Diversity of *Colletotrichum lindemuthianum* and reaction of common bean germplasm to anthracnose disease

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DECLARATION

The work contained in this thesis is entirely mine. It has never been submitted for the award of any degree or its equivalent in this university or in any other higher institution of learning elsewhere, and unless indicated, the work is entirely through my effort.

…….. …….

Mwesigwa Jasper Batureine Date

This thesis has been submitted with approval of the following University supervisor

……. …….

Tusiime Geoffrey, PhD Date

DEDICATION

To my parents Mr. and Mrs. **Batureine**, my wife **Sylvia** and my daughter **Gabriella**

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TABLE OF CONTENTS

	Declaration ……	i
	Dedication ……..	ii
	Acknowledgements ……...	iii
	Table of contents ……...	iv
	Acronyms ……..	ix
	Abstract ……..	x
	CHAPTER ONE ……..	1
	Introduction …….	1
1.1	Origin and Importance of Common Beans ……	1
1.2	Major uses and importance of beans in Uganda ……	2
1.3	Bean production in Uganda ……...	2
1.4	Bean production constraints ……..	4
1.5	Bean anthracnose as a major production constraint ……..	5
1.6	Justification ……	6
1.7	Goal ……...	10
1.8	Specific objectives of the study …….	10
1.9	Hypotheses……..	10
1.10	Research outputs ……	11
	CHAPTER TWO …….	12
	Literature review …….	12
2.1	The host (*Phaseolus vulgaris*) gene pool ……..	12
2.2	The anthracnose pathogen (*Colletotrichum lindemuthianum*) ……..	12
2.3	Classification and biology of *Colletotrichum lindemuthianum* …….....	13
2.4	Morphology and etiology of *Colletotrichum lindemuthianum* …….....	13
2.5	Epidemiology of *Colletotrichum lindemuthianum* ……..	14
2.6	Major symptoms, host range and yield losses associated anthracnose …..	16
2.7	Control strategies of bean anthracnose ……..	18

2.8	Breeding for resistance and its requirements ……	19
2.9	Pathogen variability ……..	20
2.9.1	Origins of pathogenic variability ……..	20
2.9.2	Variability of *Colletotrichum lindemuthianum* based on pathogenesis …	21
2.9.3	Determination and naming of *C. lindemuthianum* races using differential cultivars …….	22
2.9.4	Variability based on molecular characteristics ……..	24
2.9.5	Relationship between pathogenicity and molecular derived data …….	25
2.9.6	Implications of pathogenic variability in breeding for resistance …….	26
2.10	Characterization of *Colletotrichum lindemuthianum* pathotypes ……..	26
	CHAPTER THREE …….	31
	Physiological characterization of C*olletotrichum lindemuthianum* population in the major bean growing areas of Uganda …….	31
3.1	Introduction ……...	31
3.2	Materials and Methods ……..	32
3.2.1	Collection of fungal isolates ……..	32
3.2.2	Preparation of culture medium for *C. lindemuthianum* ……	35
3.2.3	Pathogen isolation from the bean pods and culturing on PDA …….	36
3.3	Raising of bean differentials …….	37
3.4	Multiplication of isolates for inoculum preparation …….	38
3.5	Preparation of spore suspension for inoculation ……	38
3.6	Inoculation …….	39
3.7	Data collection and Analysis …….	39
3.8	Results ……...	40
3.9	Discussion …….	42
	CHAPTER FOUR ……...	48
	Molecular characterization of C*olletotrichum lindemuthianum* ……..	48
4.1	Introduction ……...	48
4.2	Materials and Methods ……..	48

4.2.1	Raising of fungal cultures for DNA extraction …….	48
4.2.2	DNA extraction procedure ……	49
4.3	DNA quantification and dilution ……..	50
4.3.1	Checking for DNA quality by Electrophoresis …….	50
4.3.2	DNA quantification using a fluorometer ……..	50
4.4	Polymerase Chain Reaction (PCR) using RAPDs ……	51
4.5	Gel electrophoresis and visualization ……	52
4.6	Use of repetitive element-based polymerase chain reaction (rep – PCR)..	52
4.7	DNA Amplification with rep-PCR primers ……..	52
4.7.1	Scoring of gels ……...	53
4.8	Results ……...	54
4.8.1	RAPDs analysis …….	54
4.8.2	Wright's F statistics (FST) analysis ……..	60
4.8.3	rep-PCR fingerprinting …….	60
4.9	Analysis using a combination of RAPDs and rep-PCR data ……	65
4.10	Discussion and recommendations …….	65
	CHAPTER FIVE ……	70
	Reaction of Ugandan local and released bean genotypes to C*olletotrichum lindemuthianum* ……..	70
5.1	Introduction ……..	70
5.2	Common bean genotypes ……..	70
5.3	Raising of plants for inoculation ……...	71
5.4	Inoculum preparation …….	71
5.5	Inoculation …….	72
5.6	Data collection and Analysis …….	72
5.7	Results ……...	73
5.8	Discussion ……..	77
	CHAPTER SIX…….	79
6.1	**Conclusions and recommendations ……**	79

6.2 Summary of conclusions …….. 83
6.3 Recommendations ……. 84

List of Tables

Table 1 Analyzed isolates showing their district of origin, codes used, growth habit and gene pool of beans from which isolates were taken ……. 34
Table 2 Anthracnose differential series, resistance genes, host gene pool, and the binary number of each cultivar used to characterize races of anthracnose in common bean …… 37
Table 3 Symptom evaluation description according to Barladin et al. (1990) ….. 40
Table 4 Race, virulence phenotype and source of 47 *C. lindemuthianum* isolates analysed during the study ……. 41
Table 5 RAPD Primers that were used for analysis of molecular diversity of *C. lindemuthianum* …… 51
Table 6 Genetic characteristics of *C. lindemuthianum* isolates from four agroecologies in Uganda ……... 57
Table 7 Genetic variation among and within populations of *C. lindemuthianum* .. 57
Table 8 Genetic distance (GD) between isolates from different regions generated using RAPD analysis …… 60
Table 9 AMOVA resulting from rep-PCR analysis of *C. lindemuthianum* isolates …….. 64
Table 10 Genetic distance (GD) between isolates from different regions generated using rep-PCR analysis ……. 64
Table 11 Genetic characteristics of *C. lindemuthianum* populations in Uganda ….. 64
Table 12 Summary of ANOVA table for anthracnose severity on bean genotypes in Uganda (data shown is for both replicates) ……... 74
Table 13 Characteristics of Ugandan landraces and released bean genotypes and their reaction to races 3 of *Colletotrichum lindemuthianum* previously identified in Uganda ……. 74
Table 14 Summary of the reaction of 96 Ugandan bean varieties to race 3 of *Colletotrichum lindemuthianum* …….. 77

List of plates

Plate 1 Bean anthracnose symptoms on pods …… 18

Plate 2	Cultures of *C. lindemuthianum* growing on pure PDA media showing differences in sporulation …….	34
Plate 3	Representative gel picture showing separation of RAPDs PCR products of different isolates of *C. lindemuthianum* using OPAW 1 (left) and OPV 10 (right) primers …….	54
Plate 4	Gel run with primer OPA 9. Lanes 101 and 99 are isolates from Mbale ..	55
Plate 5	Part of a gel from Primer OPE 4: lanes 69-77 are isolates from Apac …..	55
Plate 6	Part of a gel obtained with primer OPAH 11: Lanes 66-75 are Apac isolates ……...	55
Plate 7	Part of OPAG 20 and OPG 2 gel ……..	56
Plate 8	Representative gel picture showing *C. lindemuthianum* fingerprints of selected Ugandan isolates based on BOX (left) and ERIC (right) primers	61
Plate 9	Part of a gel obtained with primer BOX ……	61
Plate 10	Part of a gel obtained with primer ERIC ……...	61
Plate 11	Symptoms of anthracnose on landraces susceptible to race 3 seven days after inoculation …….	73
	List of figures	
Figure 1	Dendrogram produced using UPGMA in the SAHN program of NTSYS-pc program showing distribution of isolates ……	58
Figure 2	A bootstrapped tree produced with Neighbour Joining using Treecon for windows program showing distribution of isolates ……..	59
Figure 3	Dendrogram produced from rep-PCR analysis showing distribution of isolates of *C. lindemuthianum* …….....	63
Figure 4	Distribution of *C. lindemuthianum* isolates on a dendrogram produced from combined RAPD and rep-PCR data …….	67
7	**REFERENCES ……**	86

ACRYNNOMS

AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
ANOVA	Analysis of Variance
BIC	Bean Improvement Cooperative
CIAT	Centro Internacianal de Agricultura Tropical
DNA	Deoxyribonucleic Acid
ERIC	Enterobacterial Repetitive Intergenic Consensus
FST	Wright's F statistics
MAAIF	Ministry of Agriculture, Animal Industry and Fisheries
MAS	Marker Assisted Selection
NARO	National Agricultural Research Organisation
PCR	Polymerase Chain Reaction
RAMs	Randomly Amplified Microsatellites
RAPDs	Randomly Amplified Polynorphic DNAs
REP	Repetitive Extragenic Palindronic
rep-PCR	Repetitive Element-Based Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RUFORUM	Regional Universities Forum (for Capacity Building in Agriculture)
UPGMA	Unweighted Pair Group Method with Arithmetic Average
ZARDI	Zonal Agricultural Research and Development Institute

ABSTRACT

The production of common bean (*Phaseolus vulgaris* L.) in Uganda is largely constrained by bean anthracnose (*Colletotrichum lindemuthianum* (Sacc.and Magn.), among other diseases. This pathogen is capable of causing total crop loss on susceptible varieties when planted in environments that are favourable to the pathogen. Previous results from Tanzania estimate that for each 1% increase in anthracnose incidence, seed yield decreases by 9kg/ha (Allen, *et al*., 1996). The most effective and appropriate control measure for this disease is the use of host plant resistance (Schwartz *et al*., 1982). Unfortunately, *C. lindemuthianum* is very variable, and this hinders the development and deployment of durable resistance (Scwartz *et al.,* 1982).

This particular work was aimed at characterizing Ugandan population of *Colletotrichum lindemuthianum* using bean differentials and molecular markers, and screening for anthracnose resistance among Ugandan local and released bean genotypes. The two methods of pathogen characterization were also compared for efficiency. Twenty one Random Amplified Polymorphic DNAs (RAPDs) markers, two rep-PCR primers and virulence on a set of 12 bean anthracnose differential cultivars were used to assess the genetic and physiological diversity of 74 *C. lindemuthianum* strains isolated from Kabale, Mbale, Apac, Mpigi and Wakiso districts. On the other hand, ninety six Ugandan local and improved bean germplasm were also screened for resistance against one highly virulent Ugandan bean anthracnose race (race 3).

Analysis of molecular data resulted into 3 major clusters (Fig. 1 and 2). Comparison of results from the race differentials with those from the molecular techniques did not show a strong relationship. This could partly be due to apparent intra-race diversity revealed using genome wide spanning molecular markers. This implies that such markers are not suitable in assessing relationships between pathogenicity and molecular marker derived fingerprints.

Pathotype characterization of 47 isolates with race differentials was carried out in the screen house at Kacwekano ZARDI, Kalengere substation in Kabale district and resulted into 21 races, of which 9 infected only Mesoamerican differential cultivars, where as 3 were virulent on Andean counterparts. Seven races infected cultivars from both gene pools, where as race 0 did not infect any of the differential cultivars. Races 1024, 1536, 1538, 1856, 1857, 1989, 3086 and 4033 were the most virulent as they incited symptoms on either or both of the highly resistant differential cultivars AB136 and G2333 with an average severity level of 4. Races 0 and 6 were the most wide spread; they were isolated from the districts of Kabale, Mbale and Apac (race 0) and Mpigi and Kabale (race 6).

Lastly, inoculum from race 3 of *Colletotrichumum lindemuthianum* was used to infect 96 bean genotypes in two seasons. Of the 96 genotypes screened, 56% (54 genotypes) were susceptible while the rest were resistant. In this experiment, Andean genotypes were the most affected (accounting for 73% of total infected genotypes) than Mesoamerican genotypes (Table 14).

CHAPTER ONE

INTRODUCTION

1.1 Origin and Importance of Common Beans

The common bean (*Phaseolus vulgaris* L) is a domesticated plant, which originated from Mexico in Central America (Purseglove, 1988). It belongs to the class Magnoliopsida in the Leguminoceae family. The crop grows in many environments ranging from tropical, subtropical to the temperate regions of the world. In East Africa, it was introduced by the Portuguese in the sixteenth century, and since then, it spread into many parts of Africa. All the bean genotypes generally originated from two gene pools; the Andean (typically large seeded) and the Mesoamerican (small seeded) gene pools. Common beans are further classified into: the dry bush type, which is the most widely cultivated, and the climbing bean type, a recent innovation with high yielding potential and suitable in areas where the land is limiting. In Africa, diversity of beans is highest in the Great Lakes Region where they are produced, marketed and consumed as complex varietal mixtures. Beans provide a cheaper source of protein than the animal form, making it highly competitive and important in dietary regimes of poor people in Africa. In East Africa, beans are rated as the second most important source of human food dietary protein and the third most important source of calories of all agricultural commodities produced (Pachico, 1993). Beans also provide valuable sources of Vitamin B complex, iron, zinc, sulfur and other essential minerals (CIAT, 1997). Bean production is associated with high human population densities (Wortmann *et al.*, 1998) and this is because they are easily grown, are suitable for intercropping and have short growing cycles ranging from 60 to 90 days.

1.2 Major uses and importance of beans in Uganda

Common beans are grown throughout the year and occupy the highest acreage among all the legumes grown in Uganda (NARO, 1998). The crop is a major component of the diet of many Ugandans. Traditionally, beans have been grown for home consumption but are now increasingly produced for income generation. Many bean parts are used for food: leaves, green pods, fresh and dry grain are the most important, of which fresh pods and dry grains are the most marketed products (*http://www.africancrops.net/rockefeller/crops/beans/index.htm*). The crop is usually grown on a subsistence scale with little or no external inputs and under poor farming conditions of pests, diseases, poor soil fertility and use of unimproved seed. The crop provides about 25% of the total calories and 45% of human protein intake in Uganda (Pachico, 1993; CIAT, 1997). It plays an important role in mitigating protein malnutrition effects since the basic foods consumed in this country are all starchy. The protein content of beans is about 22.1%, an amount far higher compared to that available in any of the staple foods in the country.

1.3 Bean production in Uganda

Beans are produced in all districts of Uganda. According to Ministry of Agriculture, Animal Industry and Fisheries (MAAIF) summary of the national agricultural statistics 2005/2006, dry bean production in Uganda between 2005 and 2006 averaged 665,000 metric tons and ranked 5th in terms of volume just after bananas, maize, sweet potatoes and cassava (MAAIF, 2006). At the current (June, 2008) domestic market price of dry beans, this is worth about $835 million. Greatest bean production is in areas with adequate and well distributed rainfall and productive soils. These include the districts of

Kabale, Kapchorwa, Mbale, Kisoro, Apac and Lira (Opio *et al*., 2001). Bean production in Uganda can be categorized into 8 major production areas, according to agroecological zones. The production areas are defined based on altitude, amount of rainfall per bean growing season, mode of rainfall and soil pH (Wortmann and Allen, 1994). These factors affect bean production in different ways leading to the observed yield variations among the districts of Uganda. Beans are a major source of both food security and income for most rural farm households that produce them (CIAT, 1997). Economically, the crop can be used either as food, sold domestically for cash or exported to earn foreign exchange (CIAT, 1997).

Since 1960s, the national beans research program has released improved bean varieties with different physiological and genetic characteristics as well as farmer preferences. Examples include NABE 1 up to NABE 6; NABE 11 to NABE 14, K20, K132, K131, all of which are bush varieties, and NABE 7C to NABE 10C, which are climbers (Opio *et al*., 2001). The bush varieties are grown in most of the bean growing areas of Uganda, either as a monocrop or intercropped with various crops like cassava, maize, bananas, cotton, sorghum, depending on the area (Opio *et al*., 2001). Bush varieties mature more quickly than the climbers. Climbers are mainly grown in the highly populated highlands of Kabale, Kisoro and Mbale. In comparison, they yield two to three times higher (up to 1.5 T/Ha) than the bush types.

Bean production in Uganda mostly occurs in traditional systems on small plots of land averaging 0.5 – 1 acres, where farmers use few modern purchased inputs. Most of the

seed is retained from previous season or is purchased from local produce markets (Mugisa-Mutetika, 1994). In addition, farmers also share bean seed with fellow farmers to ensure reciprocity (David, 1994).

1.4 Bean production constraints

Beans are vulnerable to a number of production constraints, which is culminated by the fact that most growers (small-scale farmers) lack the resources and ability to protect their crop against these many constraints that afflict them especially in tropical environments (Wortmann and Allen, 1994). Both biotic and abiotic constraints hold back common bean production. Biotic constraints in order of descending importance in Africa include angular leaf spot, anthracnose, bean stem maggot, bruchids, and root rots among others (*http://www.africancrops.net/rockefeller/crops/-beans/index.htm*). Major abiotic constraints include nitrogen and phosphorus deficiency, low pH complex and drought. Severity of root rots and bean stem maggot is often aggravated by certain abiotic stresses.

Generally, about 200 pathogens (fungal, bacterial and viral) are known to attack the common beans, but less than a dozen can cause substantial economic loss (Allen *et al*., 1998). In Uganda, bean production faces a number of constraints, among which, soil fertility, drought and pests and diseases are the most important (Opio *et al*., 2001). Hansford (1938) listed twenty diseases on beans in Uganda, out of which, ten are most important depending on ecological zone. Common bacterial blight (*Xanthomonas campestris* pv *phaseoli*), angular leaf spot (*Phaeoisariopsis griseola*), bean rust

(*Uromyces appendiculatus*) and bean common mosaic virus (bean common mosaic virus) are more serious in the low altitude high temperature areas, while halo blight (*Psuedomonas syringe* pv. *phaseolicola*), anthracnose (*Colletotrichum lindemuthianum*), ascochyta blight (*Phoma exigua var. exigua*), and root rots (*Pythium ultimatum, Rhizoctonia solani, Fusarium solani f. sp. phaseoli*) are more important in high altitude low temperature areas of Uganda. Many of the preferred landraces and popular commercial varieties are susceptible to these diseases and are targets of the breeding efforts carried out in the whole of Africa. Bean anthracnose is able to cause total crop loss on susceptible varieties in severe cases. The disease is best controlled by use of resistant varieties; however, Simbwa-Bunya (1972) demonstrated that anthracnose can be controlled chemically using Dithane M-45 at a rate of 3.4kgha^{-1}. Pyramiding genetically diverse resistance genes using MAS and deploying different gene combinations in different regions has been proposed as the most practical and realistic approach to provide efficient long-term control of bean anthracnose (Balardin and Kelly, 1998) in areas where both Andean and Mesoamerican anthracnose races were found. When selecting for resistance in a particular region, Melotto *et al*. (1999) suggested that the breeder should, if possible, choose a gene pair that, if deployed singly, would confer resistance to all known races in that region.

1.5 Bean anthracnose as a major production constraint

Bean anthracnose caused by the fungus *Colletotrichum lindemuthianum* (Sacc.and Magn.) is one of the most destructive diseases of common beans in Uganda. It infects all major bean parts like leaves, stems and pods and is seed transmitted. The disease can

result in total crop loss (Pastor-Corrales and Tu, 1989; Opio *et al.*, 2001) especially when infected seed is planted. In Uganda, the disease is particularly important in the high altitude areas that are characterized by cool temperatures and high relative humidity. It thrives best during wet conditions. The pathogen survives on infected crop debris and seeds and can survive in soil for up to three years. In the field, the disease spreads through rain splash and wind.

