg/L pimaricin, 200mg/L ampicillin, 10mg/L rifampicin, 25mg/L pentachloronitrobenzene (PCNB), 10mg/L benomyl and 50mg/L hymexazol (PARBPH) as described by Kannwischer and Mitchell (1978). The medium was dispensed into 90mm petri plates (Corning, France).

3.2.2.2 Potato dextrose agar (PDA) media

Thirty nine (39) grams of potato dextrose agar (PDA) (DifcoLaboratories, Detroit, USA) was dissolved in one litre of distilled water, autoclaved and amended as described above. The medium was dispensed into 90mm Petri plates (Corning, France).

3.2.2.3 Preparation of V8 broth

The V8 vegetable juice (CampbellSoup Co., Camden, NJ, USA) in aliquots of 50mL in falcon tubes (FisherScientific, Pittsburgh, PA, USA) was centrifuged at 13000 rpm for 5 min and 200mL of the supernatant mixed in 800 mL of distilled water and 3 g of CaCO3 were added. The broth was autoclaved at121°C and 15 psi for 15minutes and allowed to cool. The broth was sealed with parafilm and stored at 4°C till use (Jeffers, 2006).

3.2.3 Isolation and culturing of pineapple heart rot pathogens

All procedures were conducted under aseptic conditions under laminar flow hood. The leaf tissues were washed gently under running tap water to remove any adhering soil particles and thereafter surface sterilized by immersing in 70% v/v ethanol for 30 seconds and the excess ethanol was removed by immersing in sterile distilled water. Five leaf sections of about 0.5cm were excised from each sample using a sterile scalpel blade and subsequently immersed in sterile distilled water for 20 seconds to remove the excess ethanol and blot dried on sterile paper towels. Each leaf section was then plated onto a freshly prepared CMA media. All the petri plates were sealed with parafilm (Pechiney Plastic Packaging, Chicago, IL, USA) and incubated in the dark at 25°C for 72 hours (Mounde *et al.*, 2012)(Figure 5).

Pure cultures of PHRD causal organism was obtained by sub-culturing the hyphal tips of actively growing pathogens onto freshly prepared CMA media (He *et al.*, 2016). Plates were sealed and incubated as described above. To trigger rapid radial growth and vigor of the different *Phytophthora* isolates, a 5mm mycelial plug of each pureculture of each isolate growing on CMA medium was transferred onto freshly prepared PDA media; plates were sealed and incubated as described above (Gallegly and Hong,2008).

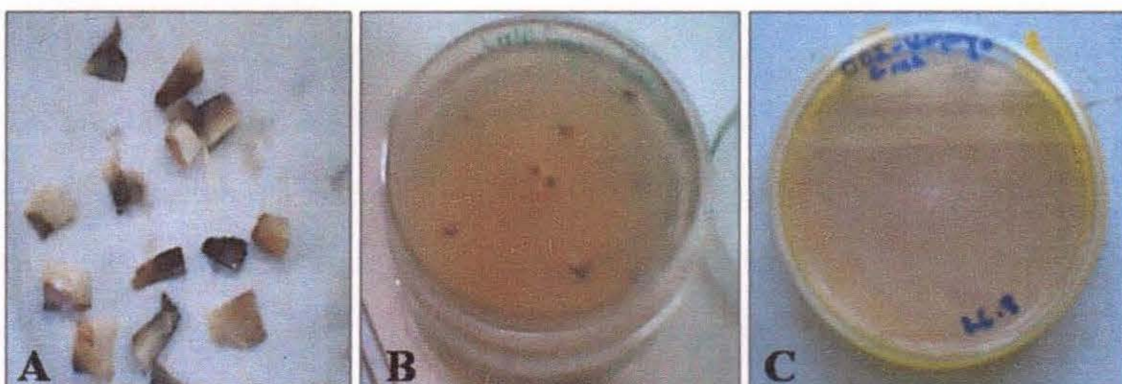

Figure 2: Isolation of pineapple heart rot disease causal pathogens: A) Leaf fragments from symptomatic and asymptomatic samples before plating onto corn meal agar. B) Leaf fragments in the corn meal agar and pure culture of PHRD causal pathogen growing on corn meal agar(C).

3.2.4 Sample selection

Eleven (11) out of 21 isolates of PHRD pathogens recovered from field samples used in this study were selected on the basis of their virulence. The isolates were group into four depending on the district of origin (Table 6 chap 4).

3.2.5. Molecular identification

From the selected isolates small pieces from the edges of young colonies grown on potatoe dextrose agar were transferred to an autoclaved liquid V8 media (800 ml/l of distilled water, 200 ml/l of V8 juice (Tymbark, Poland), 3 g/l CaCO3), and incubated at 22-25° C in the dark (Jeffers, 2006). After 3-5 days of incubation, or until mycelium filled approximately the half-filled 200ml Erlenmeyer flasks, fresh mycelia from each plate was collected, by vacuum filtration, washed in sterile distilled water and dried out on sterile filter paper.

Mycelia from each isolate were smashed in liquid nitrogen and rDNA was extracted as described by Bonants*et al.*,(2001).

3.2.5.1 .Genomic DNA extraction from *Phytophthora* isolates

Genomic DNA of each isolate was extracted from 150mg mycelium following the protocol used by Mahuku (2004) with a slight modification. Pineapple heart rot pathogens isolated from symptomatic leaf tissues were transferred from PDA plates into V8 broth in 200mL Erlenmeyer flasks (Fisher Scientific Co., Pittsburgh, PA). Flasks were plugged with sterile cotton wool, sealed with parafilm and incubated for 7 days in the dark at 27 °C in black plastic tubes (Rubbermaid, Wooster, OH, USA) after which mycelial mats were collected by vacuum filtration. The collected mycelia were then rinsed with distilled water to remove residual growth medium. Dry mycelium mats were transferred to 1.5mL screw-top micro centrifuge tubes. One hundred and fifty milligrams of fresh mycelial mat were weighed and manually macerated with a micropestle in 600μL of pre- warmed (55 °C) TES extraction buffer (0.2M Tris-HCl, pH 8; 10mM EDTA, pH 8; 0.5M NaCl; 1% w/v SDS) and acid-

washed sterilized sea sand. The macerated sample was vortexed for 30 seconds and an additional 400 µL of TES extraction buffer containing proteinase K (final concentration of 50 µg/mL) was added and vortexed for 30 seconds to thoroughly mix. After incubating the tubes on a water bath at 65°C for 15 minutes, 0.5 vol. (250 µL) of 7.5M ammonium acetate was added, gently mixed and left to stand for 10 minutes at room temperature. The tubes were centrifuged at 13,000 rpm for 15 minutes and the supernatant transferred to new tube. The DNA was precipitated by gently mixing the supernatant with an equal volume (600µL) of ice-cold isopropanol and incubating in a refrigerator at −20°C overnight. The tubes were centrifuged at 13,000 rpm at 4°C for 10 minutes to pellet the DNA and the supernatant discarded. The DNA pellet was washed with 800µL of cold 70% ethanol and dried by inverting tubes on clean paper towels for 30 minutes at room temperature. The DNA pellet was re-suspended in 100µL of nuclease-free water. The integrity of DNA of each isolate was determined using the NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific Inc., Pittsburgh, PA) and adjusted to 50ng /µL for PCR amplification (Li *et al.* 2012). The isolates were further verified using PCR amplification. For each isolate the region spanning the internal transcribed spacer (ITS1-5.8S-ITS2) region of the ribosomal DNA was amplified using the universal primers ITS4 and ITS5. The identity of 11 isolates was resolved by direct sequencing ITS-PCR amplicons. Sequences were trimmed using MEGA6 program, manually edited and ITS1, 5.8s and ITS2 regions were searched using the National Center for Biotechnology Information (NCBI) GenBank nonredundant nucleotide database in order to confirm their identity (Appendix.1).

3.2.5.2 Amplification of PCR internal transcribed spacer (ITS) region

The PCR reactions were performed in 20µL reaction volumes containing 10 ng of genomic DNA, 1U Taq DNA polymerase (Bioneer Corporation, Deajeon, Korea), 1× green reaction buffer (Promega, Madison WI, USA), 2.0 µl of 10× PCR Buffer (0.4 µl of 1 mM dNTP

mixture, 2.0 µl of 50 mM $MgCl_2$), 12.4 µl of double-distilled water, 1.0 µl of 25 µM of each primer, 0.2 µl (1.25 units) of Platinum® Taq Polymerase (Invitrogen). The amplification reactions were performed in MyGenie 32 thermal cycler (Bioneer, Daejeon, South Korea). The PCR reaction consisted of initial denaturation temperature of 95°C for 3 minutes, followed by 35 cycles of 94°C for 40 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute and a final extension step at 72°C for 10 minute before cooling to 4°C.

Table 1: Sequence of polymerase chain reaction primers used to amplify the internal transcribed spacer (ITS) region of the selected *Phytophthora* isolates from Uganda.

