DETERMINATION OF GENETIC DIVERSITY OF *PSEUDOMONAS SYRINGAE* PATHOVAR *GARCAE* AND ITS REACTION TO SELECTED COFFEE VARIETIES IN KENYA

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Determination of genetic diversity of *Pseudomonas syringae* pathovar *garcae* and its reaction to selected coffee varieties in Kenya

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A thesis submitted in partial fulfilment for the degree of Master of Science in Biotechnology in the Jomo Kenyatta University of Agriculture and Technology

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DECLARATIONS

This thesis is my original work and has not been presented for a degree in any University

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DEDICATION

To my loving wife Lydiah, my children Mark Clarence, Emmanuel, Shalom and my nephew Boniface

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LISTS OF ABBREVIATIONS AND ACRONYMS

AEZ -Agro ecological zones

ANOVA - Analysis of Variance

AFLP - Amplified Fragment Length Polymorphism

BAC - Bacterial Artificial Chromosome

BBC - Bacterial Blight of Coffee

CBD - Coffee Berry Disease

CLR - Coffee Leaf Rust

CRI/CRS - Coffee Research Institute, Coffee Research Station

CTAB - Cyltrimethylammoniumbromide

EST - Expressed Sequence Tag

ICGN - International Coffee Genome Network

ILRI - International Livestock Research Institute

KALRO - Kenya Agricultural and Livestock Research Organisation

LSD - Least Significant Difference

MLST - Multi locus sequence typing

PCR -Polymerase Chain Reaction

Pph -*Pseudomonas syringae pathovar phaseolicola*

P. s.* pv. *garcae *- Pseudomonas syringae* pathovar *garcae*

RAPD - Random Amplified Polymorphic DNA

RFLP - Restriction Fragment Length Polymorphism

rRNA - Ribosomal Ribonucleic acid

SNA - Sucrose Nutrient Agar

SSR - Single Sequence Repeats

UM - Upper midland

ABSTRACT

Bacterial blight of coffee (BBC) caused by *Pseudomonas syringae* pathovar *garcae* is a major concern in Kenya and other coffee growing countries due to high incidence and severity in Arabica coffee. Previous work to develop disease resistant coffee varieties in Kenya in order to reduce the cost of production with safe environmental management did not take resistance to BBC into consideration. The commercial coffee varieties that are resistant to the major coffee diseases in Kenya such as Ruiru 11 and Batian were not selected for BBC resistance posing a great economic threat to coffee farming in Kenya especially in the BBC hot spots of Nakuru, Nyeri and Kapsabet. The diversity of the causative agent in Kenya had not been documented. Determination of genetic diversity of *Pseudomonas syringae* pv. *garcae* and its reaction to selected coffee varieties in Kenya was therefore the objective of this research. Phenotypic characterization of 12 isolates of *P.s.* pv. *garcae* collected from different regions in Kenya was done by inoculating them on four coffee varieties to test their virulence. Twenty four coffee genotypes were further screened for BBC resistance using the 3 most virulent and the least virulent BBC isolates by inoculation with a drop of approximately 10 µl of the isolates suspension (10^9 CFU/ml) using the injection method at the shoots of 4 months old seedlings from potting. The experiment was carried out in a complete randomized design. Disease symptoms were scored using a scale of 1 to 5 and the data was subjected to analysis of variance and effects declared significant at 5% level. Molecular characterization via PCR and sequencing of the 12 isolates was done using 16S ribosomal RNA primers 8 F and 1492 R (l). The sequences were then retrieved for alignment using vector NTI software and phylogenetic analysis done by MEGA 6 using clustalW. Eleven SSR primers were used to characterize the 24 genotypes using PCR and the bands' data was used to estimate genetic distances using XLSTAT version 2014. The 12 isolates were found to have significant levels ($P>0.05$) of virulence to coffee which was closely linked with the molecular characterization using 16S gene showing the diverse nature of the pathogen and possible existence of different races of *P. s.* pv.

garcae. Rume Sudan and Hibrido *de* Timor (HDT) were found to be the most promising donor candidates for resistance to BBC and diversity was observed among the coffee genotypes via SSR molecular characterisation. This study provides new knowledge on the nature of virulence of isolates collected from different areas in Kenya and possible sources of resistance to BBC. Further research to pyramid genes related to resistance to BBC in a view of developing resistant variety while genotyping of all existing BBC isolates is recommended.

CHAPTER ONE

INTRODUCTION

1.1 Background to the study

Coffee is among the most important agricultural commodities in international trade representing an important source of income for millions of people in coffee growing countries in Asia, Africa and Latin America (Mishra & Slater, 2012). The Arabica coffee (*Coffea arabica*) varieties which are preferred for their superior beverage quality account for about 70% of the total world coffee production and are derived from either the "Typica" or "Bourbon" genetic base. The species is predominantly self-pollinating, which has resulted in low-genetic diversity among cultivated Arabicas (Gichuru *et al.*, 2008; Mishra & Slater, 2012). In contrast, *Coffea canephora* is self-incompatible and has a wide geographic distribution, extending from the western to central tropical and subtropical regions of the African continent (Mishra & Slater, 2012). Coffee production in Kenya is constrained by diseases mainly coffee leaf rust (CLR) caused by *Hemileia vastatrix*, coffee berry disease (CBD) caused by *Colletotrichum kahawae* and BBC caused by *P. s.* pv. *garcae* (Mugiira *et al.*, 2011).

BBC has become a major concern in Kenya and other countries such as Brazil, Uganda and China due to high incidence and severity in Arabica coffee (Fekadu, 2013). It is responsible for crop losses mainly in windy and cooler cultivation areas. The symptoms of the disease include dark, water-soaked necrotic lesions on leaves, tips and nodes of vegetative and cropping branches culminating in a die-back (Mugiira *et al.*, 2011). The occurrence of peak populations of *P. s*. pv. *garcae* and the high incidence of BBC coincide with periods of high relative humidity of about 80% and cool temperatures of 18-20^{o}C (Masaba, 1998). This bacterioisis is more intense on coffee grown at higher altitudes, without wind protection and in cold regions (Ito *et al.*, 2008). In Kenya, the disease was reported on coffee plantations soon after the crop was established but it was confined to the west of the Great Rift Valley (Kairu *et al.*, 1985). However, with

changing climatic conditions, the disease has spread to central and eastern Kenya. Severe epidemics of the disease as often observed in coffee plantations impact a fire scorched appearance. In areas where BBC is endemic, coffee trees are also affected by CBD (Kairu, 1999).

Coffee growers have relied greatly on copper-based formulations to control BBC which accounts for up to 30% of the total cost of production but incidences of BBC can still be observed on copper sprayed farms (Plate 1.1). The infection may be attributed to poor timing of the spraying program, micro-distribution and erosivity effects of the formulation. The chemical control manages the disease to within economic levels but does not eradicate it. The changing climatic conditions being experienced globally is posing threat of new diseases tread due to pathogen change or compromise of host plants due to change in agro-ecological factors. Emergence of diseases such as BBC in areas considered none native is therefore a great threat to the existing germplasm which necessitates initiation of mitigation and adaptation strategies that include study on pathogen diversity breeding for durable resistance. It is observed that *P. syringae* is a very stress-tolerant organism, and is the focus of many studies of stress-tolerant gene expression (Ryan O'Connor, 2011). This gram-negative plant-pathogenic bacterium comprises at least 51 pathovars that can be distinguished by their host ranges (Ma *et al.*, 2007) and the pathovars also contain several races characterized by their virulence on different host cultivars (Vinita *et al.*, 2005). The tremendous diversity of hosts and disease symptomatology found in this species presents a unique opportunity to investigate the factors that determine host specificity (Michael *et al.*, 2005).

Plate 1.1: Coffee plant showing symptoms of BBC even after spraying with copper based formulation

Disease resistant varieties not only have the potential to reduce the cost of production but also offer an environmentally safe disease management approach. Great achievements have been realized in breeding for resistance against CBD and CLR. However, resistance to BBC was not put into consideration. The commercial variety known as Ruiru 11, which was released to coffee growers in 1985 in Kenya, combines resistance to CBD and CLR with high yield, fine quality and compact growth amenable for high density planting (Omondi *et al.*, 2001). However, it has been reported to have a lower level of resistance to BBC (Ithiru *et al.*, 2013). Studies on the dynamics of *P. s.* pv. *garcae* multiplication in the foliage by Masaba (1998) indicated that the rate of multiplication, final populations, degree of colonization and severity of symptoms were lowest on coffee variety known as Catimor, intermediate on SL34 and highest on SL28, implying that Catimor is potentially resistant to BBC but only in relation to the other two varieties. SL 34 and SL 28 are traditional commercial varieties in Kenya selected on individual tree basis for their high yields, good quality and adaptable to coffee growing areas in Kenya, but they are susceptible to the fungal diseases namely CBD and CLR.