Bean anthracnose is managed through use of disease free certified seed, use of resistant varieties, crop rotation (of 2 to 3 years), field sanitation and many other cultural measures and chemical application (Tesfaye, 2003). Among these control strategies, the most effective and appropriate is one that intergrates host plant resistance (Schwartz *et al.*, 1982; Allen *et al.*, 1998). Host plant resistance is also the most affordable approach for small scale resource poor farmers (Opio *et al.*, 2001) like those in Uganda.

1.6 Justification

In Uganda today, most market-class varieties including the recently released ones are susceptible to anthracnose. In severe situations, the disease reduces a significant volume of expected harvests thereby causing food shortages and loss of income to Ugandan farmers. The blemishes on pods and the distortion and discolouration of the grain due to anthracnose all together reduce the market value of the infected crop (Luis and Bradley, 2002). In addition to the released varieties, a number of common bean breeding lines and landraces available are also susceptible to anthracnose or their reaction to the fungus is

unknown, thereby limiting their deployment in programmes for improvement of anthracnose resistance.

The pathogen exists in many physiological forms in all bean growing regions and infects bean genotypes from both bean gene pools (Mahuku and Riascos, 2004). Earlier, Leaky and Simbwa-Bunya (1972) identified five anthracnose races; later in 2006, Nkalubo acknowledged the existence of seven new and one previously identified bean anthracnose races in Uganda. These studies, however, were not comprehensive enough to give a clear picture about both molecular and physiological variability of this pathogen. The existing physiological forms (races) of this pathogen are therefore still largely unknown. This means that the fate of Ugandan useful but susceptible bean germplasm is also not known. Knowledge of the race structure of the pathogen population is a prerequisite for developing durable resistance in bean varieties.

Efforts to introduce resistance into bean varieties or to breed new ones have been ongoing in Uganda and elsewhere in the world (Opio *et al*., 2001; Alzate-Marin *et al*., 2005). More than 10 different anthracnose resistance genes have been identified in a number of bean varieties (Kelly and Vallejo, 2004). Cultivar G2333, a climber released in the great lakes region as Umubano and in Uganda as NABE 10C carries three complementary genes; Co - 4^2, Co – 5 and Co – 7 (Kelly and Vallejo, 2004) and so far is the most anthracnose resistant variety. It is currently the best choice as a donor for anthracnose resistance genes. Fortunately enough, this cultivar is widely adapted and yields heavily (Allen *et al.*, 1998). Co - 4^2 confers the broadest resistance to most known *C.*

lindemuthianum isolates. This gene could be of great value if introduced into Ugandan commercial bean germplasm.

The race structure of *C. lindemuthiaum* is highly variable and new ones reportedly keep emerging time after time (Leaky and Simbwa-Bunya, 1972; Pastor-Corrales and Tu, 1987, Nkalubo, 2006). The existence of this variability and emergence of new pathogen races of this pathogen results into continuous breakdown of resistance (Bigirimana and Hofte, 2001). This factor is a big threat against creating bean cultivars with durable resistance to anthracnose (Alzate – Marin *et al*., 2001). The possible co-evolution of the pathogen and its host in the Andean and Mesoamerican gene pools (Pastor-Corrales, 2004) provides a useful means of identifying appropriate sources of resistance since common bean genotypes originating from one gene pool are more likely to express resistance to pathogenic races from another gene pool (Allen *et al*., 1998). Durable resistance to anthracnose can therefore be achieved if appropriate genes from both centres of domestication of beans are pyramided. However, it is necessary to first characterize the *C. lindemuthianum* isolates from major bean growing zones of Uganda if durable resistance is to be developed, so that the right races are put into consideration. Such races will have to be highly virulent and/or most wide-spread in the major bean growing regions of the country.

Different tools are used for the classification of *Colletotrichum lindemuthianum* races. The common ones are the use of bean differentials and molecular markers. The use of common bean differential series is the traditional method for race identification (Pio –

Ribeiro, 1975). This procedure although useful requires strict control of conditions and is often affected by the subjective assignment of races by the evaluator (Gonzalez *et al.*, 1998). Quite often therefore, it results in misclassification of isolates (Vilarinhos *et al*., 1995). However this procedure is still very useful in studying the variability in the virulence of this pathogen to different host genotypes.

Recently, molecular markers have become very popular in pathogen characterization. They have a great advantage of being environment-neutral (Mahuku and Riascos, 2004). These markers sample a large portion of the genome and can be more informative in assessing variability within organisms (Melotto *et al*., 2000). Molecular markers are fast and safe alternative for differentiating and characterizing fungal isolates. These methods have also been employed in the characterization of *Colletotrichum lindemuthianum* (Melotto *et al*., 2000). Although molecular methods are fast, there have been contrasting conclusions from diverse workers especially on whether there is congruence between molecular and bean differential derived information (Mahuku and Riascos, 2004). Unless a reasonable relationship between the two methods is established, we may not be able to utilize the advantages of the fast molecular tools for race classification. This kind of information needs to be established for the Ugandan isolates. This classification work, therefore, will reveal the relationship between molecular derived fingerprints and the pathogenic races as determined using race differentials on one hand and any relationship with the geographical location of the strain.

1.7 Goal

The overall goal of this research is to set a foundation for developing high yielding market-class bean varieties that are resistant to bean anthracnose.

1.8 Specific objectives of the study

The specific objectives of the study, therefore, are:

1. To characterize Ugandan *Colletotrichum lindemuthianum* isolates using bean differentials and molecular markers; and establish the relationship between the two protocols.
2. To evaluate the reaction of Ugandan bean germplasm against selected races of *C. lindemuthianum*.

1.9 Hypotheses

1. Diversity of Ugandan *Colletotrichum lindemuthianum* can be revealed by both their pathogenesis on bean differentials and by DNA analysis; and molecular analysis will give better picture of pathogen variability than pathogenesis.
2. Diverse Ugandan bean genotypes react differently against Ugandan races of *Colletotrichum lindemuthianum*.

1.10 Research outputs

1. Races of *Colletotrichum lindemuthianum* in the study areas were identified

2. Molecular characteristics of Ugandan *Colletotrichum lindemuthianum* races were established

3. The reaction status of Ugandan bean germplasm against anthracnose races was documented

4. The relationship between molecular characteristics and physiological races of *Colletotrichum lindemuthianum* in Uganda was established

CHAPTER TWO

LITERATURE REVIEW

2.1 The host (*Phaseolus vulgaris*) gene pool

Wild common beans were characterized into two major gene pools, the Andean and the Middle American, based on phaseolin seed storage protein variation (Gepts and Bliss, 1986; Gepts, 1990), marker diversity (Becerra and Gepts, 1994; Koenig and Gepts, 1989), and morphology (Gepts and Debouck, 1991). The Middle American gene pool, consisting of races Durango, Jalisco, and Mesoamerica is represented by the medium and small seeded pinto, pink, black, white, and some snap beans. The Andean gene pool, consisting of races Nueva Granada, Peru, and Chile, is represented by the large-seeded kidney, cranberry, and many snap beans (Phillip *et al*., 2004). These gene pools are said to have variable resistance genes against most bean pathogens. Bean breeders have a unique opportunity to utilize resistance genes from the two major gene pools to develop complementary resistance to a range of bean pathogens.

2.2 The anthracnose pathogen (*Colletotrichum lindemuthianum*)

Anthracnose is perhaps the most economically important and widespread disease of the common beans (CIAT, 1997). Common bean anthracnose was first described and recorded in 1875 on plant specimens which had been obtained from Germany. The fungus *Colletotrichum lindemuthianum* **(**Sacc. and Magn.) Scrib. causing the disease had, however, been collected by mycologists as early as 1843. The fungus is known to have races that vary from, country, region, location, and variety, to another (CIAT, 1997). Today, the disease is reportedly one of the most important and widely distributed

throughout the world. It is found in Latin America, Asia, Europe, USA and Africa (Ansari, 2004). In Africa, it is particularly important in Uganda, Kenya, Tanzania, Rwanda, Burundi, Ethiopia and DR Congo.

2.3 Classification and biology of *Colletotrichum lindemuthianum*

C. lindemuthianum is an ascomycete and produces its conidia in acervuli. This fungus belongs to the genus Colletotrichum, order Melanconiales, family Melanconiaceae and section Hyalosporae (Alexopoulos, 1962). The fungus is found in nature in a conidial (imperfect) stage, but can overwinter as mycelia or conidia. The pathogen's perfect stage, *Glomerella cingulata* is rarely found in nature. Its conidia are oval shaped and dark brown in colour (Agrios, 1997). On the host, they form pink masses of conidia packed into the acervuli. *Colletotrichum lindemuthianum* differs from other species in this genus by its growth characteristics and a dark pigmentation on cultures (Tesfaye, 2003).

2.4 Morphology and etiology of *Colletotrichum lindemuthianum*

The conidia of *Colletotrichum lindemuthianum* are born on acervuli. The acervuli are mostly in groups, coalescing and covering lesions on infected plant parts. Setae are few, longer than the conidial mass. The conidial masses are orange to bright orange. Mycelia are scanty and white. Conidia are hyaline, oblong to dumble shaped, one-celled, straight ends rounded. Conidial size is about 9-15x3-4µm (Mathur and Kongsdad, 2000). Conidia are uninucleate, and usually have a clear vacuole-like body near the centre.

Colletotrichum lindemuthianum has a unicellular conidium which, in an aqueous environment, produces a single germ tube. The conidium germinates with in six to nine hours and produces one to four germ tubes (Zaumeyer and Thomas, 1957). Upon contact with a hard surface, the germ tube tip swells and differentiates into a thick-walled, heavily melanized appressorium. A penetration hypha arises from below the appressorium and penetrates cuticle and host cell wall during pathogenesis. Inside the cell lumen, a globose infection vesicle develops, which in turn gives rise to a primary hypha (Zaumeyer and Thomas, 1957). Following infection, the symptoms begin to show after three to seven days depending on the prevailing environmental conditions (Hirst and Stedman, 1963).

2.5 Epidemiology of *Colletotrichum lindemuthianum*

C. lindemuthianum survives in bean crop residue and seed (Barrus, 1921; Tu, 1983; Pastor-Corrales and Tu, 1989). In areas where beans are continuously cropped, previous seasons' inoculum can initiate epidemics of anthracnose (Dillard *et al*., 1993). Its longevity in infected pods and seed varies considerably depending on environmental conditions; moisture and temperature being the most important factors influencing the survival of the fungus. Anthracnose conidia are spread from one plant to another mainly by splashing raindrops (Hirst and Stedman, 1963). The average distance of conidia spread is reported to range from 3 to 4.6 m per rainstorm of 10 mm or more (Tu, 1992). A 10-h wet period in a humid (>92%) environment are necessary for *C. lindemuthianum* conidia to infect, and new lesions usually appear in 3 to 7 days (Hirst and Stedman, 1963). Although plant residues contribute greatly to pathogen survival and distribution, infected

seed serves an important role in the long distance distribution of the anthracnose pathogen (Tesfaye, 2003). In cases where poor farmers continuously exchange and use infected seed, like here in Uganda (Opio *et al*., 2001), the pathogen is capable of being distributed throughout all bean growing regions of the country (Leaky and Simbwa-Bunnya, 1972). It has been found out that different tillage methods can influence anthracnose development and distribution in the dry bean fields (Ntahimpera *et al*., 1997).

In addition to seed and plant residues, human clothing, insects, machines, and animals may also disperse *C. lindemuthianum* to healthy plants (Tesfaye, 2003). The pathogen can remain viable in seed for 3-5 years and the farmers who retain seed from previously grown crop (like those in most parts of Uganda), most probably contribute to the carry over and spread of the disease (Leaky and Simbwa-Bunnya, 1972). Once on the plant, lesions on cotyledons usually serve as sources of secondary inocula (Tesfaye, 2003). Here on the leaves, conidia are washed down in water to the hypocotyls and subsequently the stems. Rain splash from the leaves introduces the inocula to the soil, also foci of secondary infection (Tesfaye, 2003).

The spread of anthracnose from the initial infection point in the field depends on the speed and direction of wind. Prevailing wind associated with rain splash is an important factor determining spread of anthracnose (Ntahimpera *et al*, 1996). Long distance dissemination (3-5m) may develop from raindrops being blown by gusting winds (Tu, 1983). The number of foci of initial inoculum in the field also contributes, and is linearly

related to the incidence of anthracnose on plant leaves (Tesfaye, 2003). Under field conditions, anthracnose incidence is highest on leaves during the rainy season and highest on pods during the dry season (Tesfaye, 2003). The disease spreads rapidly by spores carried in splashing raindrops, or through human activities or implements that come in contact with diseased plants (Tu, 1983) and in one growing season, one diseased plant can spread the disease to other plants with in a 30m radius.

Specifically, for optimal survival of *C. lindemuthianum* and the development of anthracnose, the conditions of high relative humidity (>92%), temperature of between 18^{o}c and 22^{o}c (Tu, 1983) and moderate rainfall at frequent intervals (Tu, 1983) are required. Moderate temperatures between 13^{o}c and 26^{o}c favour infection, with an optimum at 17^{o}c. High humidity or free moisture must be present for infection to occur successfully. Local dissemination of conidia and development of anthracnose epidemics also require moderate rainfall at frequent intervals (Pastor – Corrales and Tu, 1989; Pastor – Corrales *et al*., 1995).

2.6 Major symptoms, host range and yield losses associated with anthracnose

Seedlings grown from anthracnose-infected seeds often have dark brown to black sunken lesions on the cotyledons and stems. Severely infected cotyledons die prematurely, and growth of the plants is retarded (Kelly and Vallejo, 2004). Diseased areas may girdle the stem and kill the seedling. Under moist conditions, small, pink masses of spores are produced in the lesions. Spores produced on cotyledon and stem lesions may spread to the leaves. On leaves, symptoms generally occur on the underside as linear, dark brick-

red to black lesions on the leaf veins. As the disease progresses, discolouration appears on the upper leaf surface. Leaf symptoms often are not obvious and may be overlooked when examining bean fields (Kelly and Vallejo, 2004). The most striking symptoms develop on the pods **(**Kelly and Vallejo, 2004). Small, reddish brown to black blemishes and distinct circular, reddish brown lesions are typical symptoms. Mature lesions are surrounded by a circular, reddish brown to black border with a grayish black interior. During moist periods, the interior of the lesion may exude pink masses of spores. Severely infected pods may shrivel, and the seeds they carry are usually infected. Infected seeds have brown to black blemishes and sunken lesions and are usually discoloured (Kelly and Vallejo, 2004). Characteristic bean anthracnose symptoms on pods are shown in plate 1.

Bean Anthracnose can cause losses of up to 100% particularly on susceptible varieties under favourable conditions (Pastor – Corrales and Tu, 1989), and also when infected seed is used. Production is reduced because of poor seed germination, poor seedling vigour, and low yields. There are also marketing losses, which are attributed to seed spots and blemishes, which lower their quality rating and salability. The disease is most common and severe on dry and snap *beans (P. vulgaris*) but may also affect lima bean (*P. lunatus* L.), scarlet runner bean (*P. multiflorus* Willd.), mung bean (*P. aureus* Roxb.), cowpea (*Vigna sinensis* Savi.), and broad bean (*Vicia faba* L.) (Chaves and Sherf, 1960).

Because of its importance, bean anthracnose is a major subject of research by a number of national bean programs in Africa, with the objective of developing technologies for its

control which are appropriate and acceptable to resource-poor, small scale farmers (CIAT, 1991).

Plate 1: Bean anthracnose symptoms on pods

2.7 Control strategies of bean anthracnose

No single method is effective against bean anthracnose. An integration of a number of methods is recommended. Bean anthracnose is best controlled by using disease-free seed. Seed produced under wet and humid conditions should not be planted as it is in most cases already infected. Crop rotation of at least three years also helps to eliminate or reduce the inoculum in the fields. In addition, fields should not be worked when plants are wet because fungal spores are easily spread from diseased to healthy plants under these conditions (Tu, 1986). Scouting the fields weekly for symptoms of the disease is recommended so that seed from plants that are infected with the anthracnose pathogen is not harvested as such will spread the disease in future.

Fungicides containing chlorothalonil, maneb, zineb, benomyl, captafol or folfet have also been recommended at first sight of the disease and reapplied weekly during the season

(Agrios, 1997). Chemicals like Maneb and Zineb at 3.5g/l, Benomyl at 0.55g/l (Beshir, 1997), Captafol at 3.5kg/ha, Carbendazim at 0.5kg/ha and Fentinhydroxide at 1.2g/l (Pastor-Corrales and Tu, 1989) have been reported to be effective in controlling bean anthracnose. Tesfaye (2003) demonstrated that a benomyl seed dressing followed by a foliar difenoconazole spray, or difenoconazole application alone, showed a promising anthracnose control strategy.

However, resistance is the most effective and efficient method of anthracnose management (Scwartz *et al.,* 1982; Esteban *et al.*, 2003).In Uganda, cultivar G2333 (released as NABE 10C or Umubano) is the best recommended resistant variety. It has the broadest known resistance and carries three complementary genes that confer resistance against most known races of *Colletotrichum lindemuthianum* (Young *et al*., 1997). Integrating resistance with the above described measures gives a good control of the disease. Cultivar mixtures containing at least 60% of a resistant cultivar have been reported to offer a good control of anthracnose (Tesfaye, 2003).

2.8 Breeding for resistance and its requirements

Durable resistance is the most important way of controlling *Colletotrichum lindemuthianum* (Schwartz *et al.,* 1982). The pathogen has got variable physiological races (Pastor-Corrales and Tu, 1987) and new ones keep emerging from time to time (Mesquita *et al*., 1998; Gonzalez *et al*., 1998; Bigirimana and Hofte, 2001; Ansari *et al*., 2004; Vilarinhos *et al*., 1995; Barladin *et al*., 1997*;* Tesfaye, 2003; Alzate-Marin and Aloisio Sartorato, 2004; Sartorato *et al*., 2004; Mahuku and Riascos, 2004). This is

responsible for the fast break down of resistance (Haciwa, 1991; Alzate – Marin *et al*., 2001). In Uganda, for example, the variety K20, which was released in 1978, was resistant to anthracnose at that time, but has currently become one of the most susceptible varieties in Uganda. CIAT, NARO, Makerere University and other research organizations in the great lakes region are currently doing research on bean anthracnose with the ultimate goals of coming up with resistant varieties. Durable resistance is also not easily achieved since many genes are required in the same background to guard against the variable races. Knowing the biology, ecology and diversity of *Colletotrichum lindemuthianum* races from major bean growing regions (Tesfaye, 2003) is a pre-requisite for most anthracnose resistance breeding programs (Buruchara, 1991) and this knowledge is needed to successfully develop and deploy resistance against the pathogen in particular regions (CIAT, 1996). Durable resistance can be created based on the known races by introducing the right genes to control them (Haciwa, 1991; Ogallo, 1991).

2.9 Pathogen variability

2.9.1 Origins of pathogenic variability

New races of plant pathogens arise by sexual mechanisms like recombination of nuclear genes during sexual reproduction, exchange of genetic materials in somatic cells, mutation, or by extra chromosomal variation (Singh, 1986 – Cited by Ogallo, 1991). Pathogens which reproduce sexually (like *Colletotrichum* species) are expected to produce variants more readily than those which are mostly asexual (Ogallo, 1991). Recombination of genes of two parental nuclei take place in a zygote during sexual reproduction, and the haploid nuclei or gametes resulting from meiosis are different, both

from those of the parents and from each other. Therefore, every haploid pathogen individual is generally genetically different from any other pathogen, even within the same species. It is important to note that when parasites evolve faster than their hosts (which is the case of *C. lindemuthianum*); they have an evolutionary advantage because they can quickly track the changes of the local host population, leading to their local adaptation (Barret, 1988 – Cited by Capelle and Neema, 2005). It is therefore desirable for the hosts to evolve faster than their pathogens if durable host resistance is to be achieved. Crop protection scientists and breeders should therefore devise alternative management measures as well as develop resistant bean varieties so that bean improvement matches with the prevailing rate of pathogen evolution.