Primer name	Sequence (5′→3′)	Sense	Gene	Expected amplicon size (bp)
ITS5	GGAAGTAAAAGTCGTAACAAGG	Reverse	Internal Transcribed Spacer rRNA	850
ITS4	TCCTCCGCTTATTGATATGC	Forward		

3.2.5.3 Gel electrophoresis

PCR products were subjected to electrophoresis to confirm the amplification. 10µL of amplified PCR products and a 1 Kb ladder used as a molecular marker (MBI Fermentas, Burlington, ON, Canada) were separated through electrophoresis in a 1.2 % w/v agarose gel in 1× TAE buffer (0.04M Tris–acetate, 1mM EDTA) at 100V for 2h. The gel was stained with ethidium bromide (0.5µg /mL) for 10 minutes. Gel images were captured using the GBOX Syngene gel documentation system (SYNGENE, UK). Fragment sizes were estimated based on O'geneRuler DNA ladder mix (MBI Ferment as, Burlington, ON, Canada) (Sambrook *et al*., 1989).

3.2.5.4 Sequencing of the internal transcribed spacer (ITS) region

The amplified PCR products were cleaned using the AccuPrep PCR purification kit (Bioneer Corporation, Deajeon, Korea).The cleaned PCR products were sent for Sanger sequencing

with reverse universal primers ITS5 commercially available (Bioneer Corporation, Deajeon, Korea).

The DNA sequence data was returned as FASTA files. Consensus sequences were generated from the reverse complement of the reverse read by sequence alignment using molecular evolutionary genetics analysis version 6.0 (Mega6) (Tamurra *et al.*, 2013).

3.2.5.5 Phylogenetic analysis

The ITS region of all amplified and sequenced isolates were for species identification. Sequences were aligned using multiple sequence alignment programs in MEGA 6.0 (Tamura *et al.*, 2013) under pairwise alignment parameters. Sequences were edited visually to eliminate indels and misaligned residues. Alignment gaps were treated as missing data. The evolutionary history was inferred using neighbour-joining method. The consensus sequences were queried against the National Center for Biotechnology Information; www.ncbi.nlm.nih.gov) (NCBI) Genbanknon-redundant (nr/nt) database using the basic local alignment search tool search (BLAST) (Altschul *et al.*, 1997). The matching sequences from NCBI along with their accession numbers were downloaded and aligned with consensus sequences using the Mega6 (Tamura *et al.*, 2013). Phylogenetic tree was constructed using a maximum composite likelihood model. Bootstrap analysis was performed using 500 replicates. Sequences of*Fusarium sp.* Py003 was used as an out group for the phylogenetic tree construction. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

There were a total of 338 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

3.2.5.6 Analysis of molecular variance (AMOVA)

Analysis of molecular variance (AMOVA) among the *P.nicotianae* populations was done using AMOVA input data file 1.55 programmes and generated using DCFA 1.1.The number of permutations was determined at 1000 for significance analysis. AMOVA components were used as estimates of the genetic diversity within populations.

3.3 Results

The primer combination of ITS4 and ITS5 amplified DNA of *P. nicotianae* at 55°C annealing temperature producing band pattern which was consistent with all the eleven isolates (Figure 2). Significant variations ($P < 0.001$) were observed among *P. nicotianae* isolates from the four districts.

3.3.1 PCR amplification of the internal transcribed spacer (ITS) region of

Amplification by using primer PCR generated unique DNA fragment of 850bp of isolates Lu010, Ka015, Lu011, Ka016, Lu012,Ka018, Ka019, Mu002, Ma001, Ma002 and Ka017.

In all cases a DNA banding profiles of 850bp were obtained by using ITS4 and ITS5 primer (Figure 3). ITS amplifications of *Phytophthora* isolates target conserved regions in the ITS1 and ITS2 (Figure 2)

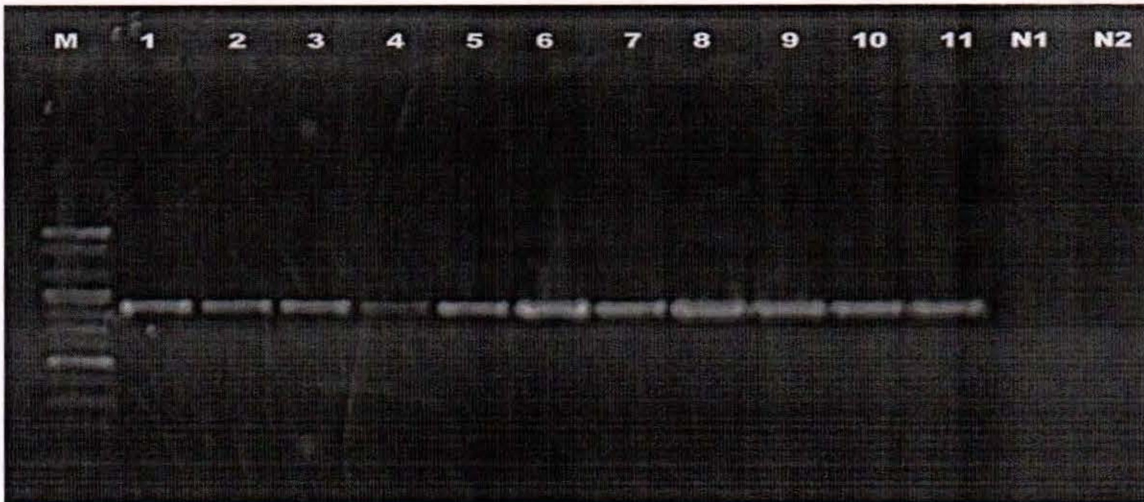

Figure 3: Amplified PCR products of the 11 test samples (Lane 1-11).

The lane marked M shows the DNA molecular weight standards (Ladder) used for estimating the band sizes of the amplified PCR products. Lane N1 and N2 are negative controls.

3.3.2 Sequencing of the internal transcribed spacer (ITS) region

ITS primers obtained partial sequence information from *Phytophthora* isolates listed in (Table 1). The match between the query sequence of the PCR products of the eleven isolates obtained from central Uganda and the database showed 74-97% identity to *P.nicotianae* and *P.parasitica*as the causalpathogen of pineapple heart rot dïsease in Uganda. However, isolate Ka015 matched *pythium glomeratum* sequence on the data base.

The trimmed and aligned amplicon was 881bp in length (Figure 4). The ITS sequences for the 9 isolates was over 74% sequence homology with the different *P. nicotianae* accessions in the database (Appendix1). Whereas isolate (Ka015) was found to be homologus to *Pythium glomeratium*, Ka018 had no matching sequence on the NCBI database. Overall, the sampled isolates consisted of 9 *Phytophthora* isolates identified as *P.nicotianae, P.parasitica* using molecular means (Appendix 1).

District	Haplotype Code	Haplotype
Kayunga	7	GCGGGAAGGTACTGCGGACGACATTACCACACCT
Kayunga	3	CCTCAGAGACTGCGGAAGACATTACCACACCTAA
Kayunga	10	GCTTTAGGGTGACTGCGGACGACATTACCACACC
Kayunga	1	CCCTGCAGCTACTGCGGAGAGACATTACCACACC
Kayunga	9	GCTTCAGAGACTGCGGAGGACATTACCACACCTA
Luwero	5	CCTTTGGGTGACTGCGGAGGATCATTACCACACCT
Luwero	4	CCTGCAGGTACTGCGGAGGACATTACCACACCTA
Luwero	2	CCGAAGTACTGCGGACGACATTACCACACCTAAA
Masak	6	CTTTGGTGACTGCGGAGGACATTACCACACCTAA
Masaka	8	GCTGAAGGTGACTGCGGAGGACATTACCACACCT

Figure 4: Multiple alignment sequence using ITS4 and ITS5 primers of *P.nicotianae* isolates from central Uganda 2016.

ITS sequence from *Phytophthora nicotianae* examined in this study ranged from 850 bp and the consensus length for the sequence alignment was 881bp.

3.3.3 Phylogenetic analysis

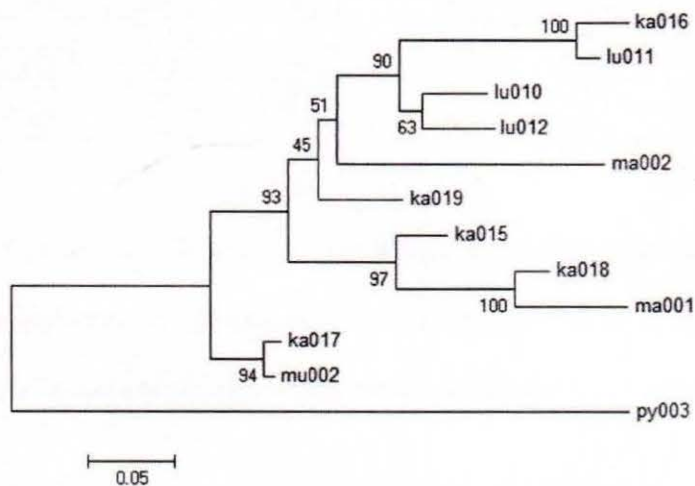

Figure 5: Phylogenetic relationships among *Phytophthora* isolates from central Uganda using ITS of rDNA sequence alignment based on Maximum Composite Likelihood model inferred by Neighbour Joining tree search.