BBC disease has been observed on Ruiru 11 hybrid, a derivative of Catimor as one of the parent, and the other Kenyan commercial varieties under field conditions in Kenya (Ithiru *et al.,* 2013).

The S_H1 gene found in the Arabica coffee genotypes Harar, Dilla & Alghe, S12 Kaffa and Geisha is thought to confer simultaneous resistance to some races of *Hemileia vastatrix* and to *P.s.* pv. *garcae* (Ito *et al.,* 2008). These genotypes are not commercially grown in Kenya and their resistance may be tested with Kenyan isolates of the BBC pathogen. Field trials with several Arabica coffee genotypes identified Geisha as the most resistant to BBC (Masaba, 1998) but Geisha is not grown commercially in Kenya. Other sources of resistance to BBC without S_H1 gene such as Icatu, Catucaí and Hibrido *de* Timor (HDT) have also been identified indicating the presence of other genes that confer qualitative and quantitative resistance (Ito *et al.,* 2008). However, some of those sources of BBC like Icatu and Catucai are not cultivated in Kenya. HDT does not meet all the qualities of a commercial variety but has been utilized in breeding programs as a donor parent for resistance to CBD and CLR. Other coffee species such as *Coffea canephora, C. liberica* and *C. racemosa* have also been sources of diseases and pests resistance traits that are lacking in *C. arabica* (Mishra & Slater, 2012).

Identification and improvement of coffee varieties with durable resistance to BBC is important to the commercial growing of coffee in Kenya and world over. This will reduce the huge cost associated with disease control or crop loss thus improving the crop profitability and reduce the risks associated with excessive use of copper sprays that include environmental pollution and phytotoxic effects on coffee trees (Kairu *et al.,* 1997). Novel methods of selection that can reduce the time taken for variety development are being explored through molecular marker approaches (Hindorf & Omondi, 2010). Species-specific microsatellite markers are desirable for genetic studies and to harness the potential of Marker Assisted Selection (MAS) based breeding for genetic improvement. Limited availability of such markers for coffee warrants newer

efforts to develop additional microsatellite markers that can effectively be deployed in genetic analysis and coffee improvement programs (Hendre *et al.*, 2008).

In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hyper variable regions that can provide species-specific signature sequences useful for bacterial identification (Clarridge, 2004). Although it was originally used to identify bacteria, 16S sequencing was subsequently found to be capable of reclassifying bacteria into completely new species, or even genera. It has also been used to describe new species that have never been successfully cultured. This makes the 16S gene an important tool for identification and classification of bacteria. This study therefore aimed at determining the genetic diversity of *Pseudomonas syringae* pv. *garcae* the causative agent of BBC and its reaction to selected coffee varieties in Kenya for further selection and improvement into commercial coffee varieties.

1.2 Statement of the problem

Coffee diseases are the major drawback of coffee farming that would either cost the farmer over 30% loss of the expected yield or 30% loss of the expected profits due to use of chemicals which also increase risk to both humans and the environment. With increasing use of copper to control BBC, excessive levels of copper deposits have been reported in both soils and plant materials in areas where BBC is endemic (Masaba, 1998). Coffee farming in Kenya is shifting from the traditionally known coffee growing areas especially central Kenya due to land scarcity and change in land utilization priorities to other regions like the west of Rift valley. However, the new areas have reported high incidences of BBC especially Nakuru, Kitale, Mt. Elgon and Kapsabet areas. So far, selections for BBC resistance have not yielded a commercial variety in Kenya. Since resistance to BBC was not factored in the breeding programs of elite varieties, the crop is subsequently faced with the challenge of infection by this disease.

1.3 Justification of the study

The economics of many coffee growing countries depends heavily on the earnings from the crop. More than 100 million people in the coffee growing areas worldwide derive their income directly or indirectly from the produce of this crop (Mishra & Slater, 2012). Improvement of coffee varieties for resistance to BBC amongst other diseases affecting coffee production in the country is imperative to the commercial growing of coffee in Kenya and world over. Better knowledge of the plant defense mechanisms is important for the transfer of useful genes and to maximize the use of available germplasm resources thus allowing development of durable and effective resistance. This will reduce the burden of cost associated with disease control and the hazards related to use of chemicals (Kairu *et al.,* 1997), market access due to maximum residue levels (MRLs) and unpredictable weather that affects performance of pesticides. The utilization of the 16S gene in characterization of the BBC isolates provides the information on their genetic diversity that may be due to functional, structural, or evolutionary relationships within the pathovar. This will enable adoption of appropriate breeding strategies that will offer durable resistance in coffee against the pathogen.

1.4 Objectives

1.4.1 General objective

To determine the genetic diversity of *Pseudomonas syringae* pv. *garcae* the causative agent of BBC and its reaction to selected coffee varieties in Kenya.

1.4.2 Specific Objectives

i. To determine the virulence of different *Pseudomonas syringae* pv. *garcae* isolates infecting coffee in Kenya.
ii. To genetically characterize the different *Pseudomonas syringae* pv. *garcae* isolates using 16S ribosomal RNA.

iii. To identify coffee varieties with resistance to *Pseudomonas syringae* pv. *garcae* in Kenya .

iv. To characterize selected coffee genotypes for BBC resistance using SSR markers

1.5. Hypotheses

1.5.1 Alternative hypotheses

H1. Different isolates of *P. s.* pv. *garcae* are different in their virulence to coffee varieties.

H2. There exist different strains of *P.s.* pv. *garcae* related to pathogenicity of BBC in coffee.

H3. There exist coffee varieties that are resistant to *P. s.* pv. *garcae*.

H4. There are SSR markers for coffee associated with resistance to BBC.

CHAPTER TWO

LITERATURE REVIEW

2.1 Coffee and its production in Kenya

Coffee is one of the most popular non-alcoholic beverages and is consumed regularly by 40% of the world population mostly in the developed world and thus occupies a strategic position in the world socio-economy (Hendre *et al*., 2008). It was introduced into Kenya by missionaries at the beginning of the 20th century. The first plantations were established at Bura in the low lying coastal region of the country (now in Taita Taveta County), but due to unfavorable climatic conditions, coffee growing was relocated to higher altitudes at Kibwezi (now in Makueni County) and Kikuyu near the capital city of Nairobi (Hindorf & Omondi, 2010). Its production is fundamental in over 50 developing countries, for which it is the main foreign currency earner (Gichimu & Omondi, 2010).

Arabica coffee (*Coffea arabica*) and Robusta coffee (*Coffea canephora*) are the two main coffee species grown for commercial production, but the former is by far the most significant, providing approximately 70% of commercial production (Davis *et al.*, 2012). The *C. arabica* varieties grown all over the world are derived from either the "Typica" or "Bourbon" genetic base and are predominantly self-pollinating resulting to low-genetic diversity among cultivated *arabicas* (Mishra & Slater, 2012). The first variety to be introduced and grown commercially in Kenya was French Mission Coffee (Hindorf & Omondi, 2010). *C. canephora* which is mainly grown in Western Kenya in low land is self-incompatible and has a wide geographic distribution, extending from the Western to Central tropical and subtropical regions of the African continent with high genetic diversity in the Democratic Republic of Congo (Mishra & Slater, 2012). Arabica coffee is grown in areas between 1330 m and 2100 m above sea level mainly in three regions that include: east of Rift Valley (comprising areas around Mt Kenya, the Aberdare ranges and Machakos), west of Rift Valley (comprising Kisii highlands, Mt Elgon area

and the North Rift valley) and Taita Hills at the coast (Plate 2). Of the estimated 109,000 hectares of land under coffee, the east of Rift Valley region accounts for about 82%, west of Rift Valley for 17% and the Taita Hills for only 1 % (Kathurima *et al.*, 2013).

Coffee growing in the world is predominantly a small holder's enterprise with an estimated 25 million farmers depending on it for their livelihoods (Waller *et al.*, 2007). Coffee is mainly grown under shade or unshaded system in three agro ecological zones (AEZ) namely; Upper Midland 1 (UM1), Upper Midland 2 (UM2) and Upper Midland 3 (UM3) (Plate 1.2). UM1 has an altitude that ranges from 1570-1810 m above sea level (a.s.l) with annual mean temperature of 18.4º C and rainfall of 1640 mm. UM2 lies between 1395-1675 m a.s.l with annual mean temperature and rainfall of 19.4ºC and 1465 mm, respectively. UM3 lies between altitude of 1330-1560 m a.s.l with annual temperature of 19.9ºC and rainfall of 1270 mm (Mugo *et.al.,* 2012). The coffee growing areas receive high rainfall yearly (over 900 mm) and have very fertile arable soils. Its production is, however, constrained by a number of major diseases, including CLR, CBD and BBC. The commercial varieties that are resistant to the major coffee diseases such as Ruiru 11 and Batian lines were not bred for BBC resistance and they have been reported to be infected by BBC.