2.9.2 Variability of *Colletotrichum lindemuthianum* based on pathogenesis

C. lindemuthianum attacks susceptible varieties grown under moderate to cool temperatures and high relative humidity (Pastor-Corrales and Tu, 1989). There are many anthracnose pathogen variants or physiological races (also called pathotypes or virulence phenotypes – CIAT, 1997) and are identified by their reactions on a set of host varieties commonly referred to as host differentials. Different variants (races) differ from each other primarily on the basis of their pathogenicity (Agrios, 1997). Pathogenic variability in *C. lindemuthianum* was first reported in 1911 (Barrus, 1911) and since then, several races of this fungus have been reported in literature (CIAT, 1997; Mesquita *et al*., 1998; Gonzalez *et al*., 1998; Ansari *et al*., 2004; Vilarinhos *et al*,.1995; Barladin *et al*., 1997*;* Tesfaye, 2003; Ana *et al*., 2004; Sartorato *et al*., 2004, Mahuku and Riascos, 2004). These researchers have shown a high variability of the pathogen using the international

set of bean differentials for the classification of *C. lindemuthianum* races (section 2.9.3). Alzate-Marin *et al.* (2004) for example identified a total of 50 *C. lindemuthianum* pathotypes in Brazil between 1994 and 2002, whereas Mahuku and Riascos (2004) identified 90 races from 200 *C. lindemuthianum* isolates collected from Andean and Mesoamerican bean varieties and regions.

Virulence diversity of this pathogen has also been reported in some areas of Africa (Tesfaye, 2003) and Europe where common bean has not traditionally been grown, and where climatic conditions differ from those of the two centres of origin of its host. Butare (unpublished data) identified 42 races of this pathogen out of 53 isolates from Rwanda. Sartorato *et al.*, (2004) used 24 races of *C. lindemuthianum* on 23 bean genotypes and only five bean genotypes were found resistant to all the races, the rest of the beans showed diverse reaction to the races. Generally, one bean cultivar may be resistant to some races, but not others (CIAT, 1997).

2.9.3 Determination and naming of *C. lindemuthianum* races using differential cultivars

Identification of a race is based on reactions of a set of differential cultivars differing in their genes for resistance or susceptibility to one or more races of the pathogen. Barrus was the first researcher to demonstrate variability with in *C. lindemuthianum* in 1911, and since then, many other researchers throughout the world came up with different magnitudes of variability within this pathogen. Because the occurrence of pathogenic variability in plant pathogens is of practical importance with direct implications in breeding for disease resistance, the methodologies employed in race determination, sets

of differential cultivars and the systems of nomenclature used by different workers should be uniform (Buruchara, 1991). This is done in order to avoid the possibility of giving the same race different names and vise versa. In order to obtain consistent and comparable results among different workers, the differential cultivars used should be internationally standardized, genetically pure and should be able to give clear resistant or susceptible reaction.

Previously, more than 40 different bean varieties were used as differential cultivars for the identification of races of *Colletotrichum lindemuthianum* (Buruchara, 1991). Besides, many different systems of race nomenclature were used. Subsequently, some anthracnose races were known by different names depending on the differential cultivars used and the system of nomenclature employed (Buruchara, 1991). For example, Goth and Zaumeyer (1965) used cultivars Michellite, Michigan Dark Red Kidney (MDRK), Perry Marrow, Cornell 49242, Sanilac and Emerson 51 to identify races Alpha, Beta, Gamma and Delta. However, Hubbeling (1976) used cultivars Michellite, MDRK, Perry Marrow and Emerson 847 to identify the same four races. This was a clear indication that these races were misclassified.

In order to curb this problem, a set of 12 differential cultivars (Table 2) and a system of nomenclature were proposed and adopted for use in identification of races of *C. lindemuthianum* by a team bean workers in Latin America during the first Latin American workshop on anthracnose held at Cali, Colombia in 1988 (Buruchara, 1991). The system of nomenclature that was adopted is called the binary system, which identifies a race by a number that is a result of sum of all binary values associated with a

cultivar when it gives a susceptible reaction to the race. A binary number is equal to 2^n, where n is equivalent to the place of the cultivar within the differential series order as given in table 2 (for example, the position of Michellite is 0 while that of G2333 is 11). The sum of all binary numbers of cultivars with susceptible reactions (i.e., $y = 2^n + 2^{(n+1)} + 2^{(n+2)} + \ldots\ldots 2^{(n+11)}$; where n=0) gives a specific race number or name (y). For example race 48 is virulent on Widusa (in position 4, binary number = 2^4 = 16) and Kaboon (position 5, binary number = 2^5 = 32). This method is currently used by all other researchers over the world in identifying races of *C. lindemuthianum* in many regions of the world (Kelly and Vallejo, 2004).

2.9.4 Variability based on molecular characteristics

Variability among races of *C. lindemuthianum* has also been studied by a number of scientists using different molecular tools. Their work have demonstrated the genetic status of the pathogen population using RAPD markers (Mesquita *et al.,* 1998, Balardin *et al*., 1997, Sicard *et al.*, 1997), AFLP markers (Ansari *et al*., 2004) or both (Gonzalez *et al.*, 1998), and RAMS and Rep-PCR (Mahuku and Riascos, 2004). Although in many cases scientists have reported great genetic variability within the pathogen stains, this has not been consistent throughout. Ansari *et al.* (2004) for example, using AFLP analysis, assigned 86 isolates from different regions of the world into 3 divergent clusters. On the other hand, Mesquita *et al*. (1998) reported that DNA – based molecular markers aided the classification of races 73, 65 and 64 of *C. lindemuthianum* in Brazil in which isolates from each race expressed genetic similarity. The genetic status of Ugandan *C. lindemuthianum* population is not known. It is also important to evaluate some of the

above techniques and establish whether they can be useful in race determination of this fungus.

2.9.5 Relationship between pathogenicity and molecular derived data

RAPD analysis of three anthracnose races 73, 65 and 64 (Mesquita *et al*., 1998) showed a complete congruence between their molecular and bean differential derived data. This work yielded three clusters each belonging to each of the three *C. lindemuthianum* races. However, the number of isolates that were analyzed their work (six from each race) was too small, and even their geographical origin was too limited to make a definite evolutionary statement. These findings, however, opened up the possibility for a rational use of molecular markers to aid the identification of races of *C. lindemuthianum* and guide resistance breeding better. Most other researchers, however, have hardly found any complete association between pathogenicity and molecular-derived data of this pathogen (Mahuku and Riascos, 2004; Balardin *et* al., 1997; Sicard *et al.,* 1997 and Fabre *et al*., 1995). For example, the work of Balardin *et al*. (1997) on 60 isolates from south, central and North America distinguished them into 15 clusters. In this particular study, no congruence between RAPD and virulence phenograms was realized, neither was there geographical nor host gene pool relationship. Later Mahuku and Riascos (2004) reported no relationship among 200 *C. lindemuthianum* isolates for geographical origin, host gene pool and virulence phenotype using random amplified microsatellites (RAMs) and repetitive-elements (Rep)-PCR primers. These differing results need further research in a different geographical location for validation.

2.9.6 Implications of pathogenic variability in breeding for resistance

Histological studies concerning host-pathogen interactions resulting from infection of a specific cultivar by a specific race of a pathogen showed that resistance to anthracnose mostly results from incompatibility brought about by hypersensitive death of host cells (Mercer *et al*., 1975). Hypersensitivity is normally associated with a high degree of race specific resistance, which tends to exert high selection pressure in favour of resistance breaking variants of the pathogens (Haciwa, 1991). Races in low proportions sometimes become more frequent and more widely distributed as a result of strong selection pressure in their favour (Pastor-Corrales and Tu, 1989). Resistance to anthracnose has therefore been short lived because it has depended on race specific resistance and the pathogen has expressed considerable variation by mutation, natural selection or other mechanisms (Pastor-Corrales and Tu, 1989). Breeders and pathologists must therefore identify broad and diverse sources of resistance which should be incorporated in their breeding programme; and to do this, uniform differentials for race classification and the methods for evaluation should be used (Haciwa, 1991). Careful deployment of such genes through pyramiding may possibly prolong resistance to anthracnose. With a large number of diverse resistance genes, there are less chances of the occurrence of mutation for virulence to match them (Haciwa, 1991).

2.10 Characterization of *Colletotrichum lindemuthianum* pathotypes

Colletotrichum lindemuthianum races are identified based on their reaction on a set of 12 bean cultivars (differentials), which differ in their genes for resistance or susceptibility to one or more races of the pathogen (Buruchara, 1991). An internationally recognized

binary system is used for this purpose (Buruchara, 1991). Differentials are inoculated with different pathogen isolates in order to characterize the latter, and races are assigned names or numbers according to the susceptible reactions on the differentials, using the binary system suggested by CIAT. Although this system is intended to establish uniform, consistent and comparable classification results for *C. lindemuthianum* races among different workers; several uncertainties still come up. For example, the environment, the number of spores, and incubation conditions used may vary considerably from one laboratory to the other, resulting into misclassification of a particular race (Ogallo, 1991). Also, the subjectivity of symptom evaluation may lead to misclassifications and to disagreements on classification of the same race by different research groups (Mesquita *et al.,* 1998*;* Vilarinhos *et al*, .1995). Characterization based on molecular differences is now encouraged alongside the traditional bean differentials.

Molecular characterization is based on molecular markers like RAPDs (CIAT, 1997), AFLPs (Ansari *et al*., 2004), RAMs and Rep-PCR (Mahuku and Riascos, 2004). Use of molecular markers has revealed various relationships between genetic, pathogenic and geographical origin of *C. lindemuthianum* (Ansari *et al., 2004;* Mesquita *et al*., 1998; CIAT, 1997). Mesquita *et al.* (1998), for example, reported three distinct groups after cluster analysis of three anthracnose races based on genetic distances revealed by RAPD analysis. These three clusters coincided with classification results based on inoculation of bean differentials. Their study underlined the usefulness of molecular procedures for the identification of races of *C. lindemuthianum*. Through their work, previously misclassified races were assigned new names and proven by inoculations on bean

differentials. Unfortunately, other studies have failed to establish a strong association between molecular and differential derived data (Sicard *et al.,* 1997 and Fabre *et al*., 1995).

Random Amplified Polymorphic DNAs (RAPDs) is a polymorphism assay which is based on the amplification of random DNA segments using sets of primers of arbitrary nucleotide sequence (Bruce, 1997). Small amounts of total genomic DNA are subjected to amplification using short oligonucleotides of random sequence. In theory, the primer anneals onto many regions of the genome simultaneously. The primers basically scan a genome for small inverted repeats and amplify intervening DNA sequences of variable length. RAPDs contradicts the general rules of DNA amplification, as unspecific primers are used, that anneal at low temperatures (35 ºC or even lower), without having prior sequence information. RAPDs have thus become widely used in molecular biology laboratories and are a powerful technique when a variety of samples have to be checked for species differentiation. However, RAPDs have some disadvantages, which require the researcher to perform a very thorough optimisation and exact reproducibility of experimental conditions, both within and between laboratories. RAPDs are dominant, so they cannot differentiate homozygotes and heterozygotes without a progeny test. RAPDs have only two alleles (amplification or nonamplification) for each amplicon locus, which is an ideal situation for genetic mapping, but on the other hand, a drawback for measures of genetic diversity that are affected by the number of alleles at a locus. They are also sensitive to small changes in PCR reaction conditions. However, because many samples can be analyzed in a short time and because RAPDs scan the entire genome, they are still a very useful fingerprinting technique.

In addition, for a RAPD assay, no previous genetic or molecular knowledge is required (Bruce, 1997). A universal set of primers of arbitrary nucleotide sequence is applied. A large number of RAPD primers are available, and only a small amount of DNA is required, making them an ideal tool for analysis. The RAPDs protocol is relatively quick, simple and cheap. Because a large number of amplicons can be screened in a relatively short period of time, RAPDs are especially useful in differentiating clonal lineages for fungi that reproduce asexually (Bruce, 1997). Furthermore, RAPD data are easy to interpret because they are based on amplification or nonamplification of specific DNA sequences (amplicons), producing a binary data set that is easy to enter into a spreadsheet for analysis.

Rep-PCR is based on the fact that in many bacteria, there are naturally interspersed repetitive DNA elements, which can serve as primer sites for genomic DNA amplification (Varsalovic *et al.*, 1991, 1994). Three families of repetitive sequences have been studied in most detail, including the 35-40bp repetitive extragenic palindronic (REP) sequence, the 124-127bp enterobacterial repetitive intergenic consensus (ERIC) sequence and the 154bp BOX elements (Versalovic *et al.*, 1994). These sequences are located in distinct, intergenic positions all around the chromosome. The repetitive elements may be present in both orientations on the chromosome, and PCR primers have been designed to "read outward" from the inverted repeats in REP and ERIC and from the boxA subunit of BOX (Versalovic *et al.*, 1994). The use of the above primers in PCR leads to the selective amplification of those distinct genomic regions located between REP, ERIC or BOX sequences. The corresponding protocols are referred to as REP-PCR, ERIC-PCR and BOX-PCR respectively, and are collectively referred to as rep-PCR. Rep-

PCR (Repetitive element-based polymerase chain reaction) technique combines the simplicity of the PCR with the polymorphism detected by restriction fragment length polymorphism (RFLP) and has been used for fingerprinting soil bacteria and plant-pathogenic bacteria (Aritua *et al*., 2007) and fungi (George *et al.*, 1998; Alves *et al*., 2004) and particularly *Colletotrichum lindemuthianum* (Mahuku and Riascos, 2004). Primers used in these studies correspond to conserved motifs in bacteria, for example, the repetitive extragenic palindronic (REP) sequence are from *Escherichia coli*, while the BOX element is from the gram-positive bacterium *Streptococcus pneumoniae*. Because of the conserved nature of these sequences, they allow amplification in many microorganisms, including fungi, and different degrees of polymorphism are detected for different genomes. This protocol is highly reproducible unlike RAPDs.

CHAPTER THREE

PHYSIOLOGICAL CHARACTERIZATION OF *COLLETOTRICHUM LINDEMUTHIANUM* POPULATION IN THE MAJOR BEAN GROWING AREAS OF UGANDA

3.1 Introduction

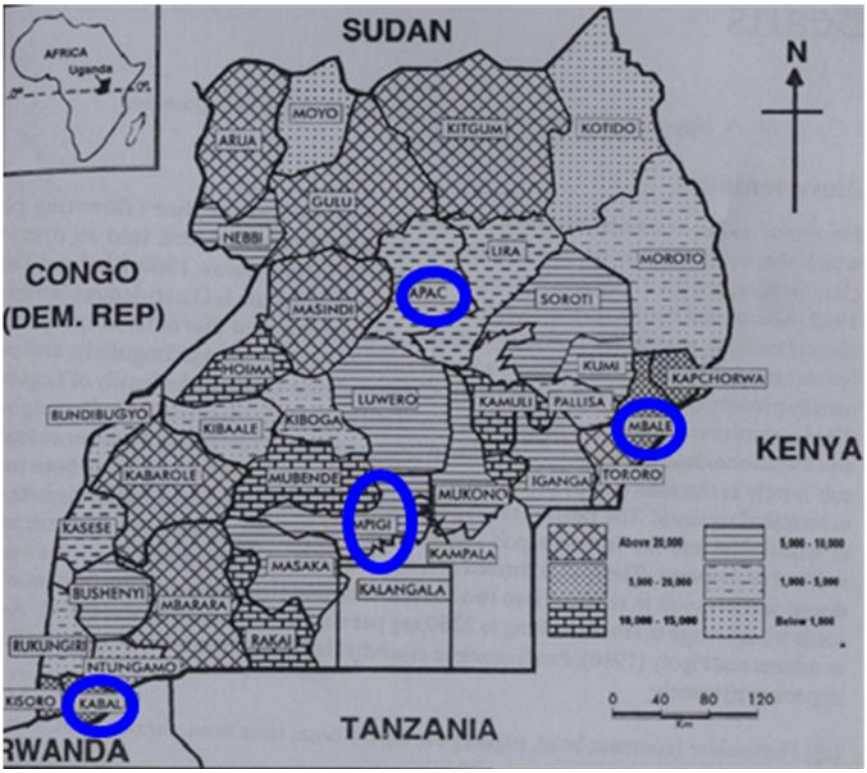
Bean anthracnose is a very destructive disease in Uganda. Its causal pathogen (*Colletotrichum lindemuthianum)* is highly variable and 12 races have previously been identified in Uganda (Nkalubo, 2006 and Leaky and Simbwa-Bunya, 1972). The pathogen has also reportedly been variable in other parts of the world (Mahuku and Riascos, 2004). Bean anthracnose disease has traditionally dominated in the highland areas because the pathogen thrives best under cool, wet and humid conditions. However, currently the disease can be seen in all regions where common bean is cultivated. The pathogen is seed borne, which facilitates long distance dispersal of inoculum. With the current trend in bean trade in the country, it is possible that the pathogen has been introduced and adapted to almost all major bean agroecologies. Use of resistant varieties is the most important and affordable management strategy of bean anthracnose to the majority of Ugandan farmers. However, adequate knowledge of the race structure of this pathogen most especially its molecular characteristics in Uganda is still lacking, yet it is a prerequisite for developing durable resistance. In order to generate reliable information about the race structure of the pathogen, good sampling methods should be employed. The spatial coverage of isolates analysed by previous researchers (Nkalubo, 2006 and Leaky and Simbwa-Bunya, 1972) was not enough to clearly represent the overall variability of this pathogen in Uganda. In this study, a set of 12 internationally recognized

anthracnose race differentials was used to characterize Ugandan isolates of *C. lindemuthianum*.

3.2 Materials and Methods

3.2.1 Collection of fungal isolates

Isolates were collected from five districts selected purposely to represent the major agro-ecologies of Uganda, the farmers' bean preferences and scale of production. These were Kabale (highland with diverse bean types), Apac (lowland, predominantly small-seeded bean types), Mbale (highland, with diverse bean types), Mpigi and Wakiso (lowland, predominant large seeded bean types). Bean pods with pronounced anthracnose symptoms were collected from naturally infected bean plants in farmers' gardens. The bean gene pools (Andean or Mesoamerican), plant growth habit (climber or bush) and variety identities from which the isolates were obtained were recorded whenever it was possible. In each district, samples were taken approximately 6km from each other. From each district, at least 3-10 sub counties were sampled from which a total of 123 isolates were gathered. From each garden, diseased pods were collected from at least 3 plants separated by 5-10 metres depending on the garden size. From each of the location, mono-conidial isolates were cultured on PDA using the procedures given below (sections 3.2.2 and 3.2.3). Below is the map of Uganda showing places from where the isolates were collected.



Map of Uganda showing districts (circled) from which Anthracnose samples were collected

From the 123 cultures isolated, 74 were selected for this study. They were selected to represent all types of the host (climber, bush, large seeded, small seeded, round seeded, seed colour), and geographical distribution. Out of the 74, 47 isolates were selected for race characterization. These were selected mainly on the basis of ease of sporulation, both on PDA and on autoclaved bean pods (Plate 2) and geographical distribution.

Each pure isolate culture was multiplied to get enough inoculum. The isolates were collected in the second season of 2005 and in the first season of 2006. The codes and origin of the study isolates are shown in Table 1.