3.3.3.1 Cluster analysis

The results of analysed ITS1 5.8s ITS2 sequenced data of 11 samples of PHRD revealed phylogenetic relationships all with some significant intra-specific variation that can be identified in the tree (Figure5). Isolate Ka017 from Kayunga Sub-population was distant from other isolates of the same Sub-pop, and also the isolate designated Mu002 from Mukono Sub-population was quite distant from the rest of isolates within the districts. Table 2: Pair wise population matrix of Nei genetic Distance between 10 PHRD isolates using Maximum Composite Likelihood model 2016.

Table 2; Pair wise population matrix of Nei genetic Distance between 10 PHRD isolates using Maximum Composite Likelihood model 2016.

	PN	Binary Distance	Kayunga	Luwero	Masaka					
Ka015	Ka016	Ka017	Ka018	Ka019	lu010	Lu011	Lu012	ma001	ma02	
0										Ka015
681	0									Ka016
566	680	0								Ka017
282	647	548	0							Ka018
616	631	646	641	0						Ka019
515	682	402	494	564	0					lu010
	546	584	573	354	603	0				lu11
676	563	691	678	570	632	650	0			lu12
627	526	667	626	537	645	484	451	0		ma01
567	772	706	592	625	602	702	672	738	0	ma02

Significant differences in genetic differentiation with distance can be seen within the sub-population, as illustrated in (Table 2). All pair wise values in this study showed a high variation between and within sub-populations.

Table 3: Analysis of molecular variance (AMOVA) for 9 *Phytophthora nicotianae* causal organisms from central Uganda 2016

Source of variation	d.f.	SS	MS	Est.Var	Total variance	p-value	PhiPT
Among population	2	500.367	250.183	0.000	0%	_	_
Within population	10	2184.933	312.133	312.133	100%	0.001	-0.068
Total	12	2685.300	562.366	312.133	100%		

d.f. – degree of freedom, SS – sum of squares, MS – mean squares, p – level of significance, PhiPT – index for genetic differentiation among populations. Probability, P (rand >= data), for PhiPT is based on standard permutation across the full data set.

PhiPT = AP / (WP + AP) = AP / TOT

Key: AP = Est. Var. Among Pops, WP = Est. Var. Within Pops.

PhiPT and AMOVA values were negative within the sub-population.

From the result, the variation between populations is 0% as the samples were picked from one region of central Uganda ecosystem/geographical region constituting one population. The variation between samples (sub-population is 100%. Most of the variance came from within samples (samples from same region).

3.4 Discussion

3.4.1 Molecular identification of pineapple heart rot(PHR) pathogen

The ability to rapidly and accurately identify pathogens causing plant disease is extremely important in disease management. Significant difference of ($p<0.001$) shows there was variation within *spp*. DNA from *Phytophthora* samples were successfully amplified and all isolates tested postive for *P.nicotianae* with ITS4 and ITS5. Variations were observed in ITS-PCR gel patterns among gels run at different times. According to Lee and Taylor (1992), minor gel-to-gel variation in the ITS-PCR patterns within a species is inevitable and should not necessarily be interpreted as intraspecific variation.

The *Phytophthora* rDNA sequences were submitted to Genebank with accession numbers Ka015 (MF350660),Ka016(MF350661),Ka017 (MF350662),Ka019 (MF350663),Lu010 (M F350664),Lu011 (MF350665A),Lu012 (MF350666),Ma001 (MF350667),Ma002 (MF35066 8),Mu002(MF350669). The closest match of sequences in NCBI Genebank identified, *P.nicotianae* var *P.parasitica* as the cause of heart rot of pineapple disease in central Uganda. This was based on ribosomal DNA sequencing using *Phytophthora* genus specific primers ITS4 and ITS5 (Table 1). *Phytophthora nicotianae var Phytophthora parasitica* is a soil-borne bi-flagellated Oomycete plant pathogen, which causes heart rot of pineapple in cultivated pineapple, black shank in tobacco and root rot, leaf necrosis, and stem lesions in a variety of plants. According to Araki *et al*., (2006), *P. nicotianae* is able to infect a wide range of hosts, spanning 255 genera in 90 different plant families. It devastates the production of a number of economically important plants, and causes millions of dollars worth of economic losses each year in the pineapple and tobacco industry alone (Araki *et al.*, 2006). Earlier Erwin and Ribeiro (1996) demonstrated that *P. nicotianae* Breda de Haan have similar genetic composition to *P. parasitica Dastur.* Our findings therefore is in agreement with Panabieres *et al.* (2016), who concluded that *nicotianae* generally considered as a root

pathogen, possessed a wide host range including herbaceous and woody plants and causes crown rots as well as may attack aerial parts such as stems, leaves and fruits. Accordingly, *Phytophthora nicotianae* may persist and develop on a broad range of plants and plant organs, but it may also survive outside of hosts (Drenth and Sendal, 2004, Panabierer *et al*,2016).

According to Hynniewta *et al*.,(2014), in order to accurately determine the extent of variability within a species or report of new or previously unreported hosts, genetic characters are used to identify individual isolates.Despite the particularly wide host range of *P.nicotianae*, there are considerable differences in aggressiveness towards particular plants among natural populations, and there is much evidence of host specialization among isolates collected on various plants (Taylor *et al*., 2012; Kamoun *et al*., 2015; Biasi *et al*., 2016 Panabierer *et al*., 2016).

Sequencing of the ITS 1 and 2 region was the most reliable method for confirming species identity (Farhana *et al* 2013, and -Elimat *et al*., 2013b). DNA samples from 11 isolates identified as *P.nicotianae* were used. However two out of these 11 isolates had ambiguous identities based on ITS patterns that is Ka015 and Ka018. Accordingly, the status of *P.nicotianae* is clarified by molecular characters of species in the complex by the Center of *Phytophthora* in Australia (Scott *et al*. 2009; Abad *et al*., 2011). Green and Scot (2015) also suggested that heart and root rots of pineapple in Hawaii are caused by several species of the plant-pathogenic water molds *Pythium* and *Phytophthora.* Phylogenetic analysis using rDNA sequences revealed that the 9 isolates of PHRD are *P. nicotianae* and yielded few clusters within the species exhibiting intraspecies variation.

3.4.2 ITS-Phylogeny

In the present study, considerable genetic diversity of *P. nicotianae* was observed with direct Sanger sequencing of rDNA of 11 isolates collected from Central Uganda. Many studies have shown that molecular markers could detect genetic variation within populations to a certain extent, including pathogenicity variation, geographical differences and host differences (Nyasse *et al.*, 1999, Li *et al.*, 2012). The genetic diversity in sub-populations was largely intraspecific variation within sub-populations of Kayunga, Luwero, Masaka and Mukono. This is in accordance with the phlogenetic cluster analysis that not all isolates from the same population clustered into one genotype (Martin,2004). The study conducted by Lamour and Hausbeck (2001) using AFLP marker to investigate the spatio temporal genetic structure of *Phytophthora capsici* in Michigan, found that population differentiation increased with distance, indicating that the genetic distances between populations correlated positively with geographical distances and that geographical separation posed an obstacle to the possibility and frequency of genetic exchanges between populations. The phylogenetic analysis of the present study revealed that some isolates from Kayunga were closely related to those from Luwero and Masaka districts. This may lead to the conclusionthat genetic distances and genetic differentiation may not directly be influenced by geographic distances/origin. This analysis provides an average ITS-based phylogeny of *P.nicotianae* one in which there is intra-specific relationships among the isolates. The result shows that clustering of *P.nicotianae* in the phylogenetic tree ocurr at random irrespective of geographical origin (Figure 5).

Results showed that ITS sequence variation was highbetween and within*P.nicotianae* isolates of the pineapple heart rot disease. This was high enough to support their separation into different clusters (Ippolito *et al.*, 2002).

The phylogenetic tree (Figure 4) was constructed from the ITS sequence data and the various molecular clusters of *P.nicotianae* formed distinct lineages. Similarly basing on sequenced rDNA ITS regions, Oliva *et al.,* (2010) argued that lineages which showed moderate levels of heterozygosity, high homology and shared a good number of allelic pairs at many loci are descended from a common origin. The intraspecific variation observed within *P. nicotianae* isolates provides a close look at micro evolutionary processes, particularly when supported by sequence data (Lassiter *et al*., 2007). However, some of the molecular groups clustered very closely suggesting recent evolvement from the ancestral origin or the ultimate merging of some of these groups example Ka015, Ka018 and Ma 001. Surprisingly, Ka018 has no match in the NCBI database implying that it may be a peculiar strain of *P.nicotianae.* Nonetheless, this was clearly demonstrated by Bastia's *et al*., (2006), who said that there are many sequences from environmental samples whose species affiliation remain unknown because they cannot be satisfactorily matched to a sequence of known taxonomic identity. As previously, indicated in the direct sequencing analysis of *P. Nicotianae*, very close relationships between molecular groups of the species were evident (Forster and Cofey, 1993). In particular, close relationships were found between Ka016 andLu011, Ka018 and Ma001 andKa017 and Mu002 with close phylogenetic distance length. The high similarity level observed in several *P.nicotianae* might also indicate a high incidence of genetic recombination (Teixeira *et al*., 2009). To date, there is relatively good knowledge on the precise molecular basis of the sequence differences responsible for the generation of new pathogen strain variation.