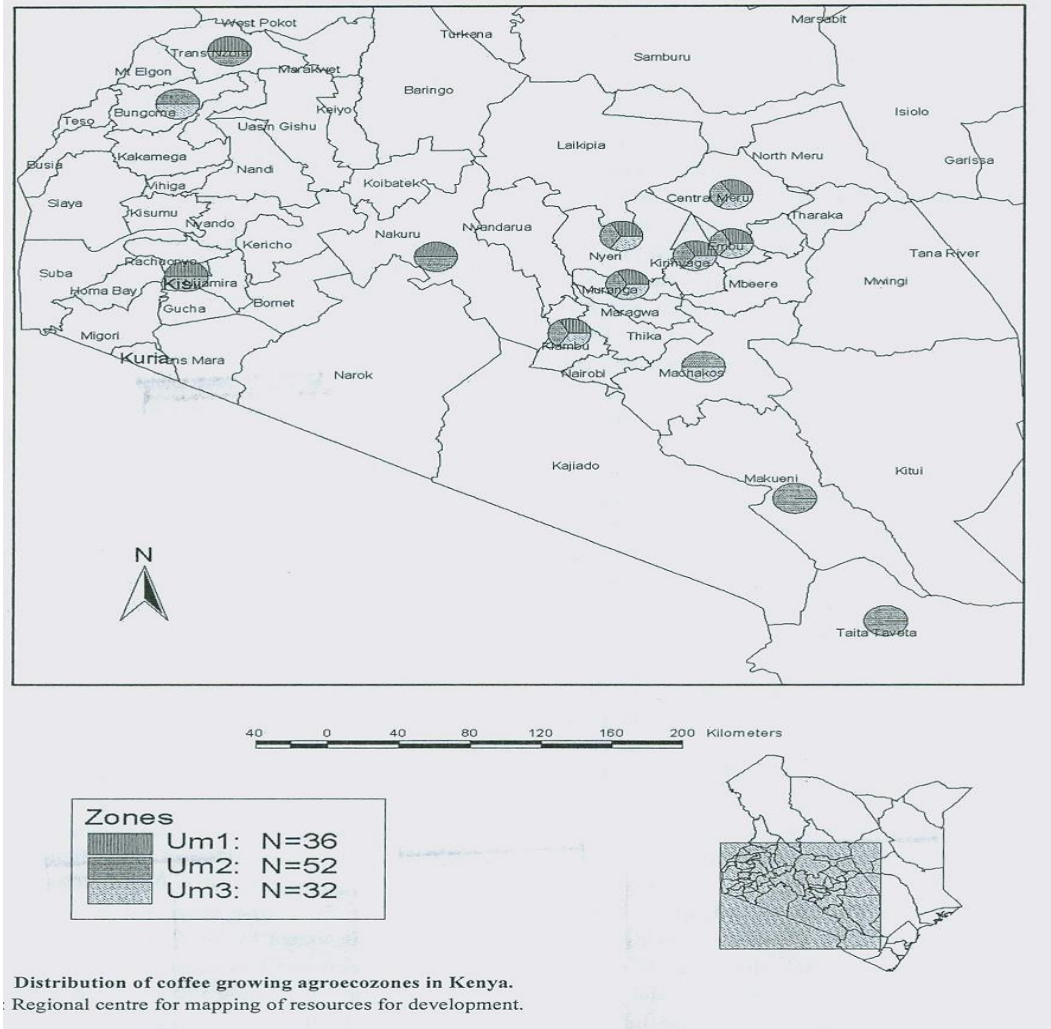

Plate 2.1: Coffee growing agro ecological zones in Kenya (Source: Mugo *et.al.*, 2012).

2.2 Economical importance of bacterial blight of coffee

The coffee bacterial blight has been reported in Brazil, Kenya, Uganda and China where it is a major concern due to its high incidence and severity (Silva *et al.,* 2006). In Kenya, the disease was reported soon after coffee plantations were introduced in 1893 but it was confined to the west of the Great Rift Valley (Mugiira *et al.*, 2011). BBC was formerly known as Elgon die-back or Solai die-back, the names signifying the areas where the disease occurred. The pathogen was reported to be confined to the high altitude zones.

Although there is variation with each pathovar, this bacterium grows optimally in cool, wet conditions from 15-25°C in temperature. Under field conditions, free moisture in the form of rain or dew, is one of the main factors influencing seasonal periodicity and distribution of epiphytic *P. s.* pv. *garcae* as well as BBC infections. The occurrence of peak populations of this pathogen and the incidence of BBC also coincide with periods of high relative humidity (about 80 %). The incubation period under such field conditions is reported as four weeks (Masaba, 1998).

BBC directly infects succulent shoot tips causing them to die-back. Hail damage has been cited as causing wounds through which *P. s.* pv. *garcae* enters the host (Masaba, 1998) thus increasing the severity of the disease. The symptoms include dark, water-soaked necrotic lesions on leaves, tips and nodes of vegetative and cropping branches culminating in a die-back (Mugiira *et al.,* 2011). It can be a serious problem in high altitudes, where plants are injured from heavy winds (Jansen, 2005) or have a protracted bimodal pattern of rainfall and often experience storms accompanied by hail (Kairu *et al.,* 1985). The inherent growth and flowering rhythms of *C. arabica* trees is controlled by the annual rainfall pattern and, greatly influence the seasonal periodicity of BBC (Ramos and Kamid 1981). Although the disease is reported as not affecting more than 5% of the crop in Kenya, it can cause total crop loss in some areas and severely affected trees sometimes have to be destroyed (Ithiru *et al.,* 2013).

Over the years, coffee growers have relied greatly on copper-based formulations to control BBC. However, excessive use of copper sprays has certain drawbacks which include environmental pollution and high costs of chemicals. Moreover, increased soil concentration of available copper may have phytotoxic effects on coffee trees which cause shortening and hardening of internodes of young shoots, chlorotic and diminished leaf area with consequent yield reduction (Kairu *et al.,* 1985). In addition, chemical control accounts for up to 30% of the total cost of production and is a major constraint to economic coffee production especially the small-holders who find the use of pesticides beyond their financial and technical capabilities (Gichuru *et al.,* 2008).

This gram-negative plant-pathogenic bacterium is the causal agent of a variety of bacterial spot, speck, and blight diseases on a wide range of plant hosts, including (but not limited to) coffee, apples, beets, beans, cabbage, cucumbers, flowers, oats, olives, peas, tobacco, tomato, and rice (Michael *et al.*, 2005).

2.2.1 Ribosomal RNA as a tool for identification and classification in prokaryotes

Ribosomal RNA is a universal tool for the phylogenetic analysis and determining interrelationships among organisms and it is an ancient, universally distributed and the most conserved region in the genome of the microorganisms (Clarridge, 2004). It is therefore a good tool for identification and characterization of bacteria. In prokaryotic ribosomes, there are three different ribosomal RNAs namely 5S, 16S, and 23S but only 16s sequence is used because nucleotides are neither less nor more in length and easy to sequence (Clarridge, 2004). The gene is large enough, with sufficient interspecific polymorphisms of 16S rRNA gene, to provide distinguishing and statistically valid measurements. About 1,500 nucleotides are present in 16S r-RNA which are determined using polymerase chain reaction (PCR) and gene sequencer. The 5S r-RNA contains only 120 nucleotides that are too small to conclude any fruitful relationship, whereas 23S contains approximately 2,900 nucleotides, it is not found suitable for experiments. In eukaryotes, 18S rRNA is used for phylogenetic studies. 16S ribosomal RNA (or 16S rRNA) is a component of the 30S small subunit of prokaryotic ribosomes. The genes coding for it are referred to as 16S rDNA and are used in reconstructing phylogenies (Woese & Fox, 1977).