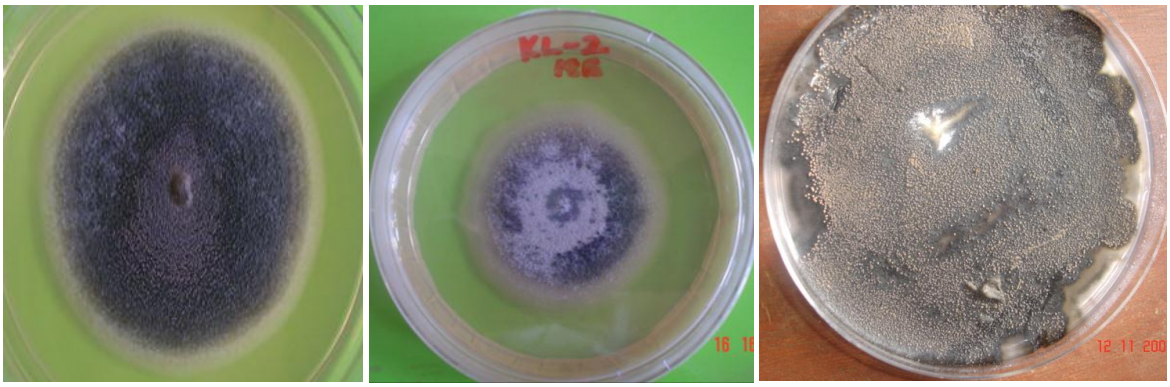


Plate 2: Cultures of *C. lindemuthianum* growing on pure PDA media showing differences in sporulation: Far right is a 6-day old culture from isolate 104, which was later characterized as race 3.

3.2.2 Preparation of culture medium for *C. lindemuthianum*

The pathogen was isolated on PDA. Standard PDA medium (24g of Potato Dextrose Broth (PDB) and 15g of granulated agar in one litre of distilled water) was prepared by mixing and autoclaving at 120^{o}C and 2 bars of pressure for 20 minutes. When it had cooled, 100µl of each of the antibiotics Chloramphenical (CAM) and Ampicilin (AMP) at concentrations of 40µl/ml and 13µl/ml respectively were added and then mixed thoroughly, and thereafter poured into 9cm Petri-dishes and allowed to set.

Table1: **Isolates that were analyzed both for physiological and molecular studies, showing their district of origin, codes used, growth habit and gene pool of beans from which isolates were taken.**

Isolate code	Growth habit	Bean Race	District of Origin
KLA	CL	Mesoamerican	Kabale
KLB	BS	Andean	Kabale
KLC	BS	Mesoamerican	Kabale
MBC	BS	Andean	Mbale
MPA	BS	Andean	Mpigi
MPB	BS	Andean	Mpigi
MPC	BS	Andean	Mpigi
MPD	BS	Andean	Mpigi
KLD	BS	Andean (Round)	Kabale

Continued from page 34

Isolate code	Growth habit	Bean race	District of origin
KLE	BS	Mesoamerican	Kabale
KLG	BS	Andean	Kabale
KLH	BS	Mesoamerican	Kabale
KLI	BS	Andean	Kabale
MBD	BS	Andean	Mbale
KLJ	CL	Andean	Kabale
MBF	BS	Mesoamerican	Mbale
MBG	BS	Mesoamerican	Mbale
MBH	BS	Andean	Mbale
WKA	BS	Andean	Wakiso
KLK	BS	Mesoamerican	Kabale
KLL	BS	Mesoamerican	Kabale
KLM	CL	Mesoamerican	Kabale
KLN	BS	Mesoamerican	Kabale
KLO	CL	Mesoamerican	Kabale
KLQ	BS	Andean	Kabale
KLR	BS	Andean	Kabale
KLS	BS	Mesoamerican	Kabale
KLT	BS	Mesoamerican	Kabale
MBI	BS	Andean	Mbale
MBM	BS	Andean	Mbale
MBO	BS	Andean	Mbale
MBP	BS	Andean	Mbale
MBR	BS	Andean	Mbale
KLV	BS	Mesoamerican	Kabale
KLW	BS	Andean	Kabale
KLX	BS	Andean	Kabale
KLY	BS	Mesoamerican	Kabale
KLZ	BS	Mesoamerican	Kabale
KLAA	BS	Mesoamerican	Kabale
KLAB	BS	Andean	Kabale
MBT	BS	Andean	Mbale
MBU	BS	Mesoamerican	Mbale
MBV	BS	Andean	Mbale
APB	BS	Mesoamerican	Apac
APC	BS	Mesoamerican	Apac
APD	BS	Mesoamerican	Apac
APE	BS	Mesoamerican	Apac
APF	BS	Mesoamerican	Apac
APG	BS	Mesoamerican	Apac
APJ	BS	Mesoamerican	Apac
APK	BS	Mesoamerican	Apac
APM	BS	Mesoamerican	Apac

Continued from page 35

Isolate code	Growth habit	Bean race	District of origin
KLAF	BS	Mesoamerican	Kabale
KLAH	BS	Mesoamerican	Kabale
KLAJ	BS	Andean	Kabale
KLAK	BS	Andean	Kabale
KLAL	BS	Andean	Kabale
KLAM	BS	Andean	Kabale
KLAO	BS	Andean	Kabale
MPF	BS	Andean	Mpigi
MPG	BS	Andean	Mpigi
MBW	BS	Andean	Mbale
MBX	BS	Andean	Mbale
MBY	BS	Andean	Mbale
MBAA	BS	Andean	Mbale
KLAP	CL	Andean	Kabale
KLAQ	BS	Andean	Kabale
KLAR	BS	Mesoamerican	Kabale
KLAS	BS	Mesoamerican	Kabale
KLAT	CL	Andean	Kabale
KLAU	BS	Mesoamerican	Kabale
KLAV	BS	Andean	Kabale
WKB	BS	Andean	Wakiso
KLAW	BS	Mesoamerican	Kabale

BS = Bush growth habit, CL = Climber

3.2.3 Pathogen isolation from the bean pods and culturing on PDA

Isolation and culturing was done aseptically in the Biotechnology laboratory at Makerere University. The pathogen was isolated from well developed pod lesions. A drop of water was put on the lesion with a fine pipette tip, allowed to soak for a few minutes, and then the spores dislodged from the lesions by gently teasing the lesion and pipetting the spore suspension onto a microscope slide. The spore suspension was then diluted by adding 100µl or more of distilled water depending on the concentration and then mixed thoroughly. 50µl of the diluted suspension of conidia were then pipetted onto the PDA medium. A sterile glass rod was used to spread conidia evenly on the surface of the

media. Petri-dishes were then incubated at approximately 23^{o}C for 2-3 days, after which, mono-conidial isolates obtained by picking pin-point colonies from the media with a sterile needle, and then sub-cultured on fresh PDA medium. These were incubated as described above.

***Table 2*: Anthracnose differential series, resistance genes, host gene pool, and the binary number of each cultivar used to characterize races of anthracnose in common bean.**

Differential Cultivar	Host Genes	Place of Cultivar	Gene Pool	Binary Number	Growth Habit
Mitchelite	- -	0	MA	1	II
MDRK	Co – 1	1	A	2	I
Perry Marrow	Co – 1^3	2	A	4	II
Cornel 49242	Co – 2	3	MA	8	II
Widusa	Co – 9	4	A	16	I
Kaboon	Co – 1^2	5	A	32	II
Mexico 222	Co – 3	6	MA	64	I
PI 207262	Co – 4^3, Co – 9	7	MA	128	III
TO	Co – 4	8	MA	256	I
TU	Co – 5	9	MA	512	III
AB 136	Co – 6, Co – 8	10	MA	1024	IV
G 2333	Co – 4^2, Co – 5, Co – 7	11	MA	2048	IV

It is not known whether all the resistance genes have been characterized. **MA**: Middle American gene pool; **A**: Andean gene pool of *Phaseolus vulgaris*. **Binary numbers**: 2^n, where n is equivalent to the place of the cultivar within the series (0-11). The sum of cultivars with susceptible reaction will give the binary number of a specific race. For example race 48 is virulent on Widusa ($2^n = 2^4$=16) and Kaboon ($2^n = 2^5$=32). Growth habit: **I** = Determinate; **II** = Indeterminate bush; **III** = Indeterminate bush with weak main stem and prostrate branches; **IV** = Indeterminate climbing habit.

3.3 Raising of bean differentials

Five seeds of each differential variety were grown in one litre plastic pots filled with sterile soil in a screen house at Kalengyere, Kabale. They were watered and NPK fertilizer applied as necessary to keep them growing vigorously well. The set of bean anthracnose differentials and their characteristics (Kelly and Vallejo, 2004) are outlined in table 2.

3.4 Multiplication of isolates and inoculum preparation

Isolates for preparing the inoculum were raised on sterile snap bean pods in order to enhance sporulation. Briefly, both ends of snap bean pods were cut off and the pods washed and autoclaved at 121^0C for 30 minutes. At the same time, potato dextrose agar was separately prepared as explained in section 3.2.2 above and about 20ml of this PDA was poured into 10cm sterile disposable plastic plates when it had cooled. Enough of the autoclaved pods were then placed horizontally in each plate. Using sterile needle or scalpel, 5-6 pieces (1-2cm diameter) of agar plugs with actively growing fungus were cut and placed in the plates containing the sterile pods. The plates were then kept in darkness for 4 days, and then exposed to light for 10 more days. Five to six cultures of each isolate were raised in order to obtain enough inoculum.

3.5 Preparation of spore suspension for inoculation

The plate cultures were flooded with known amount of distilled water, the spores scraped off using a fine brush and the suspension poured into a beaker and mixed thoroughly. This was repeated about 3 times, each time using fresh distilled water to get most of the conidia from the culture. Conidia suspensions from all cultures of the same isolate were mixed. Using a haemocytometer, the concentration of the spore suspension was standardized by adjusting it to $1.2x10^6$ spores ml^{-1}. This procedure was done for each of the 47 isolates. One drop of tween twenty was added per 100ml of inoculum and mixed thoroughly before inoculation.

3.6 Inoculation

Fourteen-day old bean seedlings of experimental plants raised at Kalengyere, Kabale district were used for inoculation. Five plants of each cultivar were inoculated. Whole plant inoculation was done using a hand sprayer. Leaves were sprayed on both the abaxial and adaxial surfaces with inoculum until runoff. All the differential cultivars in each set were inoculated with one isolate at a time. A control (plants inoculated with clean distilled water) was included for each set of the plants inoculated with an isolate. Inoculated beans were then covered with transparent polythene sheets in order to maintain relative humidity of approximately 95%. The polythene sheet was removed four days after inoculation. Symptom evaluation on inoculated plants commenced 8 days after inoculation, using a scale of 1-9 (Balardin *et al*., 1990). The experimental setup was replicated once.

3.7 Data collection and Analysis

Data was collected by observing and assessing the reaction of each bean differential variety to each of the 47 pathogenic isolates. Assessment was based on a 1-9 severity scale (Table 3), depicting the percentage of the leaf covered with anthracnose lesions as described by Balardin *et al*. (1990). For the purpose of consistency, only the primary leaves of each plant were evaluated. The scores were averaged and analyzed by the Genstat computer package to find isolate differences. Bean genotypes with scores of 1-3 were regarded as resistant; where as those with scores of 4-9 were regarded as susceptible. A variety that was considered susceptible had 60% (or 3/5) of its inoculated plants exhibiting same disease scores within the susceptibility range (Ansari *et al*., 2004). After evaluation of all varieties, each isolate was assigned a name (race number) based on

the binary nomenclature system by adding the numerical binary values of the susceptible varieties together (CIAT, 1988).

Table 3: Symptom evaluation description according to Barladin et al. (1990)

Score	Description
1	Leaf with no visible symptoms
2	Few isolated small lesions on mid-veins in the lower leaf surface
3	A higher frequency of small lesions on mid-veins in the lower leaf surface
4	Lesions in the mid-vein and occasionally in secondary leaf veins
5	Many small lesions scattered on mid- and secondary veins
6	Many small lesions as described in grade 5 in the lower and upper leaf surface
7	Large lesions scattered over the leaf blade
8	Many large, coalesced lesions accompanied by tissue breakdown and chlorotic or abscised leaf
9	Severely diseased or dead leaf

3.8 Results

The different isolates exhibited different growth characteristics on PDA. Isolates varied in terms of growth rates and extent and ease of sporulation on PDA, green pods or both. The reaction of bean differentials towards these fungal isolates was also variable. There was a case where no isolate infected any bean differential to cases where up to seven differentials were susceptible. Out the 47 *C. lindemuthianum* isolates that were analyzed in this study, a total of 21 physiological races (pathotypes) were obtained (Table 4).

***Table 4*: Race and virulence phenotype of 47 *C. lindemuthianum* isolates analysed during the study**

Anthracnose differential varieties												
MIT	MD	PM	CO	WD	KB	MX	PI	TO	TU	AB	G2333	Race name
R	R	R	R	R	R	R	R	R	R	R	R	**0**
R	S	R	R	R	R	R	R	R	R	R	R	**2**
S	S	R	R	R	R	R	R	R	R	R	R	**3**
R	R	S	R	R	R	R	R	R	R	R	R	**4**
R	S	S	R	R	R	R	R	R	R	R	R	**6**
R	S	S	S	R	R	R	R	R	R	R	R	**14**
R	R	R	R	R	R	R	S	R	R	R	R	**128**
R	S	S	R	R	R	R	R	S	R	R	R	**262**
R	R	R	S	R	R	R	R	S	R	R	R	**264**
R	R	S	S	R	R	R	R	S	R	R	R	**268**
R	R	R	R	R	R	S	R	S	R	R	R	**320**
R	R	S	R	R	R	S	S	S	R	R	R	**452**
S	R	R	R	R	S	S	S	S	R	R	R	**481**
R	R	R	R	R	R	R	R	R	R	S	R	**1024**
R	R	R	R	R	R	R	R	R	S	S	R	**1536**
R	S	R	R	R	R	R	R	R	S	S	R	**1538**
R	R	R	R	R	R	S	R	S	S	S	R	**1856**
S	R	R	R	R	R	S	R	S	S	S	R	**1857**
S	R	S	R	R	R	S	S	S	S	S	R	**1989**
R	R	R	S	R	R	R	R	R	R	S	S	**3086**
S	R	R	R	R	R	S	S	S	S	S	S	**4033**

S and R refer to susceptible and resistant reaction respectively. MIT (Mitchellite), MD (Michigan dark red kidney), PM (Perry marrow), CO (Cornel 49242), WD (Widusa), KB (Kaboon), MX (Mexico 222), PI (PI 207262), To, Tu, AB (AB 136) and G2333 are differential cultivars.

Two isolates (36 and 2) were able to infect the highly resistant genotype, G2333, though with a severity level of 4, the lowest for susceptible reactions. These were finally designated as race 3086 and 4033 respectively. Nine races fell under the Mesoamerican phenotype although some of these isolates had been obtained from Andean bean genotypes. Three races were virulent only to the Andean differential cultivars while 7 races infected both Andean and Mesoamerican genotypes. The differential cultivar Widusa had an incompatible reaction to all isolates while Kaboon succumbed to only one

(isolate 45, race 481). Of all, cultivar "To" was the most susceptible as it succumbed to 10 races. This was followed by cultivar AB 136 (8 races), Mexico 222 and Perry marrow (each succumbing to 7 races). Cultivar Perry marrow succumbed to the highest number of isolates (14 in total), six of these isolates belonging to race 6. Eight isolates were unable to be differentiated by race differentials and were subsequently classified as race 0 (zero). These were unable to cause infection or their severity scores were less than 4, hence considered resistant reactions.

In terms of origin, isolates from Kabale were the most diverse with 12 races, followed by those from Mbale (5 races). In terms of virulence, again Kabale had the most virulent isolates. The two most virulent isolates (isolate 36, race 3086 and isolate 2, race 4033) that infected G2333 were isolated from this district. Apac isolates were the least virulent and were categorized as either race 0 and or 2. Isolates from Mpigi were moderately virulent and constituted races 6 and 14. No isolates representing any single race were isolated from all the four regions. In terms of differential susceptibility, Widusa never succumbed to any single isolates used in this study where as Kaboon only succumbed to one isolate.

3.9 Discussion

Race designations give an indication of the variability of the pathogen in terms of pathogenicity. Ugandan races of *C. lindemuthianum* identified in this study were unevenly distributed among the bean growing regions from where the isolates were collected. For example, 15 races (0, 4, 6, 128, 264, 268, 320, 452, 1024, 1536, 1856,

1857, 1989, 3086 and 4033) were isolated from Kabale, five (0, 3, 262, 481 and 1538) from Mbale, two (6 and 14) from Mpigi and two (0 and 2) from Apac. Only races 0 and 6 were isolated from at least two regions. These results therefore reveal that *C. lindemuthianum* in Uganda is variable, but with distinct virulence groups existing within different bean agroecologies. Kabale and Mbale represent highland environments dominated by cool moist weather conditions suitable for anthracnose development while Apac and Mpigi are lowland areas with warm conditions. These environmental differences could have dictated the observed trend in pathogen variability. The results further demonstrate that the Ugandan *C. lindemuthianum* is composed of highly virulent races that have potential of causing much damage to the entire Ugandan common bean germplasm. This is demonstrated by the existence of two races 3086 and 4033 which were able to infect the world's most known anthracnose resistant genotype, G2333. In addition to infecting G2333, race 4033 also broke down the resistance in six other differentials (Michellite, Mexico 222, PI 207262, TO, TU and AB 136).

The differences in the pathogen populations between these regions largely reflects the differences in common bean germplasm used and the agricultural practices employed in different agroecologies. Isolates from Apac, for example, were less virulent and less diverse than other isolates analysed. This is likely due to the fact that there is less diversity of beans grown in this region, largely dominated by small seeded, bush type beans (Nkoya, 2001). On the other hand, bean diversity is very high in the highland areas of Kabale and Mbale (Nkoya, 2001). In these districts, there are small, medium and large-seeded; bush, semi-climbing and climbing bean types with wide variations in seed colour

(Opio, *et al*., 2001). Such variations within bean genotypes, coupled with the fact that the anthracnose pathogen co-evolves with its host (Esteban *et al*., 2003), may partly provide an explanation for the wide physiological diversity of the pathogen in Kabale and Mbale. On other hand, there could have been selection followed by adaptation of races in the different agroecologies. In Mpigi, however, large seeded and bush types of beans dominate (Nkoya, 2001). Previous literature on the diversity of *C. lindemuthianum* suggests that the isolates from Middle American gene pool are more diverse than those from the Andean counterparts (Mahuku and Riascos, 2004), thus confirming the observed physiological variations within Mpigi isolates. Besides, there is limited number of Andean genes for resistance to anthracnose in common beans (Kelly and Vallejo, 2004). Over all, with the current trend in bean trade in the country involving exchange of varieties within and between different regions (Nkoya, 2001), there is a possibility that all these virulence factors will be introduced to areas where they are currently absent.

The pathogen has high rates of evolution and new races keep emerging from time to time (Mahuku and Riascos, 2004; Kelly and Vallejo, 2004; Vidigal *et al*., 2007). For this reason, it is necessary to keep monitoring the pathogen population to cater for any races that may emerge to minimize the effect on the bean germplasm in the country. The differential cultivar Widusa was not infected by any isolate. This cultivar carries the anthracnose resistance gene Co-9 which originates from the Andean gene pool (Kelly and Vallejo, 2004). Previously, it was reported that cultivar Widusa could be carrying two more independent genes (one dominant and the other recessive) that confer resistance to race 38 of *C. lindemuthianum* (Ferreira *et al*., 2003). Such genes, if present, could be

responsible for the observed resistance reaction of this differential variety. These results give further clues about the importance of Widusa in controlling anthracnose.