3.4.3 Relationships between molecular clusters within *P.nicotianae*

The phylogenetic analysis based on sequence data related to ITS 1, ITS 2 +5.8S loci using maximum composite likelihood method and neighbour joining tree inference has revealed intra-species relationships and evolution of PHRD in Central Uganda. Sequence length

ITS1.5.8S, ITS2 fragments in the 11 accessions was 903bp. The phylogram presents two relatively distinct clades. All isolates except Ka017 and Mu002 occupy the same clade but different positions on the phylogenetic tree yet they come from different geographical location. This is well demonstrated by isolate Ka017 standing on a separate clade, yet it has common geographical origin with Ka015, Ka016, Ka018 and Ka019. Additionally Ma002 and Ka019 are the only monophyletic isolates. This peculiar difference is not reflected by differential virulence or geographical location. The gaps in relationships between molecular groups (Ma002 and Ka019) of the species were evident.

Py 003 falls far away from phylogeny because it originates from different genus and served to indicate the root of evolution. By majority of isolates occupying the larger clades, it was apparent that intra-specific variation was frequent within isolates of *P.nicotianae*. However, there was some significant variation in rDNA-ITS region of isolates from different location. This is contrary to Xu *et al.*(2007),who reported that *P.sojae* isolates from different geographical locations show great differences in the base constitution of ITS. Taxonomic implications of the presented phylogeny may reflects the intra species evolution of *P.nicotianae*. However, some clusters are closely related while others are not. There was no congruence between the mode of virulence habit, geographical location and the molecular grouping of the species. Isolates from the same location did not group together (Li *et al.*, 2012).

Previously the molecular variability within *P. nicotianae* was interpreted as evidence of evolution of separate clusters (Forster *et al.*,2000), has five clusters of *P.nicotianae* with each cluster having some genetic similarities based on ITS region (Forbes*et al.*, 2016). However, these clusters originate from a common ancestry triggered by intra generic factors. This matches very well with assertion that the evolutionary processes occurring in the pathogen population is attributed to underlying mutational or structural change in the genome

and length mutations in several intragenic regions (Schreier, 2013). A negative PhiPT value indicates an increase of heterozygosity and there by an increase in genetic variation, which is a result of random mating within a population and outbreeding (Doos, 2013).

AMOVA indicated that the genetic variation within *P. nicotianae* sub-populations accounts for 100%. This variation in a population may be a product of mutation, structural changes and clonal reproduction (Goodwin, 1995). This differential gene flow may lead to the introduction of new virulence combinations into existing populations as described in the next chapter (chapter four). Accordingly, epidemiological evidence for mutation leading to changes in the virulence profile of individual members of a pathogen lineage is most apparent in *Phytophthora* genus which undergoes cyclic asexual reproduction. . However, isolates or strains that have been present in the environment for a relatively long period might accumulate molecular variation and this may contribute to diversity within a species (Parkinson *et al.*, 2009 ; Kogeethavani, 2015). This is probably true for *P.nicotianae*. Ochiai *et al.* (2000) exclaimed that other factors influencing the genetic variability within a pathogen population include movements from different geographic locations or genetic variation due to recombination or mutation in response to agricultural or environmental constraints. In this study, *P.nicotianae* isolates showed a relatively high level of intra-species variation. In general, variation in a population due to mutation and Clonal reproduction in many *Phytophthora* species is the primary source of new genetic variation. Samen *et al.* (2003), unearthed a considerable level of inherent genetic variability among asexual progeny from the same parental isolate of *P. infestans* with RAPD and AFLP markers. Hao *et al.* (2003) demonstrated that variation in virulence in *P.sojae* asexual reproduction was frequently present and that the variation could not be inherited steadily after successive reproduction. Mutation in most cases could cause no observable changes in phenotypes, but it is not impossible that a portion of the genetic variation observed with these products of direct

sequencing rDNA could be due to mutations. Another potential explanation for genetic variation is hybridization. Bonants *et al.* (2000) clearly put it that hybridization, is possible between the heterothallic and the homothallic *P. Nicotianae*. That is to say possible exchange of genetic materials in hybrid Oospores may be an important factor in the evolution of natural *Phytophthora* populations. Interspecific hybrid in *P. Nicotianae* are more likely to survive if they have a fitness advantage over parent species such as increased aggressiveness or ability to exploit new host as proposed by Darine *et al.*,(2007). Brasier, (1992) indicated that the *P.nicotianae* has potential for zoospore fusion to produce hybrids and this was arrived at from successful attempts performed to create hybrids in the laboratory. Proof of hybridization was also obtained by DNA analysis (Ersek *et al.,* 1995, Bonants *et al.,* 2000). Sansome *et al* (1991) believed that although natural hybridization has never been proven but may have occurred with *P. Meadii*.

In conclusion, the clustering pattern of isolates Ka017 and Ma002 falling on a separate lineage, implies that they have undergone genetic divergence from others. This is best explained by Bonants *et al*. (2000) who pointed out that naturally; hybrids example *P. nicotianae* × *P. cactorum* hybrid isolates do not belong to a single clonal lineage and this may pose a serious threat to agriculture.

AMOVA calculated between and within sub-population from one region (Geographic locality) and one host plant, in three sub-populations. AMOVA analysis performed in DCFA 1.1 with 1000 permutations revealed that 100% of the total genetic diversity occurred within sub-populations (PhiPT = -0.068). AMOVA variation was highly significant ($p = 0.001$).

CHAPTER FOUR: PATHOGENICITY OF *PHYTOPHTHORA* CAUSING PINEAPPLE HEART ROT DISEASE (PHRD) IN UGANDA

4.1 Introduction

Pineapple heart rot disease is reported to be caused by a number of *Phytophthora* species. However, the species most commonly found associated with the disease are *Phytophthora cinammomi* and *nicotianae* (Rohrbach and Johnson, 2003). *nicotianae* is known as the most destructive plant pathogen attacking hundreds of plant species throughout the world (Hu *et al*., 2008; Sadeghy *et al*., 2014). The destructive ability of *P. Nicotiane* is due to its virulence and diversity of the pathogens strains (Panabieres *et al*., 2016). According to Agrios (2005), pathogenicity of an isolate/organism is occasioned by either its virulence (infection ability) or aggressiveness (vigour of attack). For example, Mbaka *et al.,* (2009), tested the pathogenicity of *Phytophthora* on macadamia based on their virulence on green apple fruits. Accordingly, the Koch's postulates stipulates that before any serious investment can be put on the control of any diseases, there is need to establish whether the organisms isolated from the diseased samples can cause a disease similar to that where it is isolated (Agrios, 2005). The pathogenicity of isolates also aids in the selection of the most virulent and aggressive isolates which can be used for screening for resistance to any particular disease. Therefore, understanding the variability of pathogenicity is fundamental to development of management options (Thomidis *et al*., 2002). In fact, Mbaka *et al*., (2009), demonstrated that pathogen virulence can be used in the evaluation of efficacy of soil treatment for *Phytophthora* control. Additionally, the difference in virulence of *Phytophthora* species isolated from different regions in Iran was considered in the selection of germplasm for potential resistant gene donors (Sadeghy *et al*., 2014). However, information on the pathogenicity of Pineapple heart rot (PHR) causal organisms in Uganda are limited (NARO, 2012). Therefore, the objective of this study was to ascertain the pathogenicity of *Phytophthora* species isolated from PHRD symptomatic plants from central Uganda..

4.2 Materials and methods

4.2.1 As described in previous chapter (chapter 3)

4.2.2 Pathogenicity of pineapple heart rot pathogens

The pathogenicity of pineapple heart rot pathogen was assessed on both green apple fruits and healthy pineapple leaves.

Green apples used in the study were locally purchased, surface sterilized by wiping with 70% v/v ethanol, rinsed with sterile water and blot dried with sterile paper towels. Incisions (10mm×10mm) were made into each apple using a sterile scalpel. A 5mm agar plug of the actively growing PHRD pathogens on PDA was inserted into the incision with mycelia facing inwards of the incision. Controls included apples inoculated with PDA agar discs without the pathogen. Points of inoculation were sealed with parafilm and apples kept in the dark at room temperature for 7 days (Mbaka *et al.*, 2009) (Figure7). The experiment was arranged in a completely randomized design (CRD) and replicated three times. The experiment was repeated once. The amount of disease induced by a pathogenic strain (Aggressiveness) on a susceptible green apple fruits was estimated by measuring radial expansion (diameter) of the lesion (Cooke *et al.,* 2012). Measurements were taken daily for a period of one week (Thomidis *et al.*, 2002).

4.2.2.1Pathogenecity tests using asymptomatic pineapple leaves.

Asymptomatic pineapple leaves used for pathogenicity test were obtained from healthy pineapple plants (var. Smooth Cayene) grown in a screen house at Kyambogo University to test for latent infection. The leaves were surface sterilized and blot dried as previously described. Mycelia fragments from each P.nicotianae growing on PDA was suspended in sterile distilled water for the formation of sporangia and the release of zoospores and inocula suspension adjusted to $1x10^8$zoospores per mL using haemocytometer (Rodríguez et al., 2002). The inoculum was transferred to 200 mL Erlenmeyer flasks (FisherScientific Co.,

Pittsburgh, PA) and a single cleaned leaf soaked into the inoculum. Pineapple leaves soaked in sterile distilled water served as controls.