In prokaryotes a small 30S ribosomal subunit contains the 16S rRNA. The large 50S ribosomal subunit contains two rRNA species (the 5S and 23S rRNAs). Bacterial 16S, 23S, and 5S rRNA genes are typically organized as a co-transcribed operon. The most common primer pair for 16S was devised by Weisburg *et al.*, 1991 and is currently referred to as 27F and 1492R; however, for some applications shorter amplicons may be necessary, for example in 454 sequencing with Titanium chemistry (500-ish reads are

ideal) the primer pair 27F-534R covering V1 to V3 is used. Often 8F is used rather than 27F. The two primers are almost identical, but 27F has M (which is either A or C nucleotide) instead of a C. (27F-AGAGTTTGATCMTGGCTCAG compared with 8F). In addition to highly conserved primer binding sites, 16S rRNA gene sequences contain hyper variable regions that can provide species-specific signature sequences useful for bacterial identification (GuruKPO, 2012). Although it was originally used to identify bacteria, 16S sequencing was subsequently found to be capable of reclassifying bacteria into completely new species, or even genera. It has also been used to describe new species that have never been successfully cultured. This makes the 16S gene an important tool for identification and classification of bacteria.

2.2.2 Virulence in *P. s. pv. garcae*

Individual strains of the plant pathogenic bacterium *P. syringae* vary in their ability to produce toxins, nucleate ice, and resist antimicrobial compounds. These phenotypes enhance virulence, but it is not clear whether they play a dominant role in specific pathogen-host interactions (Michael *et al.,* 2005). Most *P. syringae* strains contain one or more plasmids with cross-hybridizing replication regions and other areas of homology, and these plasmids were designated the pPT23A-like family. The majority of these plasmids encodes genes conferring epiphytic fitness or resistance to antibacterial compounds and is essential for pathogenicity or increased virulence (Sesma *et al.,* 2000). Ma *et al.,* (2007) reported that genes encoding effector proteins of importance to host-pathogen interactions and other determinants involved in virulence and epiphytic fitness of *P. syringae,* increasing strain survival on plants sprayed with agricultural bactericides have been identified on pPT23A family plasmids (PFPs) from various pathovars of *P. syringae*. Tabtoxin is a monocyclic β-lactam in which the dipeptide toxin is linked by a peptide bond to threonine. It produces chlorosis in the host plant cells after cleavage of the peptide bond, which releases the toxic tabtoxinine-β-lactam moiety (TβL). TβL is believed to inhibit glutamine synthesis or the detoxification of ammonia by irreversibly

inhibiting glutamine synthetase. Tabtoxin is historically associated with pv. *tabaci* (tobacco), *coronafaciens* (oats), and *garcae* (coffee) (Michael *et al*., 2005).

Multilocus sequence typing (MLST) was used by Sarkar and Guttman (2004) to characterize the core genome of *P. syringae* which consists of genes ubiquitously found among all strains of a bacterial species and typically includes housekeeping genes and RNAs that are essential for the survival of the organism. The core genome is less prone to horizontal gene transfer and therefore provides the best indication of the clonal evolutionary history of a bacterial species. In contrast to the core genome, the flexible genome consists of genes that vary among strains within a species and they typically encode proteins that are responsible for adaptation to specific niches and the many mobile elements that move in and out of genomes (Michael *et al.*, 2005). The flexible genome largely evolves through horizontal genetic exchange (through gene acquisition and loss).

2.3 Coffee breeding

Coffea arabica is the only tetraploid species (2n=4x=44) of the genus *Coffea* and originated from the union of the diploid genomes of *C. canephora* and *C. eugenioides*. Despite many morphological differences, *C. arabica* exhibits very low diversity at DNA level, which is attributed to its allotetraploid origin, selfing reproductive nature and recent speciation (Gichuru *et al.*, 2008).

2.3.1 Classical coffee breeding

Breeding strategy in Kenya was initially by individual tree selections, giving rise to cultivars SL 28, SL 34 and K7, which are still grown commercially. Existing plantations of French Mission and Blue Mountain coffee varieties are the original accessions planted in Kenya before the selection process commenced (Hindorf & Omondi, 2010). Although conventional breeding is mainly used for coffee improvement, it is a long process involving selection, hybridization and progeny evaluation. Further, the long

generation time of the coffee tree, the high cost of field trials, the lack of accuracy of the breeding process, the differences in ploidy level between *C. arabica* and other diploid species, and the incompatibility are all major limitations associated with conventional coffee breeding (Mishra & Slater, 2012; Hendre *et al*., 2008). Identification of coffee cultivars is mainly based upon phenotypic features, though this approach is not reliable and is subject to environmental influences.

Development of disease resistant cultivars is a major objective of many breeding programmes in order to reduce the costs of using pesticides and is safe to humans and environment. Better knowledge of the plant defense mechanisms is important for the transfer of useful genes and to maximize the use of available germplasm resources thus allowing development of durable and effective resistance (Silva *et al*., 2006). Despite the successful performance of the Ruiru 11 variety in resistance to both CBD and CLR, the major drawback has been the availability of adequate seeds to meet the high demand of growers both locally and in the region since as a hybrid variety; seed multiplication involves artificial cross pollination between the male and female parents (Hindorf & Omondi, 2010). Exploitation of genetic resources from the wild coffee coupled with efficient and reliable disease screening methods are required for successful variety development programmes. Natural interspecific hybrids such as HDT (a hybrid between *C. arabica* and *C. canephora* from Timor island), Devamachy (a hybrid between *C. arabica* and *C. canephora*), and S.26 (a hybrid between *C. arabica* and *C. liberica*, which both originated in India) are some of the sources of resistance to pests and diseases and are extensively used in *C. arabica* breeding programmes (Mishra & Slater, 2012).

2.3.2 Markers Assisted Selection in coffee

Molecular markers linked to disease resistance provide the potential to screen for resistance in a large population of plants at any stage of plant development. Where several genes confer resistance, molecular markers have the advantage over

morphological assessments, because plants carrying multiple resistances (broad-based resistance) can easily be differentiated from those carrying a single gene (narrow-based resistance) (Hindorf & Omondi, 2010). Various molecular markers, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), inter simple sequence repeats (ISSR), short sequence repeats (SSR), and expressed sequence tag derived simple sequence repeats (EST-SSR) have been used in coffee genetic diversity studies. Among the different types of DNA markers, the SSR based microsatellite markers promise to be the most ideal due to their multi-allelic nature, high polymorphism content, locus specificity, reproducibility, inter-laboratory transferability and ease of automation (Hendre *et al.,* 2008; Hindorf & Omondi, 2010). Microsatellite markers have been developed for a large number of plant species and are increasingly being used for ascertaining germplasm diversity, linkage analysis and molecular breeding (Hendre *et al.,* 2008).

Advances in DNA marker technology offer possibilities to overcome some of the conventional breeding constrains and limitations like the narrow genetic base of the Arabica genepool, long pre-bearing periods and difference in ploidy level by providing highly informative genetic markers and marker assisted breeding (Aggarwal *et al.,* 2004). Concerted efforts have been made by several laboratories across the world, under the International Coffee Genome Network (ICGN) programme, to sequence the coffee genome by using high-throughput sequencing technology which will provide key aspects of the coffee genome that may be useful for coffee genetic improvement (Mishra & Slater, 2012).

2.3.3 Genetic modification of coffee

The perennial nature of the coffee plant makes its genetic improvement through traditional breeding. Genetic transformation has tremendous potential in developing improved coffee varieties with desired agronomic traits (Mishra & Slater, 2012). It has

become an indispensable tool in plant molecular biology and functional genomics research and it is considered as an extension of conventional plant breeding technologies. The technology offers unique breeding opportunities by introducing novel genetic material irrespective of the species barrier and creating phenotypes with desired traits that are not available in the germplasm pool of crop plants.

The major objectives for using genetic engineering technique in coffee are to introduce new traits into elite coffee genotypes and develop new cultivars with desirable traits such as pests and diseases resistance, herbicides resistance, drought and frost tolerance, and improved cup quality, which are not possible to incorporate using traditional breeding techniques (Mishra & Slater, 2012). The recent developments in coffee transcriptomics and the availability of large amounts of expressed sequence tag (EST) data from both *C. canephora* and *C. arabica* as well as the development of coffee bacterial artificial chromosome (BAC) genomic libraries have opened up new possibilities in the area of coffee functional genomics.

Genetically modified coffee plants have been produced by different research groups in the world. Despite significant advances over the last 20 years, coffee transformation is far from being a routine procedure in many laboratories. Efforts have been made to identify and clone resistance genes from coffee for achieving durable resistance. Recently, the genetic and physical map of two resistance genes, that is, the S_H3 gene conferring resistance to rust and the C*k*1gene conferring resistance to CBD have been established. These genes could be used for molecular marker assisted breeding programmes (Mishra & Slater, 2012). Since transgenic coffee is at the initial stages of commercial development and needs to be integrated into the main breeding programmes for evaluation, it will take at least 15–20 years for field release of genetic modified (GM) coffee. However, the main obstacle will be consumer approval and acceptance of genetically modified coffee (Mishra & Slater, 2012).