However, cultivar PI 207262, which carries Co-4^3 in addition to Co-9 resistance genes (Kelly and Vallejo, 2004), succumbed to five races (128, 452, 481, 1989 and 4033), all of which were unable to infect Widusa. This result suggests the probable existence of an uncharacterized gene or gene combination in addition to Co-9 carried by cultivar Widusa. This revelation is similar to recent studies by Mahuku and Riascos (2004) who demonstrated that cultivar Widusa was resistant to 29 races all of which infected cultivar PI 207262. These included race 3977 from Costa Rica which also infected the highly resistant differential cultivar, G2333. Certainly, Widusa could be carrying a gene or gene combination that result in resistance to multiple anthracnose races. In this case, it could turn out to be a very good source of resistance to anthracnose in the region. Further investigation into the mode of resistance found in this cultivar is necessary since it was resistant to isolates that broke down G2333, a hitherto reported as the most resistant variety. Otherwise, Widusa is a very promising source of resistance to anthracnose that needs to be considered for use in bean improvement programmes in the region.

On the other hand, Mitchellite, which has been considered the most susceptible differential cultivar with no known anthracnose resistance gene, was surprisingly infected by a few (5 out of 47) isolates. In all cases, it was never infected alone, suggesting the possibility of Michellite hosting one or more anthracnose resistance genes that have not yet been characterized. For example, out of the 200 isolates previously characterized by

Mahuku and Riascos (2004) only 9 of them exclusively infected Michellite. Further investigation is required to explain this phenomenon.

The infection pattern shown in this research agrees partly with the previous studies in Mexico on the variability of this pathogen by Gonzalez *et al.* (1998) who demonstrated that out of 129 isolates characterized with CIAT differentials, none was capable of infecting the cultivar Widusa, among others. These findings give us an indication on the value of this cultivar in controlling bean anthracnose.

Five *C. lindemuthianum* races, namely: 1, 2, 17, 23 and 38 had been previously found in Uganda (Leaky and Simbwa-Bunya, 1972). In 2006, a study by Nkalubo (2006) revealed the existence of seven new highly virulent races (55, 102, 130, 227, 375, 511 and 767) and a less virulent one (race 23) re-isolated from Kisoro. The new races had been isolated from the Ugandan districts of Kabale, Kisoro, Bushenyi and Mpigi, and are more virulent than those reported by Leaky and Simbwa-Bunya (1972) revealing the continuous evolution of the anthracnose pathogen in the country. Our present study has come up with new and more virulent races of this pathogen (Table 4). The existing races established by this work either reflects the dynamic nature of preexisting pathotypes or it reflects the continued evolution and emergence of new pathotypes in response to the introduction of new resistance genes by bean breeders (a form of directional selection) (Fabre *et al*., 1995) and subsequent adaptation in bean growing regions. These results imply that the pathogen has been evolving over time.

In summary, a total of 21 new races of *C. lindemuthianum* were obtained from the 47 isolates tested. Diversity ranged from those isolates that were unable to cause anthracnose infection on any bean differentials to those which caused mild to severe symptoms on a few or more differentials. Two isolates, i.e., 36, race 3086 and 2, race 4033 managed to break down Co-4^2, Co-5 and Co-7 resistances (severity level 4) found in the previously most resistant cultivar G2333 in addition to others. Surprisingly, cultivar Widusa which carries the anthracnose-resistance gene Co-9 emerged exclusively resistant to all the isolates tested. Use of the gene (s) carried by Widusa in breeding programs aimed at improving resistance to anthracnose in Uganda is of paramount importance.

CHAPTER FOUR

MOLECULAR CHARACTERIZATION OF *COLLETOTRICHUM LINDEMUTHIANUM*

4.1 Introduction

In pathogen variability studies, molecular markers are very important because they are able to reveal the level of genetic differences that exists between isolates of a particular pathogen or its species. Such genetic differences are directly or indirectly responsible for pathogen characteristics such as virulence levels, adaptation or survival. Pathogen genetic characteristics can be compared to their physiological characters in order to exploit any relationship that might exist. Because molecular markers are fast and environment-neutral (Williams *et al*., 1990), they have become very popular in pathogen classification. However, previous studies that involved use of molecular markers and race differentials for characterization of *C. lindemuthianum* gave rise to contrasting conclusions especially on whether there is congruence between the two methods (Mahuku and Riascos, 2004). In this experiment, molecular markers (RAPDS and rep-PCR) were used to establish the genetic diversity of Ugandan *C. lindemuthianum* isolates. Thereafter, the results were compared with the findings from the race differential work in order to establish any relationship that may exist between molecular and differential derived data.

4.2 Materials and Methods

4.2.1 Raising of fungal cultures for DNA extraction

Most fungal cultures for DNA extraction were raised by cutting 2-3cm of actively growing fungal plugs from mother cultures with a sterile scalpel and placing them on

fresh PDA media (composed of 24g of Potato Dextrose Broth, 15g of Agar in 1litre of Distilled water). Some cultures were raised from isolates stored under mineral oil. The cultures were then placed on laboratory benches and left to grow for about 7-10 days or until there was enough mycelia to harvest for DNA extraction.

4.2.2 DNA extraction procedure

Fungal mycelia (about 2g) from actively growing cultures were carefully peeled off from the PDA surface with a sterile scalpel. The mycelia were then placed in clean pre-chilled mortars, frozen with 10-15ml of liquid nitrogen and crushed with a pestle into a fine powder. The powder was then transferred to a 1.5ml eppendorf tube and maintained at -196^{0}C in a liquid nitrogen-bath. This procedure was repeated for all isolates. The mycelial powder in each tube was suspended by adding 1ml of hot (65^{o}c) CTAB extraction buffer [2%w/v Hexadecyltrimethyl-ammoniumbromide (CTAB), 8.2%w/v NaCl (1.4M), 0.2%v/v Mecaptoethanol (98%), 1.2%w/v EDTA (20mM), 1%w/v Tris-Base (pH 8, 100mM) and 1%w/v Polyvinylpyrodilone (PVP-40)] to each tube, mixed gently by inversion followed by incubation in a water bath (65^{0}C) for two hours.

The tubes were then cooled down to room temperature, and 0.5ml of Chloroform-isoamylalcohol (24:1) was added, and the tubes shaken (100 times) by inversion. This was followed by centrifuging at 13,000 rpm for 15minutes. The aqueous top layer (about 0.9ml) was then pipetted to new tubes. Ice-cold isopropanol (0.6ml) was then added, mixed gently by inversion and incubated the tubes at -20^{o}C for 20 minutes (or overnight) in order to precipitate DNA. This was followed by spinning the DNA samples at 12,000

rpm for 10 minutes. Isopropanol was then decanted carefully leaving a DNA pellet at the bottom of the tubes. The pellet was then washed with 1ml of 70% ethanol by mixing well by inversion, and then spinned at 12,000 rpm for 10minutes. Washing was repeated two times. The supernatant was then poured off carefully and the DNA pellet dried in air (for about 2hours). Fully dried DNA pellets were dissolved in 0.15ml of TE [0.122%w/v Tris-Base (pH8, 10mM), 0.02%w/v EDTA (1mM], left to dissolve and then stored at -20^{o}C.

4.3 DNA quantification and dilution

4.3.1 Checking for DNA quality by Electrophoresis

For each sample, approximately 10µl of genomic DNA extracted above was loaded on a 1% agarose gel and run in 1x TBE buffer at 70 volts for 45 minutes. The gel was then stained by immersing it in 1% ethidium bromide solution for 30 minutes, visualized under 100% UV light and then photographed. The majority of the DNA samples produced sharp, single bands, while a few had faint bands. This was an indication that appreciable amounts of good quality DNA had been extracted from each sample.

4.3.2 DNA quantification using a fluorometer

Extracted DNA was quantified using a DQ200 fluorometer following the manufacturer's instructions. DNA quantity varied among isolates; it ranged from 23 – 1004 ngµl^{-1}. DNA from each sample was then diluted to 20 or 80 ngml^{-1} working concentrations and then stored at -20^{o}C.

4.4 Polymerase Chain Reaction (PCR) using RAPDs

Random Amplified Polymorphic DNAs (RAPDs) primers were used to amplify genomic DNA for each of the 74 fungal isolates. A total of twenty one 10-base pair primers were initially tested for their suitability. The 10 most polymorphic were finally selected and used for DNA analysis. DNA amplifications were done in either the Hybaid thermocycler (Hybaid Cycler Ltd – UK) or the GeneAmp PCR System 9700 (version 3.08 of Applied Biosystems) using a procedure described by Balardin *et al*. (1997), with minor modifications. In both cases, the results were consistent. The PCR included 2mM $MgCl_2$ (25mM), 1x PCR buffer (10x), 0.4nMdNTPs (1.25mM), 0.1µl Taq polymerase (5U/µl), 1µl BSA (1x), 1µM primer (10µM), 40-80 ng/µl genomic DNA (20-80 ng/µl) and PCR water in a 12.5µl reaction volume. The PCR conditions included one denaturation step at 94^0C for 2 minutes; followed by 3 three-step cycles (denaturation at 94^oC, annealing at 35^oC and extension at 72^oC each for 1 minute); and lastly, 37 cycles (94^oC for 10 seconds, 35^oC for 35seconds, 72^oC for 1 minute). A final extension at 72^oC for 10 minutes was included, and PCR products were held at 4^oC till electrophoresis. The primers (names and their sequences) that were screened and used in the analysis are shown in table 5.

Table* 5: RAPD Primers that were used for analysis of molecular diversity of *C. lindemuthianum

	Name	Sequence	No Bands Scored	No Polymorphic Bands
1	OPAH-11	5’ CTT CCG CAG T 3’	6	6
2	OPAQ-20	5’ TCG CCC AGT C 3’	8	8
3	OPAT-09	5’ CAC CCC TGA G 3’	4	3
4	OPAV-12	5’ ACC CCC CAC T 3’	9	9
5	OPAX-14	5’ ACA GGT GCT G 3’	11	11

Continued from page 50			No bands scored	No polymorphic bands
6	OPF-13	5’ GGC TGC AGA A 3’	9	9
7	OPG-2	5’ GGC ACT GAG G 3’	5	5
8	OPG-3	5’ GAG CCC TCC A 3’	12	12
9	OPV-7	5’ GAA GCC AGC C 3’	11	11
10	OPE - 4	5’ GTG ACA TGC C 3’	7	7

4.5 Gel electrophoresis and visualization

For both RAPDs and rep-PCR, the PCR reaction products were mixed with 2 µl of loading dye and 6.5 µl of each was loaded on a 1.2% agarose gel, which was run at 80 volts for 2 hours in a 0.5x TBE running buffer. The gel was then stained with 1% ethidium bromide solution for 30 minutes, then distained in distilled water for 10 minutes. The gel was then visualized under 100% UV light and its picture captured with Perfect Image computer program.

4.6 Use of repetitive element-based polymerase chain reaction (rep – PCR)

To have reliable results, another fingerprinting technique was employed in addition to RAPDs. This technique is called repetitive element based polymerase chain reaction (rep-PCR) (Versalovic *et al*., 1994).

4.7 DNA Amplification with rep-PCR primers

DNA sequencing of repetitive-elements (rep-PCR) was carried out using ERIC and BOX primers. The primer sequences used were ERIC (ERIC-1R: 5 -ATGTA GCTCCTGGGGATTCAC-3 and ERIC-2: 5 -AAGTAAGTGACTGGGGTGAGCG-3) and BOX-AIR (5 - CTACGGCAAGGCGACGCTGACG-3). Box-AIR is used both as

forward and reverse primer. For both primer pairs, the reactions were carried out in 12.5µl reaction volume, containing 0.2mM of each dNTPs, 1× Gitschier PCR buffer, 0.5U of Taq DNA polymerase (Perkin–Elmer, NJ, USA), 0.2mM each primer, 1.5mM $MgCl_2$, 0.16 $mgml^{-1}$ BSA, 10% DMSO and 80 ng genomic DNA. The Gitschier buffer (83mM $(NH_4)_2SO_4$, 335mM Tris-HCl (pH 8.8), 33.5mM $MgCl_2$, 33.5µM EDTA and 150mM β-mercaptoethanol and double distilled water) replaces the normal PCR buffer in rep-PCR. The PCR conditions included an initial denaturation step at 95^oC for 7 minutes followed by 30 cycles each with a denaturation stage at 94^oC for 1 minute, annealing at 51^oC for I minute and extension at 65^oC for 8 minutes. A final extension step at 65^oC for 16 minutes followed before a soaking extension of the PCR products at 4^oC until gel electrophoresis.

4.7.1 Scoring of gels

Bands were scored as 1 and 0 for presence and absence of a specific DNA fragment, respectively. Similarity matrices were generated from the RAPD and rep-PCR data using the Similarity for Qualitative Data program (SIMQUAL) in the Numerical Taxonomy and Multivariate Analysis System (NTSYS-pc). Simple matching coefficients (SM) were used to compute genetic distances and dendrograms produced. Also treecon program was used to draw bootstrapped phylogenetic tree (Figure 4). Genetic structure analysis was done using Arlequin 3.11 program (Laurent Excoffier 1998 – 2007) in order to compute genetic diversity indices as well as AMOVA. During data collection, isolates from Wakiso were combined with those from Mpigi and analysed together as one region.

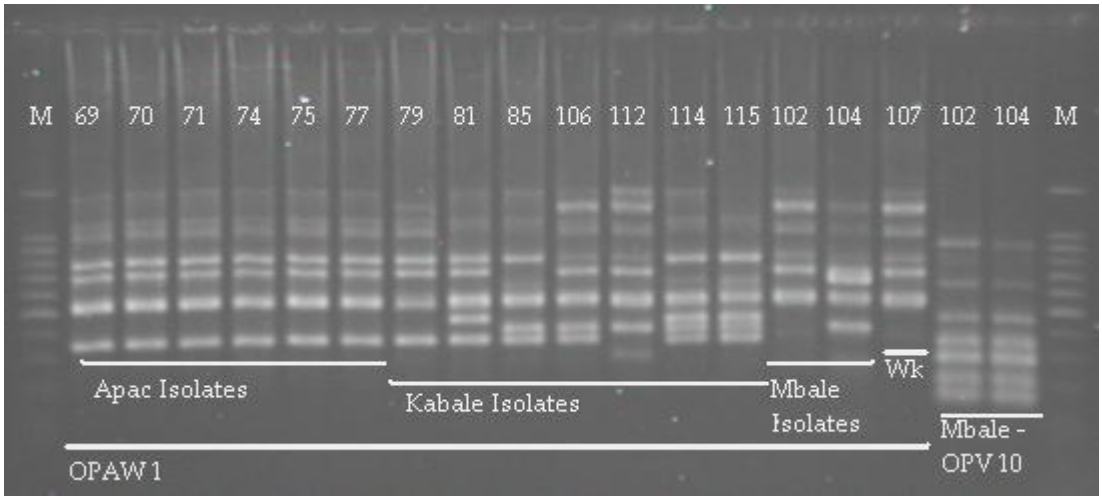
Wakiso and Mpigi districts are adjacent to each other and located in one agroecological zone in central Uganda.

4.8 Results

4.8.1 RAPDs analysis

Among the 21 primers that were initially used, 10 produced bright, polymorphic and scorable bands (plates 3-7). The rest of the primers neither had polymorphic bands nor produced bands that were capable of being used in the analysis. The ten primers that were considered include OPAX 14, OPAH 11, OPE 4, OPAQ 20, OPAT 9, OPAV 12, OPF 13, OPG 2, OPG 3, and OPV 7. The primers had variable number of bands ranging from 4 (OPAT 9) to 12 (OPG 3). Fingerprints for some isolates generated by primers OPAW 1, OPV 10, OPE 4, OPAH 11, OPAQ 20, and OPG 2 are shown in plates 3-7.

***Plate 3*: Representative gel picture showing separation of RAPDs PCR a product of different isolates of *C. lindemuthianum* using OPAW 1 (left) and OPV 10 (far right) primers**



Note that: Apac isolates were uniform for all the loci shown: Wk = Wakiso isolates; M = 100kb DNA ladder

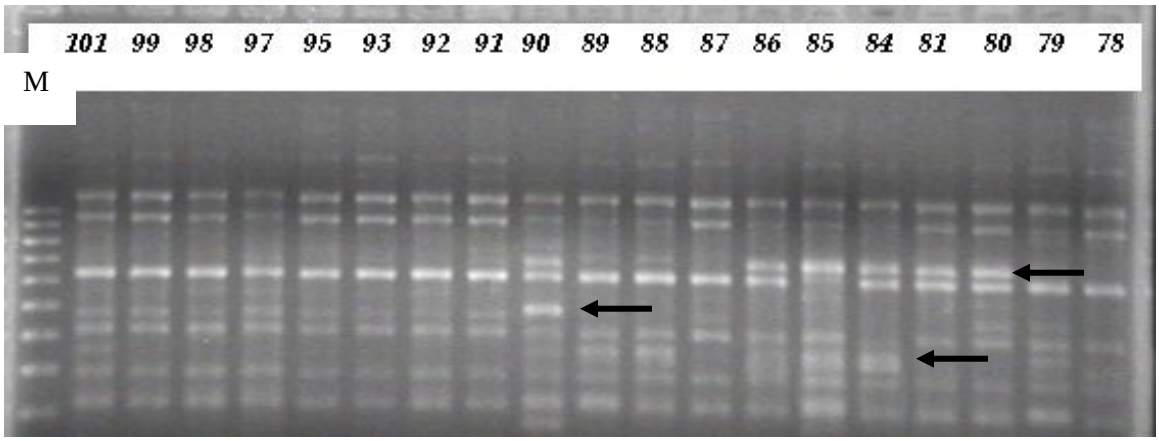


Plate 4: Gel run with primer OPA 9. Lanes 101 and 99 are isolates from Mbale, Isolates 98-91 are from Mpigi and 90-78 are isolates from Kabale. The arrows show highly polymorphic regions. M is a 100bp ladder.