4.2.3 Aggressiveness of Pineapple Heart rot Disease

Disease severity was estimated to the nearest percent (NPE), using a scale. The severity of PHR disease on inoculated green apples and pineapple leaves was assessed on scales of 0–3, where 0 = no lesion, 1 = small lesion, 2 = medium lesion and 3 = large lesion (Michalska *et al.*, 2016). Severity was measured based on proportion of apple fruits and pineapple leaves damaged.

Table 4: **Pathogenicity of *Phytophthora* isolates at Kyambogo University Laboratory, 2016**

District	Isolate	Pathogenecity
Kayunga	Ka001	++
Kayunga	Ka002	++
Kayunga	Ka010	++
Kayunga	Ka011	++
Kayunga	Ka012	++
Kayunga	Ka015	++
Kayunga	Ka016	++
Kayunga	Ka017	++
Kayunga	Ka018	++
Kayunga	Ka019	++
Luwero	Lu001	++
Luwero	Lu006	++
Luwero	Lu009	++
Luwero	Lu010	++
Luwero	Lu011	++
Luwero	Lu012	++
Masaka	Ma001	++
Masaka	Ma002	++
Mukono	Mu002	++
Mukono	Mu005	++

++ ....pathogenic

4.2.4 Data analysis

Severity data (lesion diameter (size) was done by one-way analysis. The variance, mean of disease virulence was generated using Genstat computer program. Significant difference between the means was separated using Lowest Significant test (LSD) at 5% probability level

4.3 Results

Twenty one (21) P. nicotianae was isolated from farms surveyed in this study (Table 4). All isolates (100%) were pathogenic on both green apple fruits and pineapple plants. Aggressiveness levels varied from one isolate to another. Most isolates showed firm brown rot within 48 hours of inoculation.

4.3.1 Isolation of Pineapple heart rot disease pathogens

Out of 184 samples collected, pure culture of was recovered from 21 isolates of pineapple heart rot disease. The isolates were characterised by condensed.rossette mycelial growth pattern. The highest and lowest number of pure culture recovered from symptomatic samples was from Kayunga and Luwero districts, respectively (Table 4).

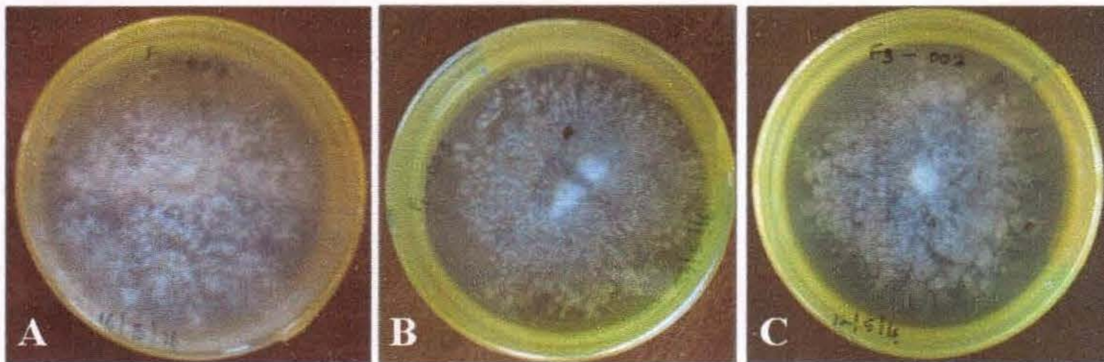

Figure 6: Pure culture of *phytophthora* growing on PDA at Kyambogo, 2016.

4.3.2 Pathogenicity of pineapple heart rot pathogen on green apples

All the pure culture of *Phytophthora* isolated from the PHRD symptomatic samples produced typical symptoms of pineapple heart rot on both green apple fruits and pineapple leaves (Figure 7).Pineapple leaves soaked in inocula showed dark brown rot at the leaf base submerged into the inocula after 48 hours.

Symptom characteristics of PHRD were observed on green apple fruits within 72 hours after inoculation. However, symptom appearance and virulence varied greatly among isolates. In the sixth day of inoculation Lu011, Ka015, Ma002 and Ka016, were the most and least virulent isolates, respectively.Nearly 100% of the apple fruits showed firm brown rots by the trials (Table 6).

Table 5: Origin of PHRD isolates used in the study, 2016

District	Isolate
Kayunga	Ka-015
Kayunga	Ka-016
Kayunga	Ka-018
Kayunga	Ka-019
Luwero	Lu-010
Luwero	Lu-011
Luwero	Lu-012
Kayunga	Ka-017
Mukono	Mu-002
Masaka	Ma-001
Masaka	Ma-002

Table 6: Pathogenicity of pineapple heart rot disease on green apples following inoculation with agar plugs containing mycelia of *Phytophthora* spp. isolates Kyambogo University 2016

Isolates	Amount of disease developed	Mean lesion diameter at sixth day
Lu011	Rapid	84
Ka015	Rapid	80
Ma 002	Rapid	76
Mu002	Rapid	75
Lu 010	Average	65
Ka017	Average	65
Ma 001	Average	67
Ka 019	Average	67
Lu012	Average	60
Ka 018	Average	55
Ka016	Slow	47
Average	_	67

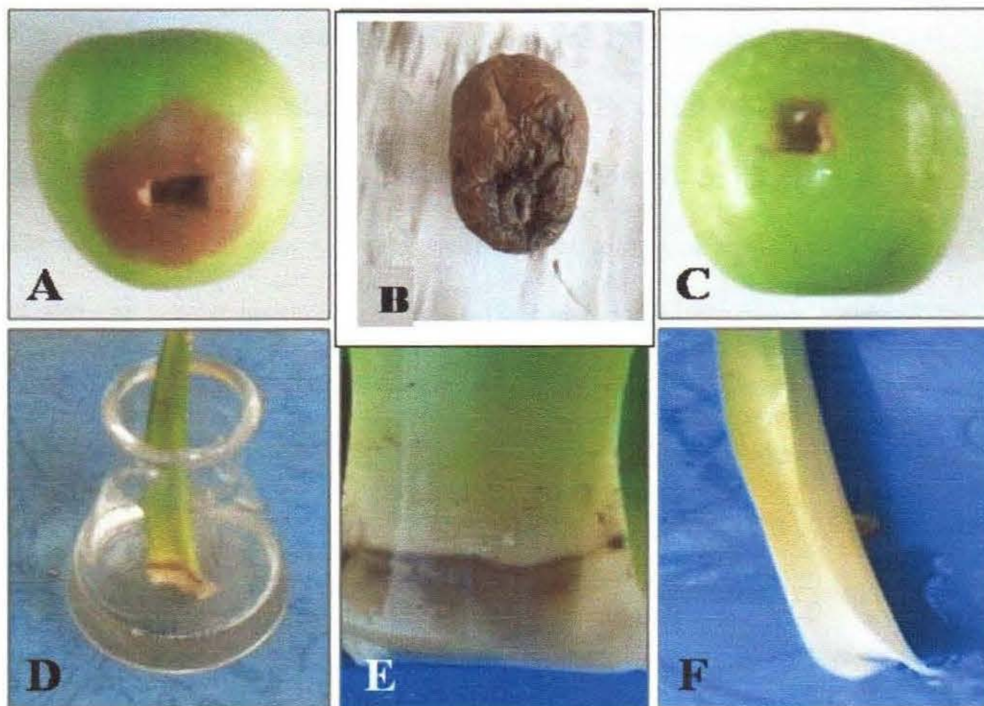

Figure 7: Pathogenicity of pineapple heart rot isolates on green apples and pineapple leaves

Apples showing a firm brown rot of pineapple heart rot disease: three days (A) and 21(B) days post inoculation. Pineapple leaf showing signs of pineapple heart rot disease three days (D) and twenty one days (E) post inoculation, Plates C and F are controls.

4.3.3 Virulence

The *Phytophthora* isolates induced hard brown necrotic lesions on green apple fruits within 48hrs (Figure 8). Lesion diameters significantly ($p<0.05$) varied among the isolates by the end of six days (Table 6). During, the first day of trial I, lesion diameters of all isolateshad covered less than 10% of the fruit surface area (Figure 9). However, by the fith day, the highest lesion diameters had covered above 70% of the fruits and the lowest was less than 70% (Figure 9) represented by73 and 45mm for isolate Lu011 and Ka012 respectively (Appendix 5). Overall, the mean lesion diameter was 13.1mm and 66.1 mm for the first and six day, respectively (Appendix 5). Additionally, 100% of the isolates had lesions covering three quarter of the apple fruits by the sixth day (Figure 9). In the second trials, the lesion diameter for all isolates was also less than 10% coverage of fruit surface (Figure 10).The highest and the lowest lesion diameter of 18.5mm and 8.1mm were recorded for isolates Ka018 and Ka019 respectively on the first day (Appendix 6). Measurement on the sixth day of the second trial indicated the highest and the lowest lesion diameter of 81.6mm and 46.3mm recorded from Ka015 and Ka016 respectively (Appendix 6). The mean lesion diameter was 13.5mm and 64.8mm for the first and six day respectively (Appendix 6).