2.4 Sources of resistance to bacterial blight of coffee

There is a strong consensus that growing genetically resistant varieties is the most appropriate and cost effective means of managing plant diseases and is one of the key components of crop improvement. It has also been recognized that a better knowledge of both the pathogen and the plant defense mechanisms will allow for the development of novel approaches to enhance the durability of resistance (Silva *et al.,* 2006). There is therefore, need to develop a breeding programme for the control of bacterial blight. Crop improvement depends on the availability of adequate amounts of genetic diversity. It is recognized that the cultivated varieties, in particular *C. arabica*, have a very narrow genetic base that greatly limits the breeding programs especially for improvement of pest and disease resistance (Anthony *et al.,* 2002, Van der Vossen, 1985). Considerable success has been obtained in the use of classical breeding to control economically important plant diseases, such as the CLR and the CBD (Silva *et al.,* 2006) but not so to BBC. Sources of resistance to BBC in Kenya need to be identified and utilized for improvement of the crop. As a prerequisite to development of a breeding programme for BBC in *C. arabica*, there is need to screen available accessions and developed varieties with resistance to the disease. Such varieties have the potential of reducing the cost of production and offer environmentally better disease management approaches. The S_H1 gene found in the Arabica coffee genotypes Harar, Dilla & Alghe, S12 Kaffa and Geisha confers simultaneous resistance to some races of *H. vastatrix* and to *P. s.* pv. *garcae*. Other sources of resistance to BBC without S_H1 gene such as Icatu, Catucaí (both genotypes not established in Kenya) and HDT have been identified indicating the presence of other genes that confer qualitative and quantitative resistances (Ito *et al.,* 2008). Field trials with several Arabica coffee varieties identified Geisha as the most resistant variety to BBC. However, Geisha is not grown commercially in Kenya because of its low yields and poor cup quality. This therefore calls for quick mitigation measures to spare the Kenyan coffee production from the effects of the bacterial disease since selections against it have not yielded a commercial variety in Kenya yet (Masaba, 1998).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Determination of *Pseudomonas syringae pv. garcae* virulence

The study aimed on determining the virulence of suspected *P. s*. pv. *garcae* isolates collected from different coffee growing zones with the history of BBC infection. The isolates were tested for virulence using SL 28, SL 34, Selection 6 and Batian 3 as described by Ithiru *et al*., (2013).

3.1.1 Sampling and Collection of *Pseudomonas syringae pv. garcae* isolates

Plant materials from identified coffee tree suspected to be infected by *P. s.* pv. *garcae* (plate 3.1)were collected from five different coffee growing zones, with the history of BBC, in Kenya.

Plate 3.1: A coffee plant with symptoms of BBC infection which include dark, water-soaked necrotic lesions on leaves and branches culminating in a die-back

A summary of these areas, from which the samples were collected, is outlined in Table 3.1. The location and altitude of the collection sites were documented using a Global Positioning System (GPS) receiver. The infected twigs or shoots were cut using a sterilized pair of secateurs, placed in well labelled khaki paper bags and stored in an ice box. The samples were then taken to the laboratory for isolation and identification of the bacteria. To isolate bacteria, 7% Sucrose Nutrient Agar (SNA) medium was used as previously described by Ithiru *et al.* (2015) prepared by dissolving 8g of nutrient agar and 20g of sucrose in 400ml of distilled water.

Table 3.1: Description of suspected *P. s.* pv. *garcae* isolates collected from different coffee regions

No.	Source/county	Altitude a.s.l	Latitudes	Lab No.
1	Kapsabet/Kericho	1959m	0.10º N, 35.747º E	28/2012
2	Kisii	1700m	0.41º S, 34.47º E	37/2012
3	Nakuru	2001m	0.153º S, 36.138º E	44/2012
4	Mweiga/Nyeri	1939m	0.365º S, 36.91º E	51/2012
5	Kisii	1700m	0.41º S, 34.47º E	58/2014
6	Kapsabet/Kericho	1959m	0.10º N, 35.747º E	59/2014
7	Nakuru	2001m	0.153º S, 36.138º E	62/2014
8	Mweiga/ Nyeri	1939m	0.365º S, 36.91º E	65/2014
9	Mweiga/ Nyeri	1939m	0.365º S, 36.91º E	66/2014
10	Mweiga/ Nyeri	1939m	0.365º S, 36.91º E	67/2014
11	Nyeri Hill/Nyeri	2200m	0.56º S, 36.93 º E	68/2014
12	Nyeri Hill/Nyeri	2200m	0.56º S, 36.93 º E	69/2014

3.1.2 Media preparation, isolation, culturing and identification of *P. s. pv. garcae* isolates

The bacterial spores from actively growing lesions were allowed to ooze out into distilled water and films of the isolates were streaked on the medium using a nichrome wire loop and incubated for 48 hours at 25^{o}C. Single colonies of the pathogen identified as opaque ash-white, levan forming and mucoid (Plate 3.2) were sub-cultured to ensure purity of cultures. After 48 hours the cultures were re-suspended in sterile water and standardized to a concentration of 10^{9} CFU/ml. A pathogenicity test was done to confirm that the isolates were *P. s.* pv. *garcae*. The cultures were replicated into two and one set used for inoculation while the other set was stored as a back-up (reference sample) in glycerine slant medium at 4^{o}C.

Plate 3.2: *P. s.* pv. *garcae* isolate growing on nutrient media

3.1.3 Inoculation to determine the virulence of the bacterial isolates

The inoculum was standardized to a concentration of 10^{9} CFU/ml by reverse dilution and culturing to count single colonies growing as shown in Plate 3.3. Actively growing bacteria colonies from each sample were collected using a sterile wire loop and put into labelled 50ml tubes containing distilled water.

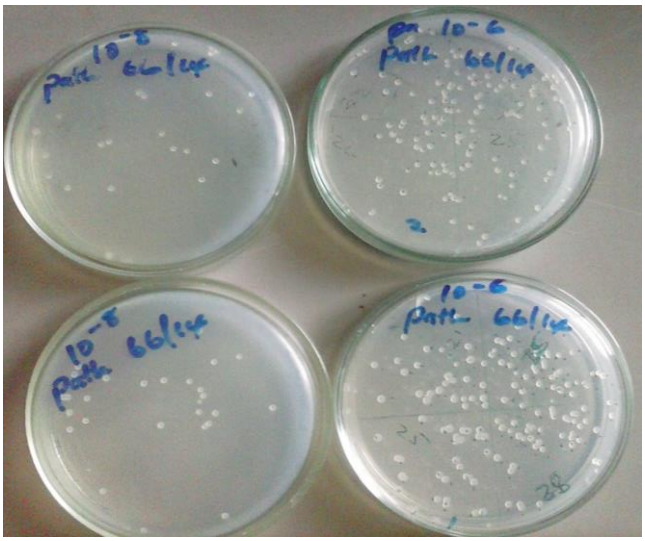

Plate 3.3: Spore count after dilution of the inoculum to determine the concentration.

A total of 4 coffee genotypes namely SL 28, SL 34, Selection 6 and Batian 3 previously reported as susceptible were inoculated with a drop of approximately 5 µl of the isolates suspension (10^9 CFU/ml) using the injection method described by Ithiru *et al.* (2013) into the 1st internode of the shoot primordial (Plate 3.4) of the selected seedlings which were approximately 4 months old with 4 pairs of true leaves. The inoculated 15 seedlings per variety (5 seedlings in 3 replications) were arranged in a complete randomized design at a temperature of 19-20^{0}C and the relative humidity maintained at approximately 70% by misting with water twice every day. It was expected that the isolates would exhibit similar characteristics if they were one and same pathovars (Kairu *et al.,* 1997). Disease symptoms were scored using a scale of 1 to 5 and data recorded for three consecutive scores from day 14 after every 7 days to determine progression of infection and severity.

Plate 3.4: Inoculation of a coffee seedling with BBC pathogen using the injection method

The data was subjected to analysis of variance (ANOVA) using COSTAT version 2012 and confidence levels set at 95%. Least significance difference (LSD5%) was used to separate the means. The seedlings that scored ≤2 were classified as resistant; those that scored >2 but ≤3 classified as moderately resistant, while the ones that scored >3 classified as susceptible. The three most virulent isolates and the least virulent isolate were selected for screening the selected genotypes for resistance to BBC.