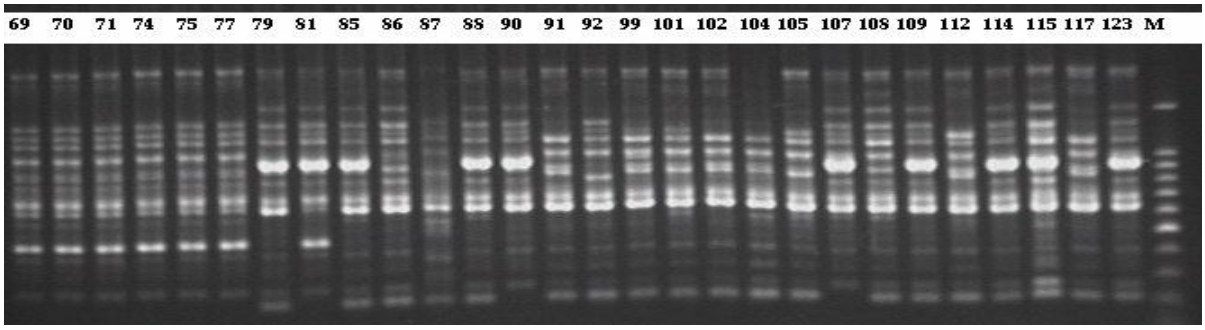


Plate 5: Part of a gel from Primer OPE 4: lanes 69-77 are isolates from Apac

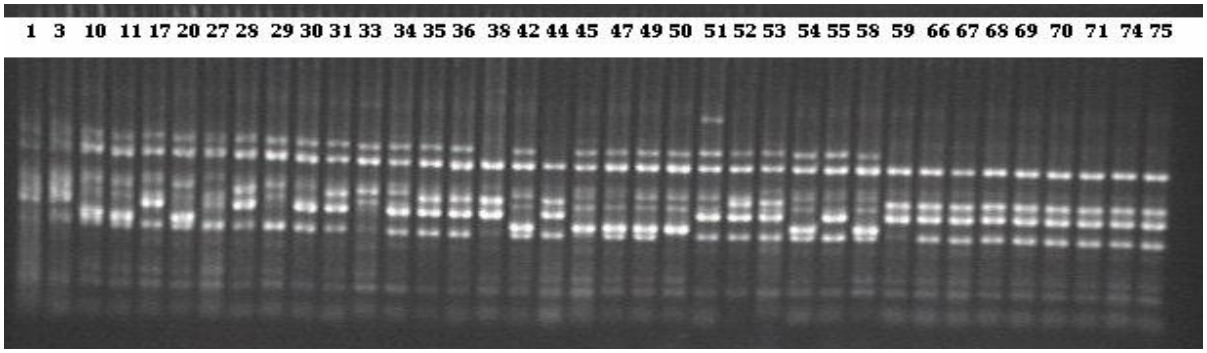


Plate 6; Part of a gel obtained with primer OPAH 11: Lanes 66-75 are Apac isolates

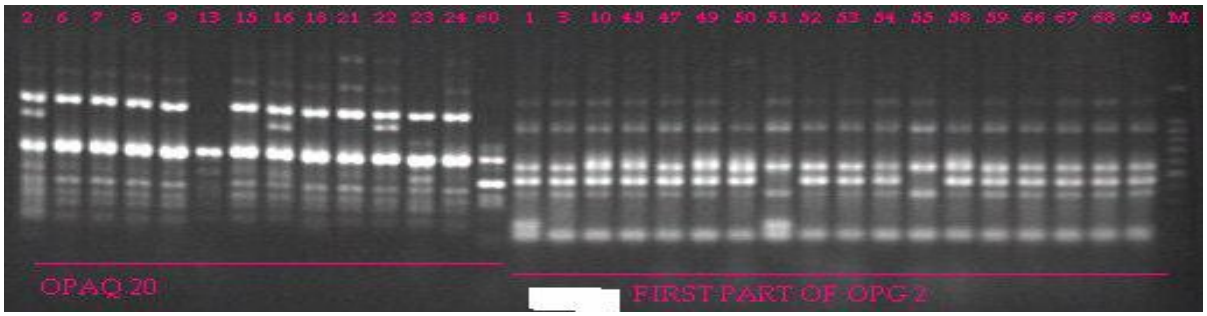


Plate 7: Part of OPAG 20 and OPG 2 gel

In total, 82 scorable bands were generated from the ten primers used. Out of these, isolates from Kabale had 77 polymorphic loci accounting for 93.9% polymorphism, Mbale isolates had 73 (89.0% polymorphism), Mpigi isolates produced 55 (67.1% polymorphic) and only 7 polymorphic loci (8.5%) emerged from Apac isolates. Similarly, average diversity over loci ranged between 0.028 (Apac) and 0.343 (Kabale), while the average fixation index over all loci (FST) was 0.237, indicating a "large" genetic differentiation (IPGRI and Cornell University, 2003) among the analysed isolates. All individual isolates, except three from Apac (namely APD, APE and APM), were able to be distinguished by RAPDs analysis. All the primers came up with different banding patterns for any one isolate; however, Apac samples were almost always uniform from one primer to another. All bands scored for the ten primers were polymorphic for all isolates analyzed, except those from OPAT 9, which had 3 out of 4 polymorphic loci.

***Table 6*: Genetic characteristics of *C. lindemuthianum* isolates from four agroecologies in Uganda**

Region	No of Polymorphic Loci	% Polymorphism	Average Diversity over Loci	Standard Deviation
Kabale	77	93.9	0.343	0.170
Apac	7	8.5	0.028	0.101
Mpigi	55	67.1	0.271	0.215
Mbale	73	89	0.336	0.181

Average fixation index over all loci (FST) = 0.237

Generally, RAPDs analysis revealed a high genetic variability of *C. lindemuthianum* in Uganda. Genetic variation was shown to occur among isolates from the same or different agroecological zones, with the exception of those from Apac. Genetic variation within-population was higher (76.34%) than among-population [(23.66%) (Table 7)]. A dendrogram constructed using RAPD data is presented in figure 1.

Table 7*: Genetic variation among and within populations of *C. lindemuthianum

Source of Variation	Among population	Within population	Total
Degrees of freedom	3	70	73
Sums of squares	213.01	856.26	1069.34
Variation component	3.79 *Va*	12.23 *Vb*	16.02
% of variation	23.66	76.34	

RAPDs analysis differentiated all the samples at 0.52 similarity coefficient (SC) cut off. At this point, the majority of the samples (95.9%) were 52-99% genetically similar (Figure 1). Three samples i.e., APD, AFE and APM from Apac were 100% genetically similar. Similarity between the most distant isolates was 52%. All the 9 samples from Apac in cluster 2 were at least 95% genetically similar. Generally, RAPDs using UPGMA generated 5 fungal clusters comprising of 1, 2, 29, 12 and 30 isolates. The last

three clusters comprising of at least 12 isolates were recognized as major clusters and were named 1, 2 and 3. The remaining 3 isolates (MBG, KLQ and MBV) were considered a minor cluster (Figure 1).

When bootstrapping was done with neighbor joining using Treecon for windows program (Van de Peer, Y and De Wachter, R, 1994), a similar trend was realized (Figure 2) with all the Apac isolates grouping themselves together (at a bootstrap value of 98, cluster labeled AP), while 50% of all the Kabale isolates dominated the right arm of the tree (labeled KL). Only one isolate from Mbale (MBF) appeared in group KL. The rest of Kabale, Mbale and all the Mpigi isolates were distributed on the remaining left part of the tree.

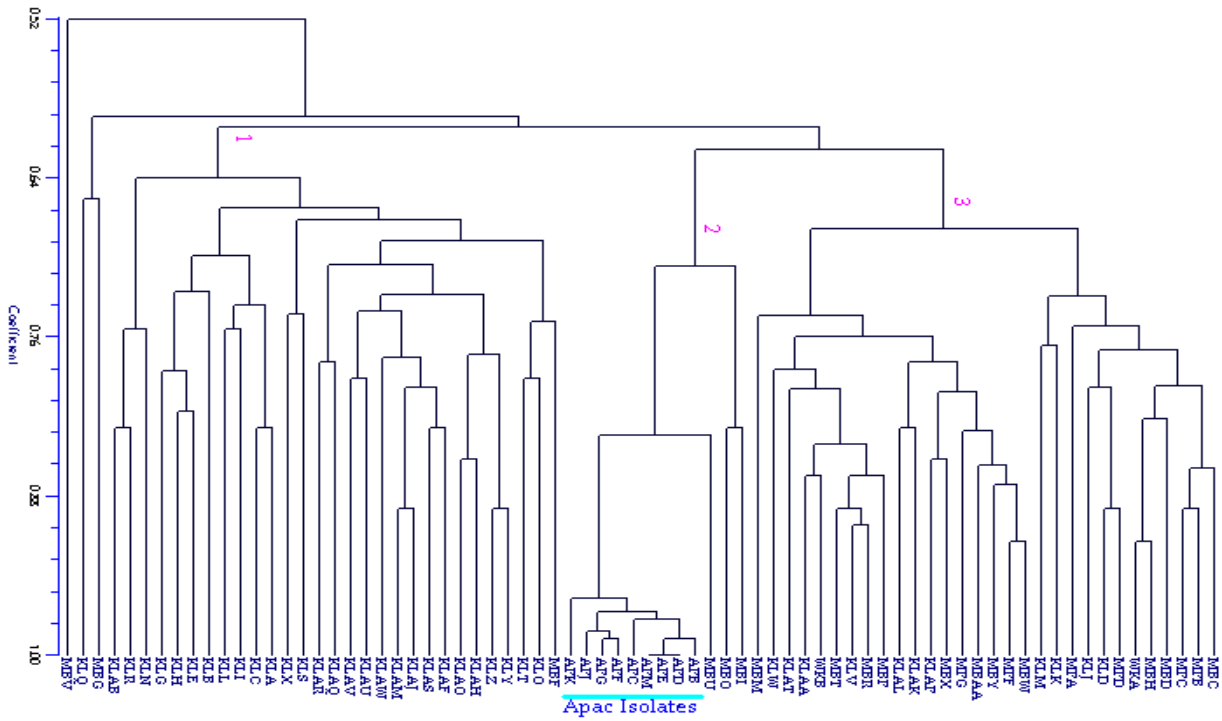


Figure 1: Dendrogram produced using UPGMA in the SAHN program of NTSYS-pc program showing distribution of isolates in three major clusters. All Apac samples clustered together. 1, 2, and 3 are major clusters. All the Apac isolates formed their own subcluster under cluster 2, while Kabale isolates dominated cluster1.

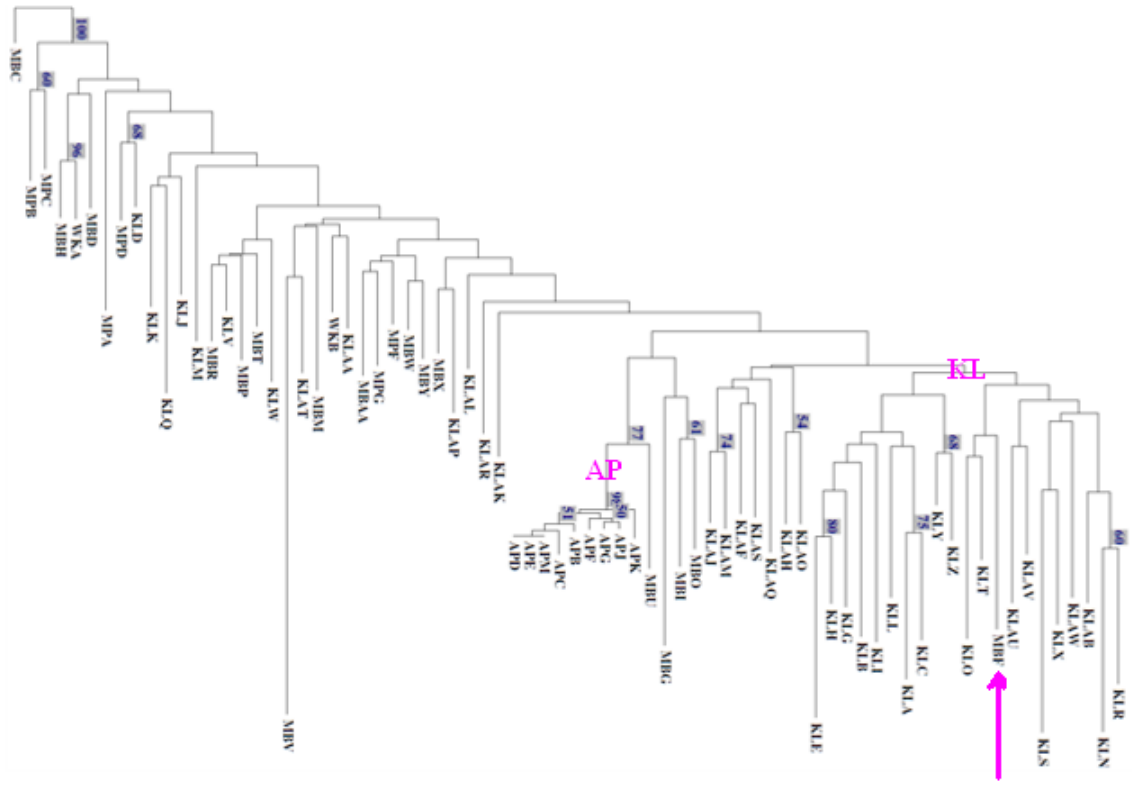


Figure 2: A bootstrapped tree produced with Neighbour Joining using Treecon for windows program (Van de Peer, Y and De Wachter, R, 1994) showing distribution of isolates. All Apac samples clustered together in the middle of the tree (labeled AP). Kabale isolates dominated the right arm of the tree (labeled KL), with one isolate from Mbale (MBF, shown with an arrow) appearing in that cluster. The rest part of the cluster had all Mpigi isolates and the rest of Kabale and Mbale.

Based on RAPD analysis, isolates from Kabale were the most polymorphic (93.9%), followed by those from Mbale (89.0%) and Mpigi and Wakiso (67.1%). All Apac samples were almost uniform (8.5% polymorphic). Cluster 1 comprised of 29 isolates and was dominated by Kabale samples with 28 out of 40 isolates. Only one isolate from Mbale (MBF) appeared in this cluster. All isolates from Wakiso and Mpigi were grouped into cluster 3 together with others from Mbale and Kabale. Cluster 2 was composed of 9 and 3 isolates from Apac and Mbale respectively. Mbale isolates exclusively appeared in all the 3 clusters with the majority (11 out of 17) in cluster 3. With the exception of isolates from Apac, there was no relationship between RAPD fingerprints and the gene pools or geographical location from which the isolates originated.

4.8.2 Wright's F statistics (FST) analysis

When pairs of populations FST values were compared by pair wise difference method, Kabale isolates appeared to be genetically closer to Mbale isolates (genetic distance or GD = 0.094), followed by Mpigi isolates (GD = 0.173). Apac isolates were the most genetically distant (0.390) from their Kabale counterparts (Table 8).

***Tabe 8*: Genetic distance (GD) between isolates from different regions generated using RAPD analysis**

	Kabale	**Apac**	**Mpigi**	**Mbale**
Kabale	0.0000			
Apac	0.390	0.0000		
Mpigi	0.173	0.6624	0.0000	
Mbale	0.094	0.4114	0.0363	0.0000

All values significant at 0.05level

4.8.3 rep-PCR fingerprinting

Rep-PCR was conducted on the isolates using BOX and ERIC primers. The majority of isolates revealed variable banding patterns. The number of bands obtained from the analysed isolates ranged between 3 and 9 for both BOX and ERIC primers. No single band was common to all isolates analysed with the BOX primer, while the 300 base pair band was the only common one among all the isolates analysed with ERIC primers.

BOX bands that were produced ranged between 800 and 1500 base pairs, while ERIC generated bands ranging between 200 and 1350 base pairs. BOX produced more bands than ERIC, although ERIC generated more polymorphism (plates 8-10).

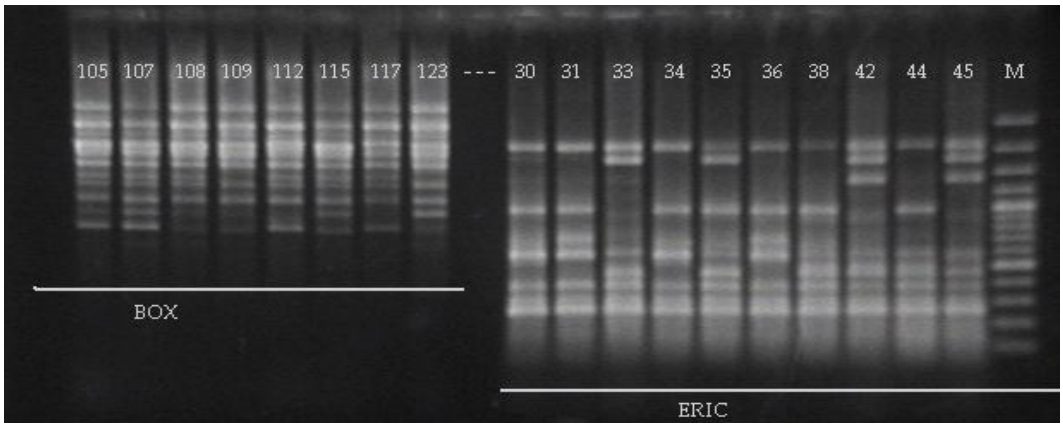


Plate 8: Representative gel picture showing *C. lindemuthianum* fingerprints of selected Ugandan isolates based on BOX (left) and ERIC (right) primers. M is a 100kb DNA ladder.

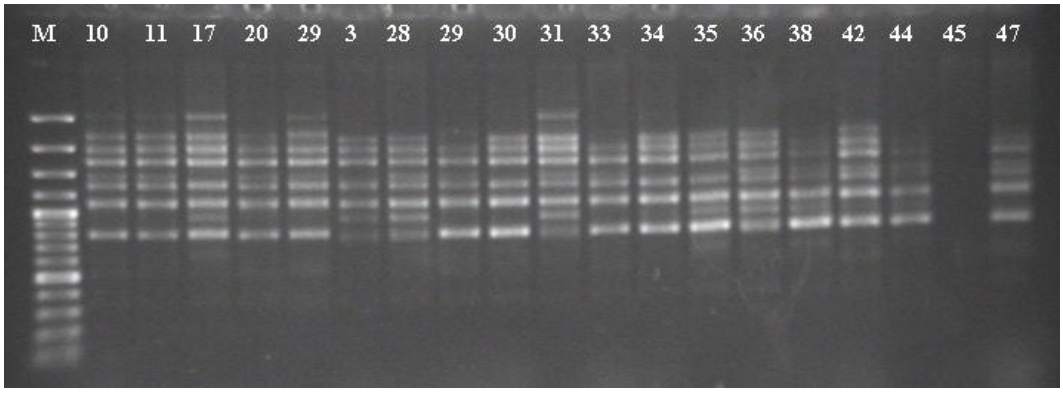


Plate 9: Part of a gel obtained with primer BOX. Faint bands as in case of the 800bp band in lane 3 were not considered during scoring. Scorable bands ranged between 800-1500 base pairs. M is 100kb DNA ladder

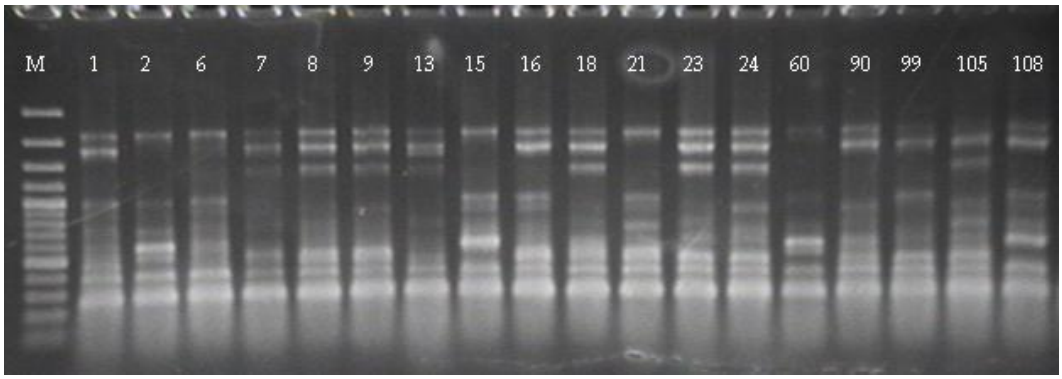


Plate 10: Part of a gel obtained with primer ERIC. M is the 100kb DNA ladder. Scorable bands ranged between 200-1350 base pairs.

Analysis of rep-PCR data using UPGMA generated a dendrogram, which divided all the study samples into 2 clusters: a minor one (I) and a major one (II) at 53% similarity. The minor cluster I was composed of 4 isolates, three of which were from Mbale and one from Kabale. The major cluster II consisted of 70 isolates. The larger cluster was further subdivided into two sub clusters, namely 1 and 2 at 0.63 similarity coefficient. The sub cluster 2 constituted all the Apac isolates, 67.5% of all the Kabale isolates and only 29% of Mbale isolates. All Mpigi and Wakiso isolates were grouped under sub cluster 1 of the major cluster II.