4.3.4 Aggressiveness

.The statistical analysis of the aggressiveness tests (data not shown) showed a significant difference in leision diameter ($P < 0.05$). The result (Figureigure 8) showed the first eleven isolates were more aggressive than the rest of the remaining isolates. Measurements of symptom appearance on different green apple fruits showed that isolate Ka019, Mu-002 ,

Luo11, Ka015,Ma001, Ma002 had shorter latent periods and formed larger lesions within 12 hours from time of inoculation than any other isolate.(Table 6, Appendices 5 and 6)

Table 7: Summary of ANOVA for the daily change in lesion diameter of apples inoculated with isolates of *Phytophthora* species at Kyambogo University 2016.

Source of variation	D.F	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Lesion diameter	10	32.0**	149.6**	191.3**	287.3**	396.0**	417.3**	529.2
				Trial II				
Lesion diameter	10	34.3**	153.0**	185.0**	270.0**	354.1**	411.8**	527.9

** means siginificance at <0.001

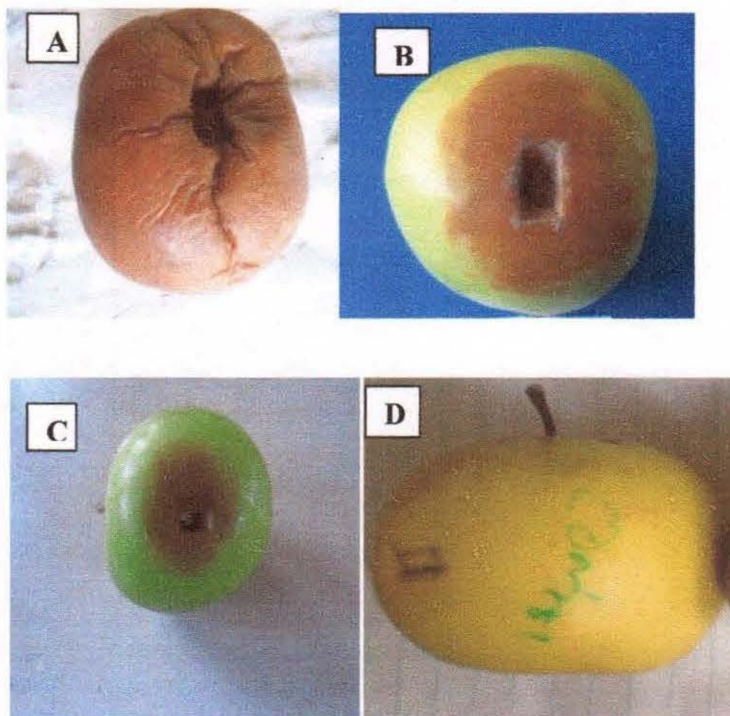

Figure 8: Brown rot induced by *Phytophthora* species on apples in different days. A) Day six. B and C) Day three. D) Control.

Trial I

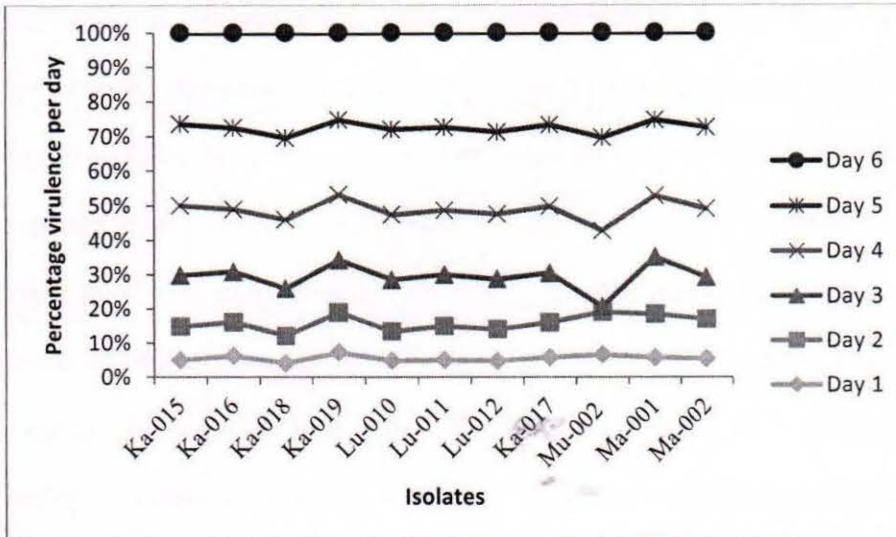

Figure 9: Mean lesion diameter caused by *Phytophthora* species on apples on a period of six days at Kyambogo University, 2016

Trial II

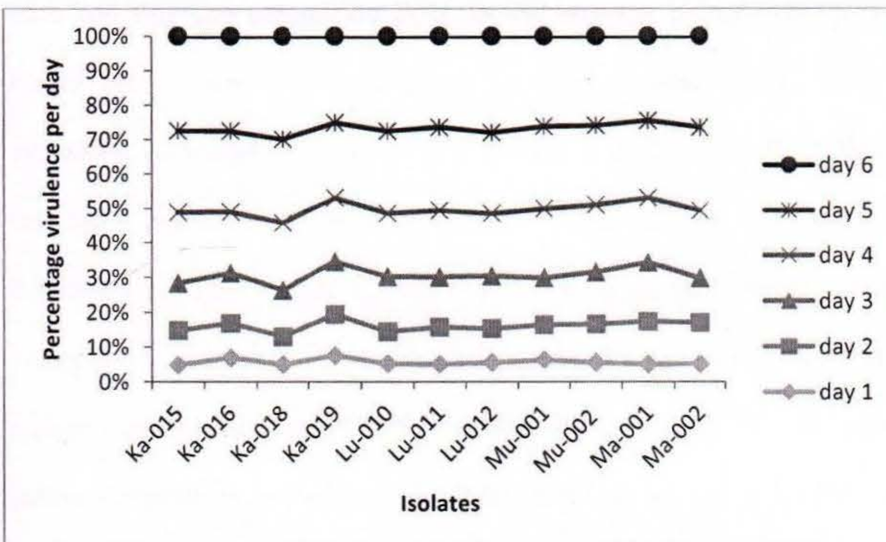

Figure 10: Mean lesion diameter caused by *Phytophthora* species on apples on a period of six days at Kyambogo University, 2016

4.4 Discussion

The objective of this study was to assess pathogenicity of *Phytophthora* isolates recovered from symptomatic pineapple leaf samples. Isolation from plant leaves produced a *phytophthora* that slowly forms sparse mycelia in 5 to 10 days. Isolating *phytophthora* from symptomatic and asymptomatic pineapple leaves was equally very difficult. All isolates were virulent (Table 4). This compares well with (Mbaka, 2009, Milenković *et al*., 2014), who reported several isolates were virulent to apple fruits causing significant firm brown rot to the apple fruits. Generally all isolates were able to infect pineapple and green apples with varying levels of virulence and aggressiveness as observed by Michalska *et al.* (2016). Higher rate of virulence exhibited by more than 50% of isolate in (table 6) is an indication of pathogens ability to overcome the host resistance. The same was observed by McDonald andLinde (2002) and Petersson ((2015) of P.infestant pathogen. Therewas varyin aggressiveness of all isolates on green apple and pineapple hosts. Matheron and Matejka (1990) argued that phytophthora species is a multi host pathogen that can survive in an ecosystem for longer time and this can complicate management strategy of the pathogen. Rapid expansion of brown hard lesion was experienced by some isolates while others had slower rate of expansion. Similarly (Thomidis *et al*., 2002; Mbaka *et al*., 2009; Petersson, 2015), agreed that rapid expansion of the lesion (hard brown rot) was indication of aggressiveness of P.nicotianae. The results showed that all pineapple plants used were susceptible. Accordingly, susceptibility of pineapple to infections by Phytophthora species was previously reported elsewhere (Jung (2009), Milenković *et al*., (2014).The significant difference in lesion diameter is probably a result of variation in soil conditions from the distrcts of Kayunga, Luwero, Masaka and Mukono where the samples were picked. This result compares very well with Mounde *et al*., (2012) in Kenya who reported significant differences between the isolates of *Phytophthora* as a result of isolate origin. In Italy, Ippolito *et al*., (1992) attributed varying infection rates to temperature of samples at the time of collection,

during storage and during incubation period of isolates in the agar media. Similarly, Matheron and Matejka (1990), Thomidis *et al.*, (2002) attributed the difference in virulence to the nature and type of the host plant. Additionally the difference in virulence could be attributed to variation in the virulence gene and physiological races of the isolates within the pathogen population (Jaarsveld *et al.,* 2002, Leah *et al*., 2012). This observation tally with Liu *et al* (2016), who declared that physiological races of *P. nicotianae*, races 0 and1in China are predominantly observed in cultivated tobacco fields around the world. Accordingly, the varying level of virulence of *P.nicotaianae* confirms the threats it poses to pineapple industry in Uganda.

CHAPTER FIVE: GENERAL DISCUSSIONS, CONCLUSIONS AND RECOMMENDATIONS

5.1 GENERAL DISCUSSIONS

Pineapple Plants are generally susceptible to a range of pathogens including *phytophthora* with overlapping host ranges frequently observed. Nevertheless, from the result of this study, it became apparent that *P. nicotianae* is the cause of pineapple heart rot disease in Uganda. According to Panabieres *et al.*(2016),,*Phytophthora nicotianae* is a common and destructive pathogen of numerous ornamental, agronomic and horticultural crops, such as tobacco, tomato and citrus.