3.2 Characterization of different *Pseudomonas syringae pv. garcae* isolates

This was to determine the level of molecular diversity of the 12 isolates used in the virulence test in Table 3.1 via polymerase chain reaction (PCR) targeting the 16S rRNA primers.

3.2.1 DNA extraction from bacteria

Under aseptic conditions a single colony of each of the *P. s.* pv. *garcae* isolates growing on glycerine slant medium was picked and streaked on SNA media and incubated for 48 hours at 24^0C. The bacteria cells were then collected using sterile ear buds and put in a 2ml tubes with distilled water. DNA isolation was done using the QIAprep Spin miniprep kit (Qiagen, USA) according to the manufacturer's instructions. The samples were centrifuged at 10000 rpm for 15 minutes before discarding the supernatant to retain the pellet containing the bacterial cells. The pelleted bacterial cells were transferred into micro-centrifuge tubes and re-suspended in 250µl buffer P1 containing LyseBlue and RNase A solution. A 250µl of buffer P2 was added and mixed thoroughly by inverting the tubes 4-6 times until the solution turned clear. Lysis was allowed to take place for 5 minutes and the solution turned blue. Then, 350µl of buffer N3 was added and mixed immediately by inverting the tube 4-6 times and the solution turned colourless. It was centrifuged for 10 minutes at 13000 rpm. The supernatant was applied to the QIAprep spin column by pipetting then centrifuged for 60 seconds and the flow was discarded. The QIAprep columns were washed by adding 500µl buffer PB which helps in binding the DNA on the column. Centrifugation was done for 60 seconds and the flow was discarded. The QIAprep spin columns were washed by adding 750µl of buffer PE then added 220 µl of absolute ethanol. Centrifugation was done for 60 seconds and the flow discarded. The step of centrifugation was repeated again to remove residual wash buffer. The QIAprep spin columns were placed in a clean 1.5ml micro centrifuge tube. Finally, 50µl of Buffer EB (elution buffer) was added to the center of the QIAprep spin column and allowed to stand for 1 minute.

3.2.2 Bacterial DNA Quantification

Agarose gel of 1% concentration was prepared and 2µl of Ethidium bromide added before pouring into an electrophoresis tank with combs. After polymerization, the combs were removed and 5 µl of each DNA sample plus 2 µl of loading dye was loaded on the

gel wells. The electrophoresis was set at 100V for 30mins after which the gel was viewed using an illuminator and results documented using a camera. The quantification of the bands was done by comparing with the intensity of the marker.

3.2.3 Bacterial DNA Amplification

The following master mix for 14 reactions (to cater for pipetting errors) was prepared as shown in Table 3.2. Kapa Taq polymerase with Magnesium Chloride and dNTPs with a concentration of X1 was added to PCR water containing the 16S forward and reverse primers with a concentration of 0.5µM. The 16S primers were, 8 Forward - AGAGTTTGATCCTGGCTCAG and 1492Reverse (l) - CGG TTA CCT TGT TAC GAC TT.

Table 3.2: PCR master mix for bacteria DNA amplification

Ingredients	**Final concentration**	**Volume/ reactions(µl)**	**Volume in 14 reactions**
Kapa Taq polymerase (with MgCl2 &dNTPs)	X1	10 µl	140µl
Primer F	0.5 µM	1 µl	14 µl
Primer R	0.5 µM	1 µl	14 µl
DNA template (1ng/ µl)	50ng/ µl	2 µl	28 µl
PCR water		6 µl	84 µl
Total		**20 µl**	**280 µl**

The conditions of the amplification were set for 30 cycles at 70ºC for lid heating to avoid evaporation, 94ºC for 30 seconds denaturation, annealing temperature at 50ºC for 30 seconds, final temperature at 72ºC for 2 minutes , hold at 72ºC (final elongation) 5 minutes and cooling at 4ºC. A sample of 5 µl of each PCR products was used for running in horizontal electrophoresis unit with agarose gel at 110V for 30 minutes and viewed under an U.V trans illuminator.

3.2.4 Purification of bacterial PCR products and sequencing

This was achieved using a PCR purification kit (Qiagen, USA). A volume of 1:250 of pH indicator was added to buffer PB and the colour turned yellow. Each PCR product of the isolate containing 55 µl was put in 275µl of the binding buffer (PB) and the mixture put into a column and spun for 60 seconds. Elution of the DNA from the column was done with PCR water. A sample of 5 µl of each isolate was loaded on 1% agarose gel with 2 µl of loading dye and electrophoresis done at 110V for 30 minutes and gel viewed under a U.V Trans illuminator. The DNA samples were sent for sequencing at BeCA, ILRI using 16S primers (8 F- AGAGTTTGATCCTGGCTCAG and 1492 R (l) - CGG TTA CCT TGT TAC GAC TT).

3.2.5 Data analysis on characterization of the 12 different *P.s. pv. garcae* isolates

Sequences were retrieved and used for BLAST analysis, at NCBI, to obtain related sequences. They were then used for multiple sequence alignment using vector NTI software version 11.0 (Invitrogen, USA). The alignments were then used to determine the genetic evolutionary relatedness using clustalW in Molecular Evolutionary Genetic Analysis 6 (MEGA 6) (Tamura *et al*., 2013). The Neighbor-Joining algorithm was used to determine pairwise distances and the evolutionary history inferred using the Maximum Likelihood method based on the Tamura and Nei (1993) model. A phylogenetic tree was constructed using Boostrap method with 500 replications with gap opening penalty of 15 and gap extension penalty of 6.66. All positions containing gaps and missing data were eliminated (Felsenstein, 1985).

3.3 Identification of coffee varieties with resistance to *P. s.* pv. *garcae* in Kenya

The study was carried out at the Coffee Research Institute (CRI), Kenya. Four BBC isolates selected from the previous test on virulence of the BBC pathogen were used for screening selected coffee genotypes. Three of the isolates were the most virulent and one of them was the least virulent among the 12 isolates.

3.3.1 Preparation of coffee genotypes for inoculation

The test plant materials consisted of 24 coffee genotypes (Table 3.3) drawn from commercial and non-commercial varieties in Kenya.

Table 3.3: Description of coffee varieties inoculated against selected *P. s.* pv. *garcae* isolates

Sn	Variety	Description
1	Batian 1	Commercial variety tolerant to CBD and CLR
2	Batian 2	Commercial variety tolerant to CBD and CLR
3	Batian 3	Commercial variety tolerant to CBD and CLR
4	SL 28	Commercial variety susceptible to CBD and CLR
5	SL 34	Commercial variety susceptible to CBD and CLR
6	K7	Commercial variety moderately tolerant to CBD and CLR
7	Ruiru 11	Commercial hybrid variety resistant to CBD and CLR
8	Robusta	Commercial *C. canephora* resistant to the CLR races affecting *C. arabica*
9	HDT	Spontaneous hybrid of *C. arabica* and *C.canephora*. Source of resistance to CLR
10	Rume sudan	Source of resistance to CBD
11	Catimor	Parent of Ruiru 11, resistant to CLR and known to be tolerant to BBC
12	Dilla alghe	Known to be tolerant to BBC
13	Bourbon	Known to be tolerant to BBC
14	Harar	susceptible to CBD and CLR
15	*C.eugeniodes*	wild type species resistant to CBD and CLR
16	Wild type	Newly collected from West Pokot
17	Selection 6	S.274 x Kents a commercial variety introduced from India –ex Robarbica
18	Selection 5a	(Devamachy x S.881) introduced from India where it is a commercial variety
19	Sarchimor	Introduced from Colombia-Villarsachi x Hybrido *de* Timor
20	Geisha	Known to have some resistance to BBC
21	Caturra	Compact growth with increased productivity
22	Mokka 5	Dwarf, lowly productive and susceptible to CBD and CLR
23	San ramon	Dwarf, lowly productive and susceptible to CBD andCLR
24	Dilla	Has S_H1 gene known to confer resistance to *P. s.* pv. *garcae*.

Coffee seeds from each selected genotypes were planted in germination boxes containing sterilized sand (Plate 3.5a) and watered regularly at room temperature. The germinated seedlings at the eighth week when they had attained the cotyledonous leaves (Plate 3.5b) were transplanted into black perforated polybags measuring 5" x 9" inches containing a potting medium composed of soil, sand and manure at a ratio of 3:2:1, respectively. Triple super phosphate (TSP) fertilizer (125 g/15 kg of potting mixture) was added into the medium. The seedlings were watered regularly and relative humidity was maintained at 80-90% in the nursery (Plate 3.6a) to enable the seedlings to produce a new pair of true leaves approximately every four weeks until they were 4 months old (Plate 3.6b).