In this analysis, Apac isolates were evenly distributed throughout sub cluster 2 unlike the case with RAPDs. The majority (53%) of Mbale isolates were grouped under sub cluster 1 of the major cluster II. The similarity between the most distant isolates was 53%, while 14 isolates were 100% genetically uniform although they were distributed in different sub clusters. These isolates grouped as A, B, C, D and E having 4, 3, 2, 3 and 2 isolates, respectively. Sub clusters C and E comprised of only Kabale isolates while D comprised of only Apac isolates. Sub clusters B and A had isolates from all regions with the exception of Apac. Generally, the 74 isolates did not cluster by region. Some isolates from different regions exhibited similar DNA fingerprints with genetic similarity of over 80% compared to those isolated from the same sampling regions.

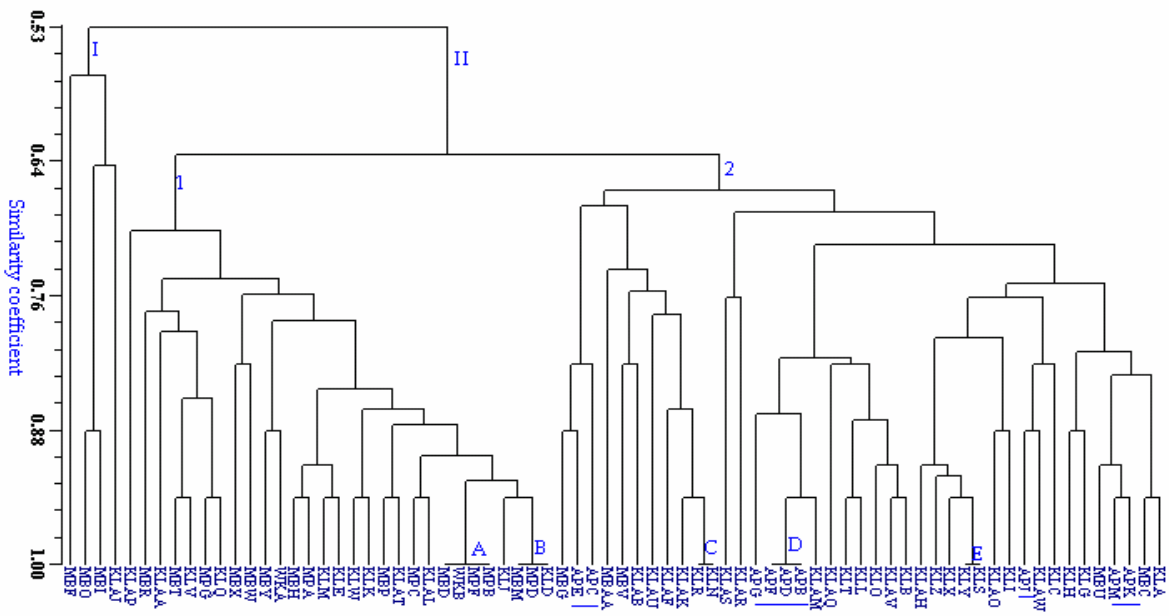


Figure 3: Dendrogram produced from rep-PCR analysis showing distribution of isolates of C. *lindemuthianum* in I and II clusters. 1 and 2 are subclusters of II. A, B, C, D and E are groups of isolates that shared 100% genetic similarity, while the blue lines show the distribution of Apac isolates.

Analysis of molecular variance (AMOVA) resulted into 11.3% and 88.7% genetic variation among- and within-populations respectively, with a fixation index (FST) value of 0.113 (Table 9), implying a "moderate" genetic differentiation (IPGRI and Cornell University, 2003) of *Colletotrichum lindemuthianum* population in Uganda. When pairs of populations of *C. lindemuthianum* isolates were compared, Kabale isolates were closer to Mbale counterparts, followed by Apac and Mpigi isolates with genetic distances of 0.051, 0.072 and 0.197 respectively (Table 10). The trend here is generally similar with the RAPDs result except for genetic distance between Kabale and Mpigi isolates.

Table 9*: AMOVA resulting from rep-PCR analysis showing the genetic variation within and among populations of *C. lindemuthianum

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of Variation
Among populations	3	22.709	0.324 Va	11.3
Within populations	70	178.197	2.546 Vb	88.7
Total	74	200.905	2.870	
Fixation Index	(FST)	=		0.113

***Table 10*: Genetic distance (GD) between isolates from different regions generated using rep-PCR analysis generated by pair-wise difference method**

	Kabale	Apac	Mpigi	Mbale
Kabale	0.0000			
Apac	0.0721	0.0000		
Mpigi	0.1965	0.4410	0.0000	
Mbale	0.0514	0.1313	0.0908	0.0000

A combination of ERIC and BOX primers gave rise to 18 bands (or loci). According to this combined data, Mbale isolates were the most polymorphic (94.4% polymorphism) followed by Kabale (88.9%), Apac (50%) and lastly Mpigi (44.4%). On the basis of individual population comparisons, AMOVA also revealed moderate genetic differentiation (FST = 0.113) for Ugandan *C. lindemuthianum* sub populations (Table 11).

***Table 11*: Genetic characteristics of *C. lindemuthianum* populations in Uganda**

	Percentage polymorphism	Population Specific FST Indices	Expected Heterozygosity
Kabale	88.9	0.107	0.304
Apac	50.0	0.129	0.216
Mpigi	44.4	0.144	0.141
Mbale	94.4	0.106	0.326

Total number of loci considered = 18.

4.9 Analysis using a combination of RAPDs and rep-PCR data

Because both RAPDs and rep-PCR data is collected in a similar manner, data matrices generated by scoring gels from both PCR techniques were combined and analyzed. The dendrogram that was produced separated the 74 *C. lindemuthianum* isolates into 2 clusters at the 55% genetic similarity cut off. The clusters were named 1 and 2, and consisted of 1 and 73 isolates respectively. Using both RAPDs and rep-PCR data, all the 74 isolates were differentiated. The most similar isolates (KLY and KLZ, both from Kabale) were 99% genetically similar (Figure 3). The most distant isolates (KLA and KLR, also from Kabale) were 55% genetically similar. The major cluster 2 was as before divided into sub clusters A and B. Sub cluster A consisted of the majority (58.8%) of Mbale isolates, 66.7% of Apac isolates, the 2 Wakiso isolates, 2 from Mpigi and only six isolates from Kabale. Sub cluster B was dominated by Kabale isolates which accounted for 82.5%. Some Mbale isolates were grouped together in small clusters (MB1 and MB2) comprising of 9 and 3 samples respectively (figure 3). Similarly, Kabale isolates formed small clusters, namely, KB1, KB2 and KB3 consisting of 10, 3 and 6 isolates respectively. Generally, the combination of rep-PCR and RAPD matrices presented the overall variability of *Colletotrichum lindemuthianum* isolates from the four regions of the country. The combined matrix was able to distinguish all (except 2) isolates.

4.10 Discussion and recommendations

RAPDs analysis differentiated all the isolates except three from Apac, which emerged as 100% genetically similar. Clustering of isolates using UPGMA resulted into all isolates being evenly distributed on the dendrogram, except those from Apac, which formed their

own subcluster. All isolates from Apac were collected from small seeded bean varieties. Large seeded beans are rarely grown in this part of the country (Nkoya, 2001). It is worth noting that isolates from small seeded beans in the other regions shared a few similar fingerprints with those from Apac. Apac is located in mid-northern Uganda where small-seeded bean types are the most preferred (Opio *et al.*, 2001; Nkoya, 2001). Consequently, there has been limited introduction of other bean varieties into this region (Nkoya, 2001). On the other hand, the rest of the locations grow mixed bean types – large, medium and small seeded. Although there is substantial trade in beans in Uganda, the trend has been movement from north to south and not vice-versa (Nkoya, 2001). These results point to a possibility of adaptation of the anthracnose pathogen with its host. A similar conclusion was arrived at by Ferreira *et al*. (2003).

When results from the two molecular techniques are compared, RAPDs analysis seems to be more informative since it was able to differentiate the majority of the isolates, except three from Apac. RAPDs are capable of sampling the whole genome of an organism (Bruce, 1997) unlike rep-PCR technique which selectively amplifies distinct regions of the genome (Versalovic *et al*., 1994).

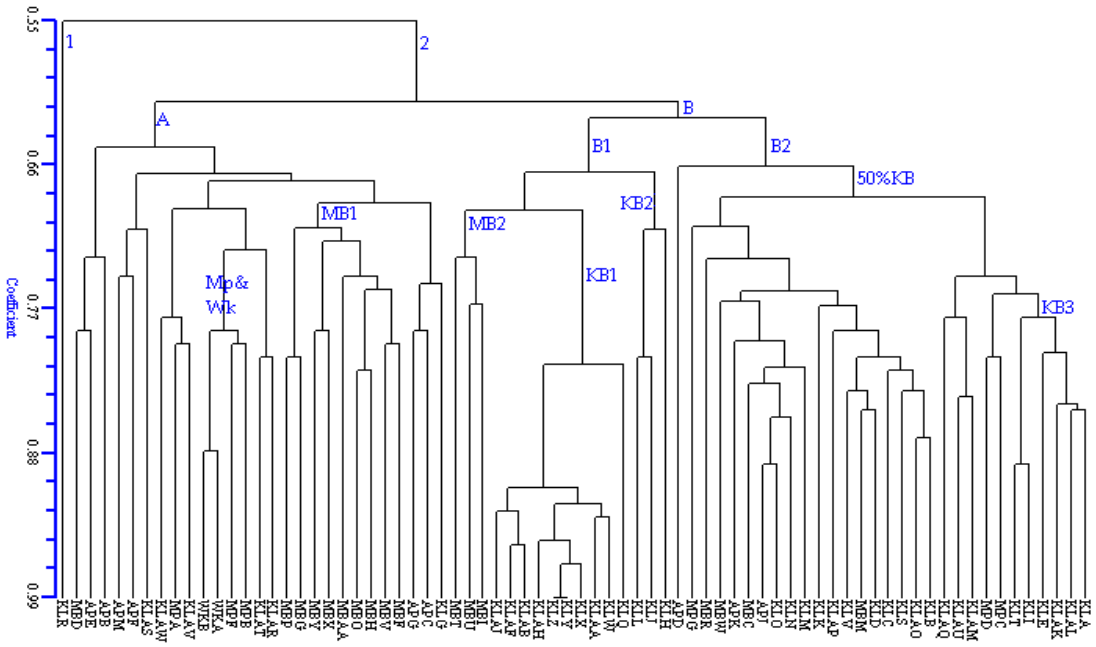


Figure 4: Distribution of *C. lindemuthianum* isolates on a dendrogram produced from combined RAPD and rep-PCR data. 1 and 2 are the minor and major clusters respectively. A and B are subclusters of the major cluster 2. B1 and B2 are branches of subcluster B. Small clusters consisting of only Kabale isolates (KB1, KB2 and KB3), Mbale isolates (MB1 and MB2) and Mpigi and Wakiso isolates (Mp and Wk) are also shown

The genetic variability revealed by RAPDs was greater (FST =0.237) than that shown by rep-PCR (FST =0.113). On the basis of clustering of isolates on the dendrograms, RAPDs produced more genetic variation compared to rep-PCR. Similar reasons given above are responsible for this. From both techniques, it could still be concluded that *Colletotrichum lindemuthianum* population in Uganda is highly variable. Elsewhere in the world, Mahuku and Riascos. (2004) reported a high genetic variability in this pathogen, and it is thought that it is due to gene flow, mutation or parasexual reproduction. The movement of bean seed between farmers in and across regions coupled with selection over time could be responsible for the introduction and subsequent maintenance of variable genetic factors of the fungus in the different bean growing areas. New races of the pathogen with variable genetic and virulence factors are constantly

introduced or transferred to new places since the pathogen co-evolves with its host (Ferreira, 2003). It has been reported that the perfect stage of this pathogen is very rare or absent in nature (Vidigal *et al*., 2007), therefore, it is most likely that the current variability displayed by *C. lindemuthianum* is not due to sexual reproduction.

In all the analyses, the 74 isolates did not cluster by region or bean gene pool from which the samples were isolated. This is in agreement with the results from previous work by Mahuku and Riascos (2004) who found out that *C. lindemuthianum* (represented by 200 isolates from around the world) is not specialized on a particular *P. vulgaris* gene pool. The study also revealed no total geographical differentiation of the isolates; although the greatest pathogen variability was observed from regions that normally grow mixed bean genotypes with high proportions of small seeded bean types. These findings present useful information regarding deployment of resistance genes and directing of programmes aimed at developing anthracnose resistant cultivars.

In conclusion, therefore, this study established enough evidence that Ugandan *C. lindemuthianum* population is highly variable. Variability within this pathogen was clearly demonstrated by RAPDs and rep-PCR analyses. RAPDs analysis revealed more polymorphism than rep-PCR protocol. Kabale isolates were more polymorphic, followed by those from Mbale and Mpigi, while the Apac isolates were generally genetically uniform. Again, no clear geographical differentiation of the pathogen was realized. Similarly, there was no clear specialization of the pathogen on either of the host's gene pools.

Comparison of the two protocols for pathogen variability assessment revealed that the molecular techniques were more informative than the pathogenicity method. However, for the purpose of race determination, the molecular technique hardly revealed the specific races existing in bean agroecologies, therefore does not give clear information regarding which resistance genes are to be deployed for the control of anthracnose. Also, this method does not tell the level of virulence of the isolates. It is therefore important to always combine the two methodologies if adequate information about pathogen variability is required.

CHAPTER FIVE

REACTION OF UGANDAN LOCAL AND RELEASED BEAN GENOTYPES TO *COLLETOTRICHUM LINDEMUTHIANUM*

5.1 Introduction

Anthracnose is one of the most important diseases of beans in Uganda. Its causal pathogen (*Colletotrichum lindemuthianum*) is seed borne, highly variable and widely distributed in almost all bean agroecologies. It is best controlled through growing resistant varieties. The national beans programme has in the past released a total of 17 improved varieties. In addition, the programme collected over 400 local landraces. Varieties that were previously known to be resistant (for example K 20) have currently succumbed to the disease in all regions where they are grown. The reaction of other varieties and market-class landraces to anthracnose in Uganda is not documented. If such information was known, it would ease the identification of adapted sources of resistance genes for breeding purposes and also directly help farmers improve bean yields by selecting and growing resistant varieties. In this experiment, 17 released bean varieties and 79 local landraces were screened for resistance to anthracnose using race 3 of *C. lindemuthianum* with the aim of identifying sources of resistance for future breeding work.

Materials and methods

5.2 Common bean genotypes

The common bean germplasm used in this experiment comprised of 96 genotypes. Out of these, 17 were improved (and released) while 79 were landraces. The released varieties

included 5 climbers and 12 bush varieties. Six of the released varieties were small-seeded while the rest were large-seeded varieties. The landraces were all bush, 18 of which were small seeded while 61 were large seeded. The characteristics of each genotype as well as their reaction to race 3 of *C. lindemuthianum* are given in Table 13.

5.3 Raising of plants for inoculation

Five seeds of each variety were planted on raised beds in a screen house at Kalengyere, Kabale district. They were planted in 8 rows spaced at 20cm. The plants were spaced at 5cm and 10cm between plants and genotypes respectively. The plants were continuously watered as necessary in order to maintain vigorous growth, until they were ready for inoculation at 3 fully expanded trifoliate leaf stages.

5.4 Inoculum preparation

Twenty culture plates of isolate 104 (race 3) of *Colletotrichum lindemuthianum* were multiplied on autoclaved fresh bean pods placed on PDA medium in 9cm petri-dishes. Isolate 104 (race 3) was selected on the basis that it had infected the differential cultivar MDRK (the largest seeded anthracnose differential variety) to total death and also incited substantial infection (severity level 4) on cultivar Michellite (small seeded) in our previous experiments. It was therefore capable of screening the majority Ugandan Andean bean types as well as the small seeded ones. In addition, this race was fast-growing on PDA media with or without bean pods and sporulated within 3-5 days. A few other isolates took long to grow on and even then, their sporulation was scanty, while the rest of the isolates never sporulated at all on both PDA or on bean pods. The cultures

were incubated at 19-21^{o}C for 14 days or until there was enough sporulation. Inoculum was prepared by harvesting spores from the cultures using a fine brush, followed by filtration and standardization to 1.2x10^{6} spores per mL of distilled water. About 20 drops of tween twenty were added per litre of the inoculum and the mixture shaken gently for 10 minutes before inoculation.

5.5 Inoculation

The fresh spore suspension was gently sprayed onto both the upper and lower surfaces of three well developed trifoliate leaves and the hypocotyls of 28-day old plants using a hand sprayer. The inoculated seedlings were then covered with a transparent polythene sheet (Tu, 1995) for 5 days and thereafter the sheet removed. Inoculation was repeated two more times at a seven-day interval. Upon removal of the polythene sheet, the plants were left in the screen house awaiting symptom development and evaluation. This experiment was replicated five times (five seedlings per genotype) and repeated once i.e., 3rd October – 20th November and between 28th November – 30th December, 2007.

5.6 Data collection and analysis

The percentage of leaf area diseased was scored 7 days after the second inoculation regime using a 1-9 severity bean anthracnose scoring scale. Symptom evaluation was again repeated 7 days later. On the basis of virulence, the Ugandan genotypes were then grouped into resistant (scores of 1.0-2.9), less susceptible (3.0-4.9), moderately susceptible (5.0-6.9) and highly susceptible (7.0-9.0). Data was then subjected to

ANOVA in Genstat computer programme, version 7.2 DE (Lawes Agricultural Trust - Rothamsted Experimental Station, 2007) in order to partition variances.

5.7 Results

Typical bean anthracnose symptoms were obtained on the susceptible genotypes (Plate 11). Symptoms appeared on all vegetative parts of the susceptible seedlings including stems, leaves and leaf petioles and ranged from very small to large extensive lesions, with some of them showing pathogen sporulation (Plate 11). Highly susceptible reactions resulted into complete death of the test plants, while others shed most or all of the leaves.

Plate 11: Symptoms of anthracnose on landraces susceptible to race 3 seven days after inoculation

There was a significant (P = <0.001) genotype effect on mean final anthracnose severity (Table 12). Severity scores ranged from 1 (no visible anthracnose symptoms) to 9 (highly susceptible) (Tables 13 and 14). Of the 96 genotypes tested, 54 (56%) were susceptible while 44% were resistant. Among the susceptible genotypes, 47 (87%) were landraces while only seven were released varieties. Among the susceptible varieties, 40 of them (over 74%) were highly susceptible, 9 moderately susceptible and 5 were less susceptible (Table 14).