Additionally, the results showed clear differences in the population in both the aggressiveness and the genetic diversity. Finding by Sjöholm *et al.,* (2013), Petersson, (2015) and Liu *et al.*, (2016) are in tandem with our findings. This is because the virulence and aggressiveness vary greatly from one species to another, depending on a range of variables, such as pathogenic ability and environmental parameters including geographical location, soil status or cultural practices used in crop protection. The results indicated that Kayunga Sub-population comprised more genotypes than members of the other sub-populations. Generally speaking, the isolates from the same sub-population (district) were divided into the different cluster

Sexual recombination can result in the occurrence of more virulent strains (Petersson, 2015). This has been seen in the Netherlands after the appearance of the A2 mating type (Drenth *et al.*, 1994). In this study a higher aggressiveness of the isolates from Kayunga compared to the isolates from Luwero was observed when referring to the latency period and significant differences in the lesion growth rate were found within the population.

On the basis of prevalence, incidence and severity of disease symptoms, *P. nicotianae* may be locally and/or temporarily a secondary pathogen behind other pathogens that generally

display more restricted host ranges, like *P. infestans* on tomato, *P. capsici* on pepper, *P. citrophthora* or *P. palmivora* on citrus, *P. cactorum* on fruit trees (Erwin and Ribeiro, 1996), and *P. erythroseptica* on potato (Taylor *et al.*, 2008). Nevertheless, *P. nicotianae* displays several characteristics that may allow it to outcompete these other *Phytophthora* species.

Pineapple heart rot disease (PHRD) caused by *P.nicotianae* was first reported in central Uganda especially districts within the Lake Victoria cresent basin with only one soil condition (Bua *et al.*, 2013). However Rohrbach and Apt (1986) and Jung *et al.*, (2013), have divergent views and observed that *P. nicotianae* is found in a wider range of soil conditions.

Phytophthora nicotianae var *parasitica* is a diverse fungal species causing disease on a broad range of both temperate and tropical crops. As such this has been one of the most limiting factors to pineapple production in Uganda. This has been affirmed by (Li *et al.*, 2012) who asserted that fungal pathogen has been reported to cause destructive disease on a variety of hosts including pineapple.For effective control and management strategy of the disease, there is need for better understanding of host ranges and genetic diversity of this plant pathogen (McDonald and Linde, 2002).

Although more has been unravelled about the genetic structure of *P. nicotianae*, so far, little is known about its pathogenicity and diversity in Uganda. According to many studies, molecular markers could detect genetic variation within populations to a certain extent, including pathogenicity/virulence variation and geographical differences (Nyasse *et al.*, 1999; Zhang *et al.*, 2007; Li *et al.*, 2012; Farhana *et al.*, 2013).

In the present study, considerable genetic diversity of *P.nicotianae* was observed with ITS regions, based on the 11 isolates collected from central Uganda. The differences in genetic diversity of the selected isolates were largely due to genetic variation within populations. This finding is in harmony with Darine *et al.* (2007) who recognised that the genes occur in

multiple arrays and mutation in their non coding regions occur at a rate that approximate the rate of species emergence. The same author asserted that such mutation over time becomes fixed through unequal crossing over and gene deletion. This was in accordance with the ITS cluster analysis that not all isolates from the same geographical locations clustered into one genotype. Interestingly, the results of the present study also showed that there was correlation between the genetic clusters of the ITS region and the pathotypes of the isolates studied (Salati *et al.,* 2012) or similar to that reported of *Phytophthora capsici* (Salati *et al.*, 2010;Quesada-Ocampo *et al.*, 2011).

The PHRD was detected in four major pineapple growing areas in central Uganda. Pathogenicity on green apple fruits showed that symptom appearance (latent period) and virulence varied greatly among isolates. Ka019, Mu002 and Ma002 were among isolates that had the largest lesion diameter within the first 48 hrs.On the sixth day Lu011, Ka015, Ma002 were the most virulent isolates and Ka016 was the least virulent for the first trial. This is in line with earlier report of Matheron and Matejka (1990) which revealed that isolates of *P.parasitica* of Rosemary were all pathogenic to sweet orange but varies markedly in virulence. Considerable differences in aggressiveness of some isolates towards the apple fruits may point to an evidence of host specialization among isolates collected from pineapple of various geographical locations (Kamoun *et al.*, 2015; Biasi *et al.*, 2016; Panabieres *et al.*, 2016).

The study to assess the pathogenicity of *P. nicotianae* in pineapple was to help identify potential threat of *P.nicotianae* to commercial pineapple production in Uganda so that appropriate disease management strategy can be implemented. The results from this study indicated that *P. nicotianae* is the main causal agent of pineapple heart rots disease in central Uganda because 90% of the strains isolated from infected pineapple plants were recognized after molecular analysis as *P. Nicotianae* (Burgess (2015; Blaya *et al.*, 2015). Sequencing of

the ITS region was a successful approach to differentiate *P. nicotianae* from other species. Although ITS region is not always sufficiently diverse to allow the identification of closely related taxa, it successfully differentiated *P. nicotianae* from other *Phytophthora* species (White *et al* 1990).

Phytophthora nicotianae was isolated consistently from the leaf tissues of infected pineapple plant with pineapple heart rot disease. Pathogenicity tests showed that *P. nicotianae* causes heart rot disease of pineapples both in the laboratory, field and under screen house conditions. Re-isolation of *P. nicotianae* from symptomatic tissue and molecular characterization confirmed the identity of the organisms as causal pathogens of PHRD.

According to Erwin and Ribeiro (1996), *Phytophthora* is one of the most harmful genera of plant pathogens worldwide known to cause seed rot, root and heart rot, seedling damping-off, rots of lower stems, tubers and corms and soft rots of fleshy fruits in contact with the soil. Phylogenetic analysis of the ITS rDNA region clearly showed the genetic distance between all the isolates. The ITS rDNA region sequences, indicated that *P.nicotianae* are distinct species responsible for heart rot disease of pineapple in Uganda.

5.2 CONCLUSIONS

Basing on the molecular characteristics of the 11 isolates, it was concluded that 9 of them are *P.nicotianae* the causal organism of pineapple heart rot disease in Uganda. However, significant variations were observed among *P.nicotianae* isolates fromKayunga, Masaka, Luweero and Mukono, the major pineapple growing areas in Uganda. All the isolates were pathogenic on both green apple fruits and pineapple plants.

5.3 RECOMMENDATIONS

Isolate Ka018 which occupied the same cluster with Ma001 had no genetic match in the genetic database. Therefore, additional studies are recommended toconclusively identify isolate Ka018. This can then be followed by depositing the unique sequence associated to this

isolate in NCBI. In addition, further studies should be done on the mating types to consolidate the confirmation of the identity of *P.nicotianae* as the causal organisms of PHRD in Uganda. Furthermore, studies should be conducted to determine the molecular mechanisms underlying the difference in virulence and aggressiveness between isolates of the same species and geographical location. Variation in isolates virulence from different regions in central Uganda suggests that appropriate integrated disease management package needs to be developed if PHRD diseases is to be successfully managed. Above all a study should be conducted to determine the epicenter of origin of *P.nicotianae* in Uganda.

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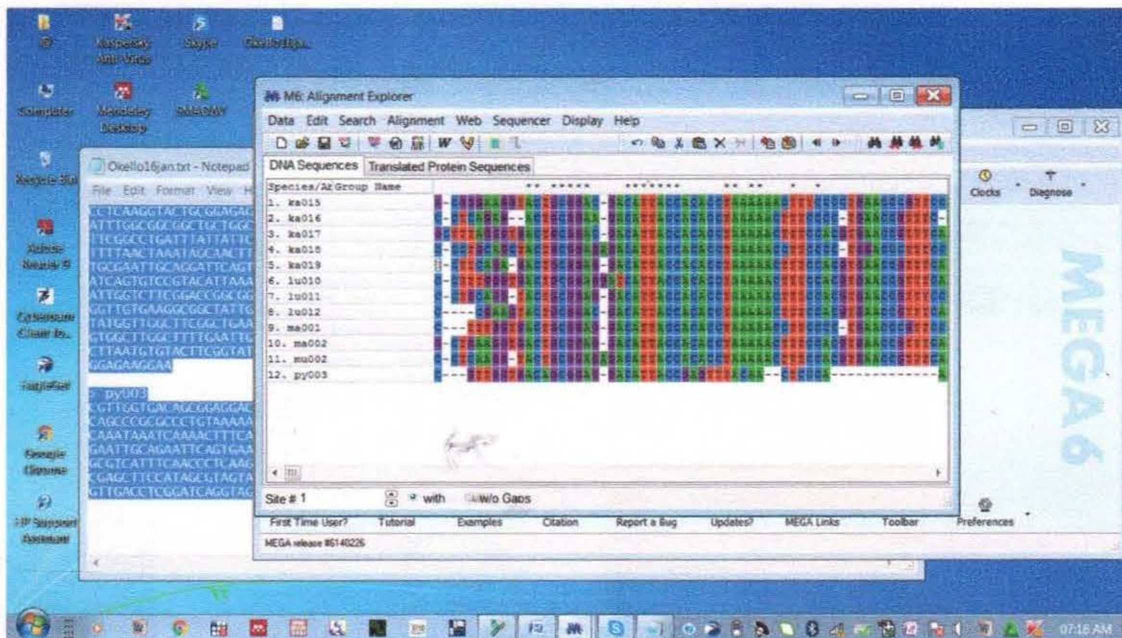
APPENDICES