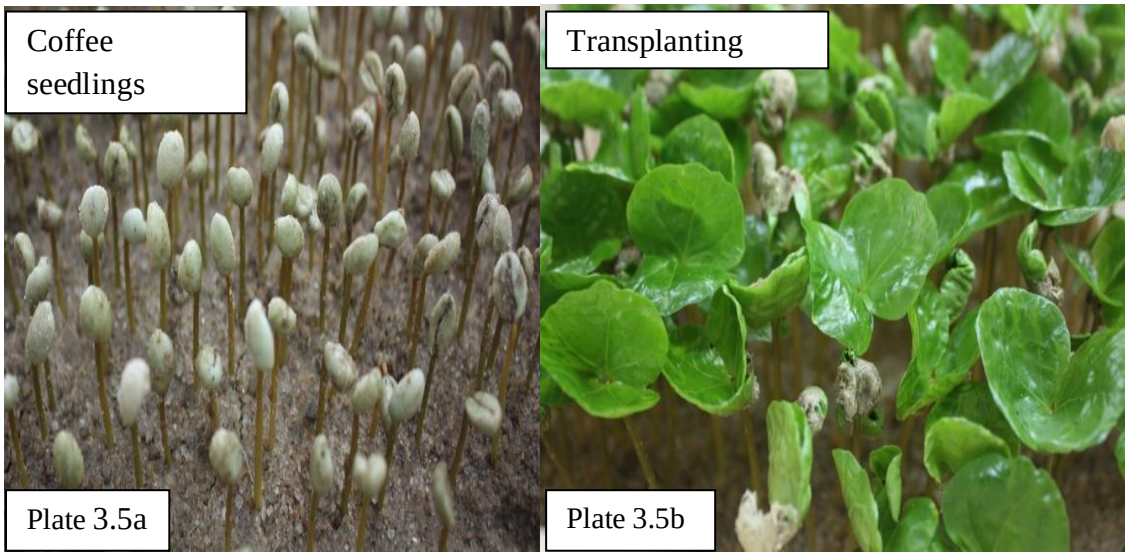

Plate 3.5: Germinated coffee seedlings at the soldier stage (3.5a) and transplanting stage with cotyledons (3.5b) growing on sand.

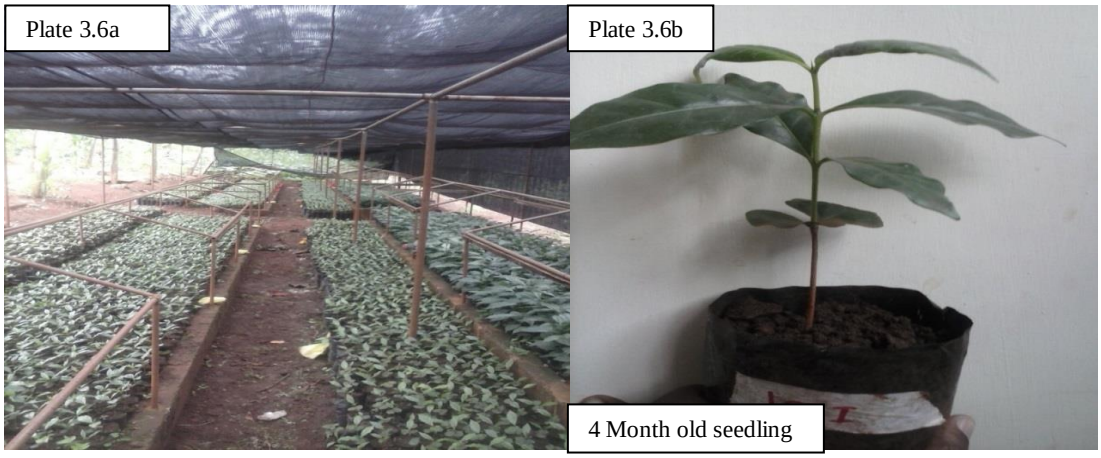

Plate 3.6: A-Coffee seedlings at the nursery stage ; B -a 4 month old seedling ready for inoculation

3.3.2 Experimental design for inoculation of coffee genotypes

After four months the seedlings were arranged in a complete randomized design (CRD) to test them for resistance to BBC. The experiment was arranged in three replications each replication having 10 seedlings per variety. The 3 most virulent and 1 least virulent *P. s.* pv. *garcae* isolates were used in the inoculation using the injection method described by Ithiru *et al.* (2013) with a few modifications where the injection was done on the shoot primordium between the 1st internode. The BBC isolates (Table 3.1) were cultured in Sucrose Nutrient Agar (SNA). The isolates were coded as follows: Isolate 1- 58/014, Isolate 2- 62/014, Isolate 3- 66/014, Isolate 4- 69/014 and Isolate 5- Control (ddH_2O). Approximately 10µl the BBC inoculum (10^9cfu) was introduced on the seedlings' meristematic tips using a clinical needle with a syringe (Plate 3.4). The inoculated seedlings were put in a controlled room at a temperature of 19-20ºC and the relative humidity maintained at approximately 75% by misting with water twice every day.

3.3.3: Data analysis on identification of coffee varieties with resistance to *P. s. pv. garcae* in Kenya

Disease symptoms were scored using a scale of 1 to 5 and the scoring was repeated three times to determine progression of infection and severity. The data was subjected to analysis of variance (ANOVA) using COSTAT version 2012 and confidence levels set at 95%. Least significance difference (LSD5%) was used to separate the means.

3.4 Characterization of selected coffee genotypes for BBC resistance using SSR.

The aim of this work was to determine there exist any SSR marker associated with BBC resistance in the selected coffee genotypes. All the genotypes used in screening for resistance to BBC were used. Young clean mature leaves from the selected coffee genotypes were collected for DNA extraction and 11 SSR markers used in applification.

3.4.1 DNA extraction from coffee

Leaves from sample seedlings from each genotype were excised using a clean blade and DNA extracted using MATAB according to Diniz *et al.*(2005) with minor modification. A quantity of 0.5g of fresh leaf sample was ground with motor and pestle in liquid nitrogen and 2.0 mls (1ml lysis + 1ml extraction) buffer was added during grinding before transferring into two 2ml eppendorf tubes and incubated at 62^{o}C for 30 minutes. An equal amount of Chloroform/Isoamyl alcohol (24:1) was then added mixed thoroughly and centrifuged at 13000rpm for 10 minutes. The supernatant was transferred into a clean 2ml tube, added equal volume of pre-chilled Iso-propanol and allowed to precipitate then centrifuged at 13000rpm for 10minutes. The supernatant was decanted and the DNA pellet washed with 400µl of 70% ethanol, then dried in vacuum concentrator for 15mins at 30^{o}C. The pellet was dissolved in 50µl 1X TE and left overnight in a refrigerator at 4^{o}C.

3.4.2 Coffee DNA quantification and amplification using Microsatellite (SSR) markers

Quantification of DNA was done in concentration of 1% agarose gel using known DNA ladder. Eleven SSR primers were used to characterise the genotypes. A volume of 2 µl of DNA extract was diluted with 7 µl of ddH_2O and added 3µl of loading dye before loading for electrophoresis set at 50v for 1 hour. PCR master mix was prepared for 28 reactions using Kapa Taq polymerase pre-mixed with both Magnesium Chloride and dNTPs at a concentration of X1 to aliquot 6 µl per reaction. The concentration of the primers used was 0.5 µM (1µl per reaction) and DNA template of 1ng/ µl concentration (10 µl per reaction) as shown in Table 3.4. Amplification was then done using a PCR machine with initial denaturation at 95^{o}C for 5mins, 30 cycles with denaturation temperature at 94^{o}C for 1minute, annealing temperature at 60-55^{o}C (reducing) with extension at 72^{o}C then hold at 4^{o}C. Eleven SSR primers (Table 3.5) were used in amplification and the PCR products electrophoresed alongside standard markers using 1% agarose gel.

Table 3.4: Microsatellite reaction mix for amplification of coffee genotypes' DNA

Reagents	**Final concentration**	**Vol/rxn (µl)**	**Vol in 28 rxns**
Kapa Taq polymerase (with MgCl2 &dNTPs)	X1	6 µl	**168µl**
Primer F	0.5 µM	1 µl	**28 µl**
Primer R	0.5 µM	1 µl	**28µl**
DNA template (1ng/ µl)	10g/ µl	10µl	
Total		**18 µl**	**224 µl**

3.4.3 Data analysis on characterization of selected coffee genotypes for BBC resistance using SSR

Scoring was done for the presence or absence of amplified fragments. Clear and well-resolved bands were coded in a binary form by denoting '1' and '0' for presence and absence of band respectively along each row for each genotype. In order to describe genetic diversity among the different coffee genotypes, the bands' data was used to estimate genetic distances using XLSTAT version 2014.