***Table 12*: Summary of ANOVA table for anthracnose severity on bean genotypes in Uganda (data shown is for both replicates)**

Source	D. Freedom	S.S	M.S	V.R	F Probability
Repeat	1	545.73	545.73	396.15	
Genotype	94	5108.22	54.34	39.45	< 0.001
Residual	786	1082.78	1.39		
Total	881	6416.62			

CV%= 38.1, LSD (at 5% level) = 1.03

Table 13: Characteristics of Ugandan* landrace*s and released bean genotypes and their reaction to races 3 of Colletotrichum lindemuthianum previously identified in Uganda

Bag Code	Name	District of Origin	Sample Number	Seed size	Growth Habit	Average Severity	
						Rep 1	Rep 2
	Landrace genotypes						
1	Special K132	Kayunga	6	LG	Bush	8.3	3.2
2	Nambale	Mukono	4	LG	Bush	4.3	2.5
3	Red	Mukono	2	SS	Bush	1.0	1.0
4	Red	Kabale	5	SS	Bush	1.0	1.0
5	Red/Maroon	Masaka	1	SS	Bush	1.0	1.0
6	Kanyebwa Omuwanvu		71 – 1	LG	Bush	6.8	6.0
7	Kayinja	Kayunga	2	MD	Bush	7.7	3.5
8	Nambale Omuwanvu	Mbale	48	LG	Bush	7.0	4.0
9	Like K132	Mukono	1	LG	Bush	1.0	1.0
10	White	Kayunga	9	SS	Bush	1.0	1.0
12	Kanyebwa		36	MD	Bush	9.0	7.3
13	Kakhi Mixture	Hoima	3	MD	Bush	1.0	1.0
14	White	Hoima	8	SS	Bush	1.0	1.0
15	Nambale	Kayunga	4	LG	Bush	7.1	2.2
17	Nambale mix	Hoima	1	LG	Bush	7.4	2.8
19	Nambale	Bushenyi	21	LG	Bush	5.8	1.6
20	Nakyewogola	Bushenyi	6	LG	Bush	1.0	1.0
21	Kahura	Mbarara	18	SS	Bush	2.8	1.1
22	Kayinja	Mbarara	9	MD	Bush	9.0	3.5
23	Yellow	Mukono	17	MD	Bush	6.3	6.3
24	Kanyebwa white	Bushenyi	1	MD	Bush	1.0	1.0
25	Red mixture	Mukono	3	SS	Bush	1.3	1.0
28	Nambale	Mbarara	21	LG	Bush	8.8	8.6
29	Yellow	Mbarara	4	MD	Bush	8.9	9.7
30	Kanyebwa	Bushenyi	25	MD	Bush	8.9	7.0
31	Yellow	Kayunga	1	MD	Bush	9.0	4.2
32	Kanyebwa	Masindi	1	MD	Bush	8.9	3.6
34	Pink	Mubende	4	SS	Bush	1.0	1.0
35	Kayinja Owamabara	Mukono	6	MD	Bush	6.3	6.5

Continued from page 74

36	Red	Kiboga	3	SS	Bush	1.0	1.0
37		Hoima	10	SS	Bush	6.8	1.0
39	Kanyebwa	Mbarara	20	MD	Bush	9.0	3.6
40	White	Hoima	5	SS	Bush	1.0	1.0
41	Kahura	Bushenyi	10	MD	Bush	9.0	5.7
42	Manyigamulimi	Mukono	7	LG	Bush	2.6	3.8
43	Kanyebwa	Mbarara	14	MD	Bush	8.6	8.4
44	Kanyebwa		54 – 2	MD	Bush	9.0	6.5
45	Yellow	Kiboga	5	MD	Bush	9.0	4.2
46	Yellow	Mukono	20	MD	Bush	9.0	2.9
47	Purple stripped	Mukono	14	LG	Bush	8.0	2.0
48	Kanyebwa white black stripes	61–5 1st		LG	Bush	8.2	4.9
49	Kanyebwa	Mukono	16	LG	Bush	8.9	4.6
50	Kayinja	Mubende	13	MD	Bush	1.0	1.0
51	Yellow	Masaka	7	MD	Bush	9.0	3.6
52	White	Mbarara	17	SS	Bush	1.0	1.0
53	Kahura	Mbarara	19	MD	Bush	9.0	2.8
54	Special K132	Mukono	18	LG	Bush	8.5	6.5
55	Nkulyembalukye	Mubende	4	SS	Bush	1.0	1.0
56	Yellow	Kiboga	4	MD	Bush	9.0	7.1
57	Large coffee	Bushenyi	7	LG	Bush	8.6	4.4
58	Kanyebwa white	Bushenyi	11	MD	Bush	7.8	4.1
59	Kanyebwa	Mbarara	8	MD	Bush	3.2	1.0
60	Kahura	Mbarara	3	MD	Bush	3.0	1.4
61	Manyigamulimi	Mukono	12	LG	Bush	1.0	1.2
62	Kanyebwa	Mbarara	11	MD	Bush	5.8	4.0
63	Yellow	Hoima	7	LG	Bush	1.0	1.1
64	OBA 1-like	Kiboga	6	MD	Bush	6.5	5.2
65	Yellow	Bushenyi	9	LG	Bush	9.0	5.6
66	Yellow	Mbarara	6	MD	Bush	9.0	6.6
67	Kanebwa	Masaka	5	MD	Bush	9.0	7.1
68	Special K132	Mukono	15	LG	Bush	9.0	5.4
69	Nambale omumpi	Masindi	2	MD	Bush	9.0	3.1
70	Kanyebwa	Kayunga	12	MD	Bush	9.0	3.8
71	Kanyebwa omuwanvu	Bushenyi	5	LG	Bush	1.8	2.0
72	Calima	Kiboga	2	LG	Bush	6.0	4.2
73	Kahura	Kabale	12	SS	Bush	1.0	1.0
74	Kanyebwa	Masindi	3	MD	Bush	9.0	4.0
75	Manyigamulimi	Masaka	8	LG	Bush	1.0	1.0
76	Cream	Hoima	4	SS	Bush	1.3	1.0
77	Kanyebwa (long)	Bushenyi	18 2nd	LG	Bush	1.0	1.0
78	Large yellow	Hoima		LG	Bush	1.0	1.0
79	Coffee	Bushenyi		SS	Bush	1.0	1.0
80	Ngori	Apac		LG	Bush	9.0	7.1
81	Purple	Bushenyi		MD	Bush	1.0	1.0
82	Large yellow	Bushenyi		LG	Bush	8.3	5.5
83	Apac white	Apac	29	SS	Bush	1.0	1.0

Continued from page 75

84	Carolina	Mpigi	LG	Bush	1.0	1.0
85	Red	Mukono	SS	Bush	1.0	1.0
86	Nakawunde (black striped)	Mpigi	LG	Bush	1.5	1.2
	Released varieties					
101	NABE 1	OBA 1	LG	Bush	7.3	3.1
102	NABE 2	MCM 1015	SS	Bush	1.0	1.0
103	NABE 3	MCM 2001	SS	Bush	1.0	1.0
104	NABE 4	POA 2	LG	Bush	6.0	3.5
105	NABE 5	SUG 73	LG	Bush	9.0	5.0
106	NABE 6	UBR (92) 25ML	SS	Bush	1.0	1.0
107	NABE 7C	VUNIKINGI	SS	Climbing	2.3	1.8
108	NABE 8C	NGWINURARE	LG	Climbing	1.5	1.0
109	NABE 9C	GISENYI	LG	Climbing	1.0	1.0
110	NABE 10C	G2333(UMUBANO)	SS	Climbing	1.0	1.0
112	NABE 12C	MAC 31	LG	Climbing	1.0	1.0
113	NABE 13	RWR 1946	LG	Bush	4.3	1.0
114	NABE 14	RWR 2075	LG	Bush	3.6	4.3
131	K131		SS	Bush	1.0	1.0
132	K132	CAL 96	LG	Bush	9.0	7.1
120	K20		LG	Bush	9.0	7.0

On the basis of bean gene pools, only one small seeded (and landrace) genotype was found to be susceptible (Table 14). The remaining 23 small seeded genotypes were highly resistant to isolate 104 (race 3) of *C. lindemuthianum*. On the other hand, 40 out of the 72 large seeded genotypes tested were found susceptible to this race. Of all the landraces tested, only 32 were resistant among which, 15 were large seeded while 17 were small seeded (Table 14). All the five Ugandan released climbing bean varieties tested were resistant to this race. They include NABE 7C, NABE 8C, NABE 9C, NABE 10C (G2333) and NABE 12C with severity scores ranging from 1-2.3. The most susceptible released varieties were NABE 1, NABE 5, K132 and K20 (Table 13).

Table 14*: Summary of the reaction of 96 Ugandan bean varieties to race 3 of *Colletotrichum lindemuthianum

Description	Resistant (score 1.0-2.9)	Susceptible			Total
		Less (score 3.0-4.9)	Moderate (score 5.0-6.9)	Highly (score 7.0-9)	
Genotypes Tested	42	5	9	40	96
Large seeded	19	5	8	40	72
Small Seeded	23	0	1	0	24
Landraces	32	3	8	36	79
Released varieties	10	2	1	4	17

5.8 Discussion

Differences in response to anthracnose among bean cultivars were clearly detected under screen house conditions. Out of the 96 bean genotypes that were assessed, the majority (54) were susceptible. Of the 54 susceptible genotypes, 53 were from Andean origin while only one was Mesoamerican. A similar observation was previously realized in a study by Pathania *et al*. (2006) in which 35 out of 49 bean varieties were susceptible to race 3 of *C. lindemuthianum*. In their study, most of the genotypes resistant to race 3 were either improved varieties or exotic in India. In Ethiopia, a related study by Tesfaye (2003) had also revealed a variety of reactions of 200 bean genotypes to 8 races of anthracnose. Likewise, a study on the reaction of 26 Brazilian landraces to 9 *C. lindemuthianum* races showed that Andean genotypes were generally more susceptible to anthracnose than their Mesoamerican counterparts (Vidigal *et al*., 2007). Race 3 had been isolated from highland areas of Mbale, and according to the reaction of the differential cultivars (chapter 3); race 3 was virulent to two of the 12 differentials, i.e., Michellite and MDRK. Mitchellite is Mesoamerican and has no known anthracnose resistance genes

while MDRK is Andean and carries Co-1 anthracnose resistance gene (Kelly and Vallejo, 2004). This race, therefore, is capable of overpowering beans from both gene pools. This implies that the Ugandan bean genotypes under production, especially the large seeded ones, are at stake of being wiped out by the anthracnose pathogen. This work also confirmed that the previously resistant bean variety K20 currently succumb to anthracnose. Besides, the status of K 132 had not been documented, but was confirmed susceptible in the present study. Majority of the released varieties were resistant and showed superiority over landraces.

All in all, this study showed that common bean genotypes resistant to race 3 of *C. lindemuthianum* exist in Uganda. Genotypes that were found resistant to this race could therefore be used in breeding programmes aimed at improving resistance to both Andean and Mesoamerican races of *C. lindemuthianum* (Vidigal *et al*., 2007). An integrated disease management strategy involving use of the resistant genotypes identified in this study plus other cultural management options could help farmers in controlling bean anthracnose in areas where it is a big problem. Nabe 8C, Nabe 9C and Nabe 12C, all of which are climbers were the only large seeded released varieties that were resistant to anthracnose. Since large seeded varieties are preferred by majority of bean growers, production of the above three varieties should be extended to areas of high anthracnose incidence. All the released small seeded varieties (Table 13) were resistant to race 3 and introduction of these varieties into anthracnose-stricken areas is highly recommended.

CHAPTER SIX

6.1 GENERAL FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

The focus of this research was to determine the diversity of the bean anthracnose pathogen, *Colletotrichum lindemuthianum* using pathogenic isolates collected from five major bean agroecologies in Uganda and to compare the methodologies used in pathogen variability assessment. This was aimed at setting up a foundation for developing high yielding, market-class bean varieties that are resistant to anthracnose. Results indicate that Ugandan population of *C. lindemuthianum* is highly variable, both physiologically and genetically within and between different regions. Physiological diversity was expressed by occurrence of 21 races of the pathogen from 47 different isolates. Both highly virulent as well as less virulent races were realized. For example, two isolates (36 and 2) which were finally designated as races 3086 and 4033 respectively were able to infect (severity level 4 under a 1-9 anthracnose severity scale) the highly resistant cultivar, G2333. On the other hand, Eight isolates were unable to be differentiated by race differentials because either they were unable to cause infection or their severity scores were less than 4; hence considered under resistant reactions and were subsequently classified as race 0 (zero). In terms of differential susceptibility, cultivar Widusa never succumbed to any single isolates used in this study, Kaboon only succumbed to one isolate and the rest succumbed to 2 – 10 races.

Ugandan *C. lindemuthianum* is not only variable physiologically but also highly genetically variable. This was revealed when molecular analysis was done. Use of RAPDs analysis differentiated all but 3 isolates (APD, APE and APM), all of which

originated from Apac. Analysis of 74 isolates using UPGMA resulted into a dendrogram with 3 major clusters, on which all Apac samples formed their own subcluster. Isolates from other regions were uniformly distributed around the dendrogram. Based on RAPD analysis, isolates from Kabale were the most polymorphic (93.9%), followed by those from Mbale (89.0%) and Mpigi and Wakiso (67.1%). All Apac samples were almost uniform (8.5% polymorphic). Rep-PCR was also conducted on these isolates using BOX and ERIC primers. In this analysis, the majority of isolates revealed variable banding patterns. Analysis of rep-PCR data using UPGMA also generated a dendrogram, which divided all the study samples into 2 clusters: a minor one (I) and a major one (II) at 53% similarity. The minor cluster I was composed of 4 isolates three of which were from Mbale and one from Kabale. The major cluster II consisted of 70 isolates.

All isolates from Apac were collected from small seeded beans varieties. Large seeded beans are rarely grown in this part of the country (Nkoya, 2001). Apac is located in mid-northern Uganda where small-seeded bean types are the most preferred (Opio *et al*., 2001; Nkoya, 2001). Consequently, there has been limited adoption of large seeded bean varieties into this region (Nkoya, 2001).

On the other hand, the rest of the locations grow mixed bean types – large, medium and small seeded. Although there is substantial trade in beans in Uganda, the trend has been movement from north to south and not vice-versa (Nkoya, 2001). These results show some possibility of adaptation of the anthracnose pathogen with its host. A similar conclusion was arrived at by Ferreira *et al*. (2003).

When results from the two molecular techniques were compared, RAPDs analysis seemed to be more informative since it was able to differentiate the majority of the isolates, except three from Apac. RAPDs are capable of sampling the whole genome of an organism (Bruce, 1997) unlike rep-PCR technique, which selectively amplifies distinct regions of the genome (Versalovic *et al*., 1994).

The genetic variability revealed by RAPDs was greater (FST =0.237) than that shown by rep-PCR (FST =0.113). On the basis of clustering of isolates on the dendrograms, RAPDs produced more genetic variation compared to rep-PCR. Similar reasons given above are responsible for this.

From both techniques, it could still be concluded that *Colletotrichum lindemuthianum* population in Uganda is highly variable. The ultimate source of variability could be due to gene flow, mutation or asexual reproduction. Exchange of bean seed between farmers in and across regions together with selection over time could also be responsible for the introduction and subsequent maintenance of variable genetic factors of the fungus in the different bean growing areas. New races of the pathogen with variable genetic and virulence factors are constantly introduced or transferred to new places since the pathogen co-evolves with its host on top of being seed borne (Ferreira, 2003). It has been reported that the perfect stage of this pathogen is very rare or absent in nature (Vidigal *et al*., 2007), therefore, it is most likely that the current variability displayed by *C. lindemuthianum* is not due to sexual reproduction.

When the two protocols of pathogen variability assessment were compared, the molecular techniques were more informative than the pathogenicity method. However, for the purpose of race determination, the molecular technique hardly revealed the specific races existing in bean agroecologies, therefore does not give clear information regarding which resistance genes are to be deployed for the control of anthracnose. Also, this method does not tell the level of virulence of the isolates. It is therefore important to always combine the two methodologies if adequate information about pathogen variability is required.

Finally, 96 common bean genotypes comprising of 17 released varieties and 79 land races were screened for resistance to anthracnose using isolate 104 (race 3) of *Colletotrichum lindemuthianum*. Of these, 54 genotypes (56%) were susceptible while 44% were resistant. Among the susceptible genotypes, 47 (87%) were landraces while only seven were released varieties. Among the susceptible varieties, 40 of them (over 74%) were highly susceptible, 9 moderately susceptible and 5 were less susceptible (Table 14). Bean anthracnose symptoms appeared on all vegetative parts of the susceptible seedlings including stems, leaves and leaf petioles and ranged from very small to large extensive lesions. Some lesions involved pathogen sporulation (Plate 11). Highly susceptible reactions resulted into complete death of the test plants (plate 12), while other susceptible plants resulted into shedding of most or all of the leaves. Out of the 24 small seeded genotypes, only one (and landrace) was found to be susceptible (Table 14). On the other hand, 40 out of the 72 large seeded genotypes tested were found susceptible to this race. Of all the landraces tested, only 32 were resistant among which, 15 were large seeded

while 17 were small seeded (Table 14). All the five Ugandan released climbing bean varieties tested were resistant to this race. They include NABE 7C, NABE 8C, NABE 9C, NABE 10C (G2333) and NABE 12C. The most susceptible released varieties were NABE 1, NABE 5, K132 and K20 (Table 13). Overall, large seeded bean genotypes were more susceptible to race 3 of *C. lindemuthianum* than the small seeded genotypes.

6.2 Summary of conclusions

- Ugandan *Colletotrichum lindemuthianum* is highly variable, physiologically and genetically
- Geographical distribution of the pathogen is not clearly reflected by either the pathogen's genetic or physiological characteristics
- Similarly, there is no specialization of the pathogen on the two common bean gene pools
- Highly virulent isolates capable of breaking down strong resistances exist in Uganda
- Variation within the pathogen is due to mutation, gene flow and non-sexual reproduction
- Introduction and maintenance of seed of new varieties into new areas is responsible for genetic and physiological distribution of variable pathogenic isolates
- Variability of the anthracnose pathogen in Uganda is directly related to diversity within the common bean genome in a given region
- Large seeded bean genotypes emerged more susceptible to anthracnose infection than the small seeded counterparts

- RAPDs analysis still remains a fast and easy means of detecting the genetic variability within *C. lindemuthianum* population as compared to rep-PCR
- Cultivar Widusa is highly resistant to Ugandan bean anthracnose isolates
- Land race bean genotypes are generally more prone to anthracnose infection than the improved released varieties

6.3 Recommendations

- I recommend that Ugandan population of *Colletotrichum lindemuthianum* be regularly monitored for emergence of variable pathotypes since the current study revealed new races that were never found by previous researchers (Nkalubo, 2006 and Leaky and Simbwa-Bunya, 1972)
- I also recommend that a comprehensive study to dissect and ascertain the anthracnose - resistance genes other than Co-9 carried by cultivar Widusa be done
- Cultivar Widusa showed significant capacity to control bean anthracnose, therefore, it should be incorporated in breeding programs aimed at improving beans for resistance to anthracnose
- Ugandan isolates of *C. lindemuthianum* from the West Nile bean agroecology were not included in this study. A more comprehensive study involving isolates from all bean growing regions of Uganda should be carried out. After that, Ugandan isolates should be analyzed together with those from other neighbouring countries where bean trade or exchange has occurred over time. In this case, a comparative study between Ugandan isolates, those from Rwanda, Kenya, Tanzania, D. R. Congo and

Sudan should be done so that any existing trend regarding source of variable pathogenic isolates within and between these countries is discovered

- Regarding the protocols used for genetic variability analysis, no problem was encountered using RAPDs. In fact the technique was easy and quick as compared to rep-PCR protocols. In addition, RAPDs revealed more polymorphism than rep-PCR protocols. I therefore recommend continued use of RAPDs when dealing with this kind of work
- As regards to the bean genotypes, generally the small seeded varieties were more resistant to race 3 of bean anthracnose than the large seeded types. Introducing them or increasing their production in areas prone to anthracnose is recommended. Also, the resistant genotypes could be used as sources of resistance for the improvement of the popular but susceptible large seeded varieties.
- The Ugandan bean genotypes that were found resistant should be analyzed so that the genes involved in resistance are established. Since such genes are adapted to Ugandan farming conditions, they can be used for improvement of locally demanded and market-class but susceptible varieties
- Since anthracnose currently exists in all bean agroecologies, I recommend that variety evaluation trials put bean anthracnose in consideration before releasing new bean varieties.

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