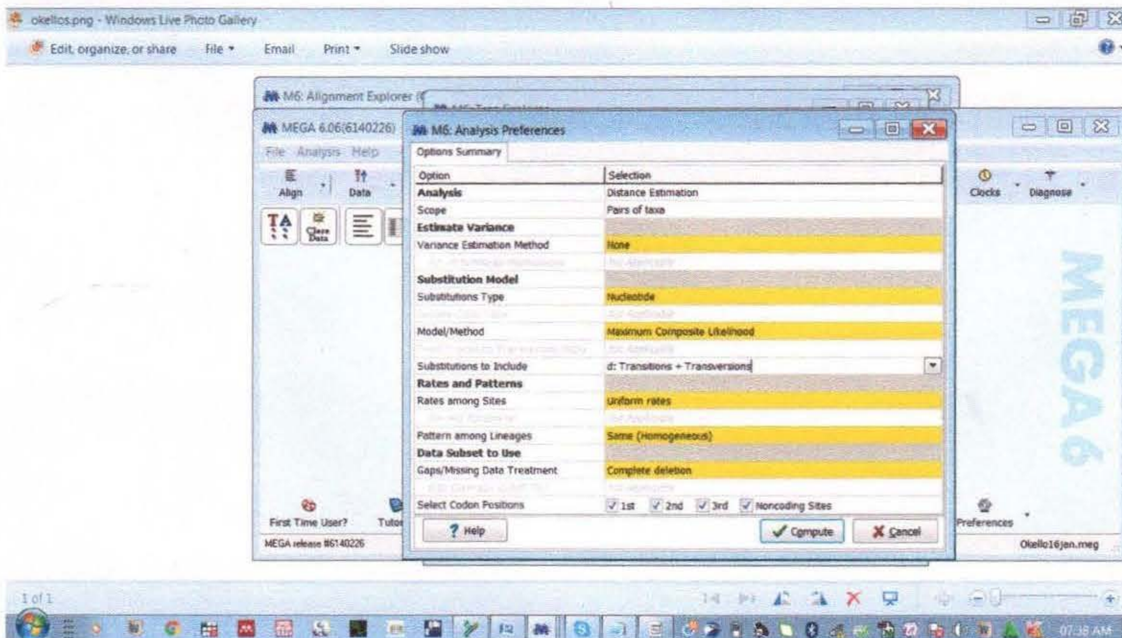
Appendix 1: Genetic identity of pineapple heart rot disease isolates from central ***Uganda***

S/No.	Isolates	% Query Coverage	E-value	% Identity	GI-Acession	Identification
1	Ka015	93	3e4	97	KU210439.1	*Phythium glomeratum*
2	Ka016	96	6e81	74	JQ911773.1	*Phytophthora parasitica*
3	Ka017	96	0.0	96	KJ494913.1	*Phytopthora nicotianaee*
4	Ka018					
5	Ka019	83	0.0	87	GU931702.1	*Phytopthora nicotianaee*
6	Lu010	89	9e104	77	KT148936.1	*Phytopthora nicotianaee*
7	Lu011	94	2e84	74	KT148935.1	*Phytopthora nicotianaee*
8	Lu012	71	1e116	81	KU248812.1	*Phytopthora nicotianaee*
9	Ma001	91	8e70	74	KJ506732.1	*Phytopthora nicotianaee*
10	Ma002	62	6e88	89	GU931702.1	*Phytopthora nicotianaee*
11	Mu002	97	0.0	95	KU248811.1	*Phytopthora nicotianaee*
12	Py003	99	0.0	99	KT582068.1	Fusarium sp.

Appendix 2: ***Output1: Multiple sequence alignment of phytopthora nicotianaee in central Uganda 2016.***

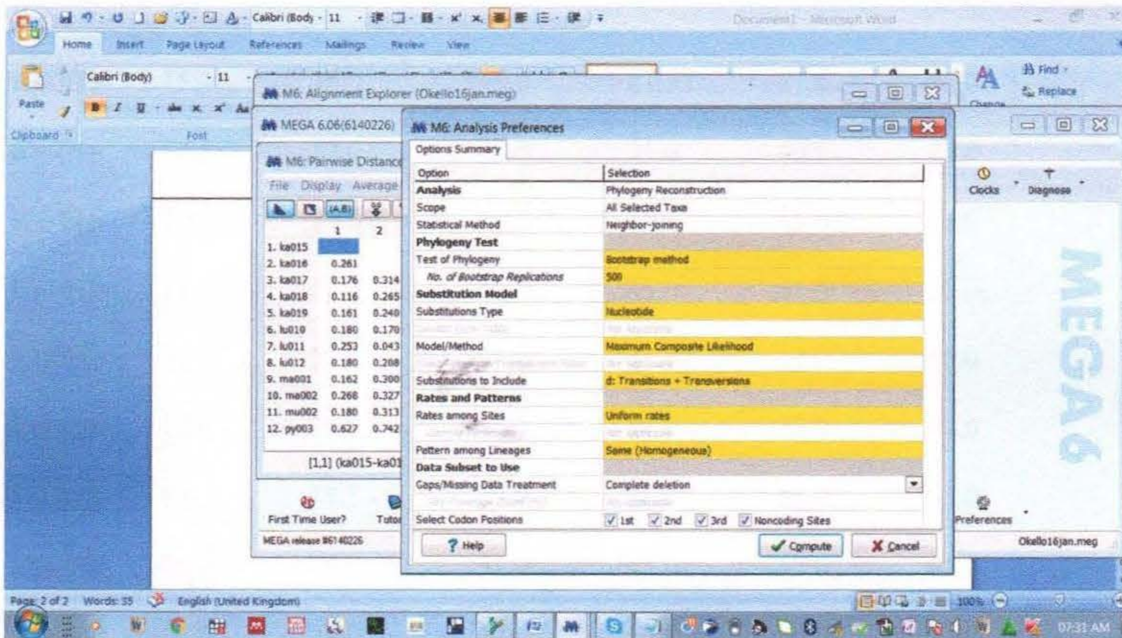


Appendix 3: ***Output 2; Alignment parameters***



Appendix 4: Output 3*: **Phylogenetic analysis parameter***

(TEST: Bootstrap, MODEL: Maximum composite Likelihood model, NO. OF BOOTSTRAP REPLICATIONS/BOOTSPRAP PARAMETER: 500 and STATISTICAL METHOD: Neighbour joining)



Appendix 5 Lesion diameter caused by *Phytophthora nicotianae* on apples on a period of seven days at Kyambogo University, 2016

			Trial I			
Isolate	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Ka-015	15.0	30.0	45.0	61.33	71.0	80.0
Ka-016	10.5	17.0	25.0	31.0	40.0	47.0
Ka-018	7.0	14.0	24.0	35.0	41.0	53.0
Ka-019	17.0	28.0	36.0	45.0	51.0	60.0
LU-010	11.0	20.0	35.0	43.67	57.0	65.0
Lu-011	15.0	30.7	45.7	57.0	73.0	84.0
Lu-012	9.0	17.7	28.0	36.0	45.0	55.0
Mu-001	14.0	25.0	35.0	47.0	57.0	65.0
Mu-002	16.0	31.0	4.0	54.3	66.0	75.0
Ma-001	15.0	34.0	44.0	47.0	58.0	67.0
Ma-002	15.0	32.0	34.0	55.0	65.0	76.0
Mean	**13.1**	**25.42**	**43.7**	**46.4**	**56.7**	**66.1**
LSD $_{(5\%)}$	**0.21**	**1.28**	**35.9**	**2.5**	**2.3**	**2.0**
		Appendix6	**Trial II**			
Ka-015	14.3	29.6	40.3	61.33	70.3	81.6
Ka-016	11.5	16.7	24.3	30.0	39.5	46.3
Ka-018	8.1	13.3	22.1	32.2	40.0	49.5
Ka-019	18.5	28.9	37.1	45.5	53.4	61.4
Lu-010	11.9	21.3	36.5	42.5	55.0	63.6
Lu-011	15.3	32.4	43.9	58.9	73.5	80.5

Lu-012	10.7	18.9	29.4	35.3	45.4	54.4
Mu-001	15.2	24.3	32.5	48.4	57.5	63.2
Mu-002	15.6	31.3	42.3	55.5	65.1	73.5
Ma-001	13.2	32.5	44.6	49.6	59.0	64.6
Ma-002	14.3	33.1	35.7	54.8	67.3	74.0
Mean	**13.5**	**25.7**	**35.3**	**46.7**	**56.9**	**64.8**
$LSD_{(5\%)}$	**0.4**	**2.1**	**2.5**	**2.9**	**3.0**	**3.1**