Table 3.5: SSR Primers used in amplification of coffee DNA

Locus	Genebank ac/no	Forward primer	Reverse primer
Sat 11	AJ250252	ACCCGAAAGAAAGAACCAA	**CCACACAACTCTCCTCATTC**
Sat 32	AJ250258	AACTCTCCATTCCCGCATTC	**CTGGGTTTTCTGTGTTCTCG**
Sat 172	AJ308753	ACGCAGGTGGTAGAAGAATG	**TCAAAGCAGTAGTAGCGGATG**
Sat 207	AJ308753	GAAGCCGTTTCAAGCC	**CAATCTCTTTCCGATGCTCT**
Sat 227	-	TGCTTGGTATCCTCACATTCA	**ATCCAATGGAGTGTGTTGCT**
Sat 229	-	TTCTAAGTTGTTAAACGAGACGCTTA	**TTCCTCCATGCCCATATTG**
Sat 240	-	TGCACCCTTCAAGATACATTCA	**GGTAAATCACCGAGCATCCA**
Sat 254	-	ATGTTCTTCGCTTCGCTAAC	**AAGTGTGGGAGTGTCTGCAT**
Sat 255	-	AAAACCACACAACTCTCCTCA	**GGGAAAGGGAGAAAAGCTC**
Sat 262	AY705498	CTGCGAGGAGGAGTTAAAGATACCAC	**GCCGGGAGTCTAGGGTTCTGTG**
Sat 283	AJ308772	GCACACACCCATACTCTCTCTT	**GTGTGTGATTGTGTGTGAGAG**

CHAPTER FOUR

RESULTS

4.1 Determination of *P. s. pv. garcae* virulence.

P. s. pv. *garcae* was identified as a Gram-negative, rod-shaped, obligate aerobe and flagellated motile bacteria. Twelve (12) isolates (Table 3.1) were positively identified and were used in virulence determination. Disease symptoms started to be visible after 5 days from inoculation with observations of wilting and water soaking (Plate 4.1) and later lesions were observed on infected seedlings.

Plate 4.1: Seedlings inoculated with BBC isolates showing early symptoms of infection

The symptoms were scored using a scale of class 1 to 5, from the lowest to the highest, based on the degree of necrosis reached as described by Ito *et al.* (2008) with modification, where: 1 = absence of the dark necrotic lesions, 2 = small black lesions; 3 = black lesions coalescing 4 =black coalesced lesion over 50% and 5 = complete girdling around the meristem. Isolate Kisii-58/014, Mweiga-66/014, Nakuru-62/014 and

Nakuru-44/014 in that respective order were the most virulent. Isolates 69/014, 28/012 and 37/012 caused the least infections which closely compared to the control. The analysis reviewed significant difference ($P<0.05$) among the 12 isolates used (Table 4.1). Isolates from the same region appeared to differ in virulence. The two isolates from Kisii: Kisii-58/014 (4.60^{a}) and Kisii-37/012 (1.85^{efg}) were different in virulence and similarly, the five isolates from Nyeri region were found to be quite different with isolate Nyeri-69/014 (1.57^{fg}) causing the least infection while isolate Nyeri-66/014 (4.13^{ab}) was among the most virulent. The two isolates from Kapsabet [Kap-59/014 (3.48^{bc}) & Kap-28/012 (1.82^{efg})] were different in their virulence. However, the isolates Nakuru-62/014 and Nakuru-44/012 had no significant difference in their virulence.

The number of seedlings inoculated per isolate (15seedlings x 4 coffee genotypes) were categorised into resistant seedlings (class 1 and 2) and susceptible seedlings (class 3, 4 & 5). The isolates from Nyeri-68/014, Nakuru-62/014, Kisii-58/014, Mweiga-66/014, Mweiga-67/014, Kap-59/014 and Nakuru-44/012 had over 50 seedlings which translated to 83% of seedlings scoring between classes 3-5 of the disease symptoms (Figure 4.1).

Table 4.1: Disease severity of the BBC isolates against the four selected coffee genotypes

No.	Isolate	Disease severity
1	Kisii-58/014	4.60 [a]
2	Mweiga-66/014	4.13 [ab]
3	Nakuru-62/014	4.08 [ab]
4	Nakuru-44/012	3.82 [ab]
5	Mweiga-67/014	3.62 [bc]
6	Kap-59/014	3.48 [bc]
7	Nyeri-68/014	2.95 [cd]
8	Mweiga-65/014	2.43 [de]
9	Mweiga-51/012	2.33 [def]
10	Kisii-37/012	1.85 [efg]
11	Kap-28/012	1.82 [efg]
12	Nyeri-69/014	1.57 [fg]
13	Control	1.48 [g]
	LSD(0.05)	**0.84**
	CV (%)	**35.23**

*Means followed by the same letter indicate no significance difference according to Duncan's test

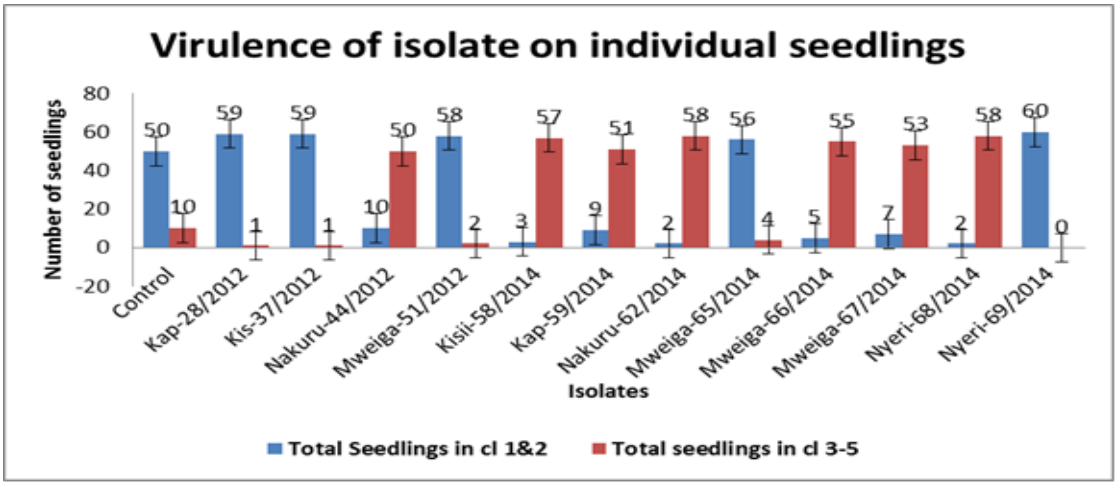

Figure 4.1: Categorisation of resistant and susceptible seedlings of the four coffee genotypes inoculated with the 12 BBC isolates**.**

Seedlings in class 1 and 2 were resistant while those in classes 3, 4 and 5 were susceptible. The more seedlings that scored class 3 to class 5, the more virulent an isolate was considered to be.

Isolates Kap-28/012, Kisii-37/012, Mweiga-51/012, Mweiga-65/014 and Nyeri-69/014 had an average of 97% of the total seedlings inoculated scoring class 1 and 2 representing a low severity of disease. The interactions between the coffee varieties and the isolates had no significant difference. However Batian 3 which is a commercial variety in Kenya scored a disease mean of 2.72 which was the lowest level of infection followed closely by Selection 6 which was introduced from India with a mean of 2.81. The other Kenyan commercial varieties SL 28 and SL 34 had a mean of 3.06 and 3.15 respectively. The two genotypes are also susceptible to the other major coffee diseases in Kenya. Though the action of the 12 BBC isolates against the coffee four varieties appeared to be different, the coffee varieties had no significant difference (Table 4.2 and Figure 4.2).

Table 4.2: Reaction of the 4 genotypes used to determine virulence of the 12 isolates

Rank	Genotype	Mean
1	**SL34**	**3.15** [a]
2	**SL28**	**3.06** [a]
3	**Sel 6**	**2.81** [a]
4	**Batian 3**	**2.72** [a]

*Means followed by similar letter indicate that there is no significant difference in Duncan's test 95% level of confidence.

Half of the seedlings inoculated with the isolates were classified as susceptible. Isolates Kap-28/012, Mweiga-65/014 and Nyeri-69/014 caused minimum disease infection on Batian 3. However, isolates 58/014 and 62/014 and 67/014 were the most virulent to Batian 3 with all seedlings scored in classes 3 to 5 (Figure 4.2). Isolates Nyeri-69/014 had no seedlings scored in class 1 or 2 in all the four varieties.

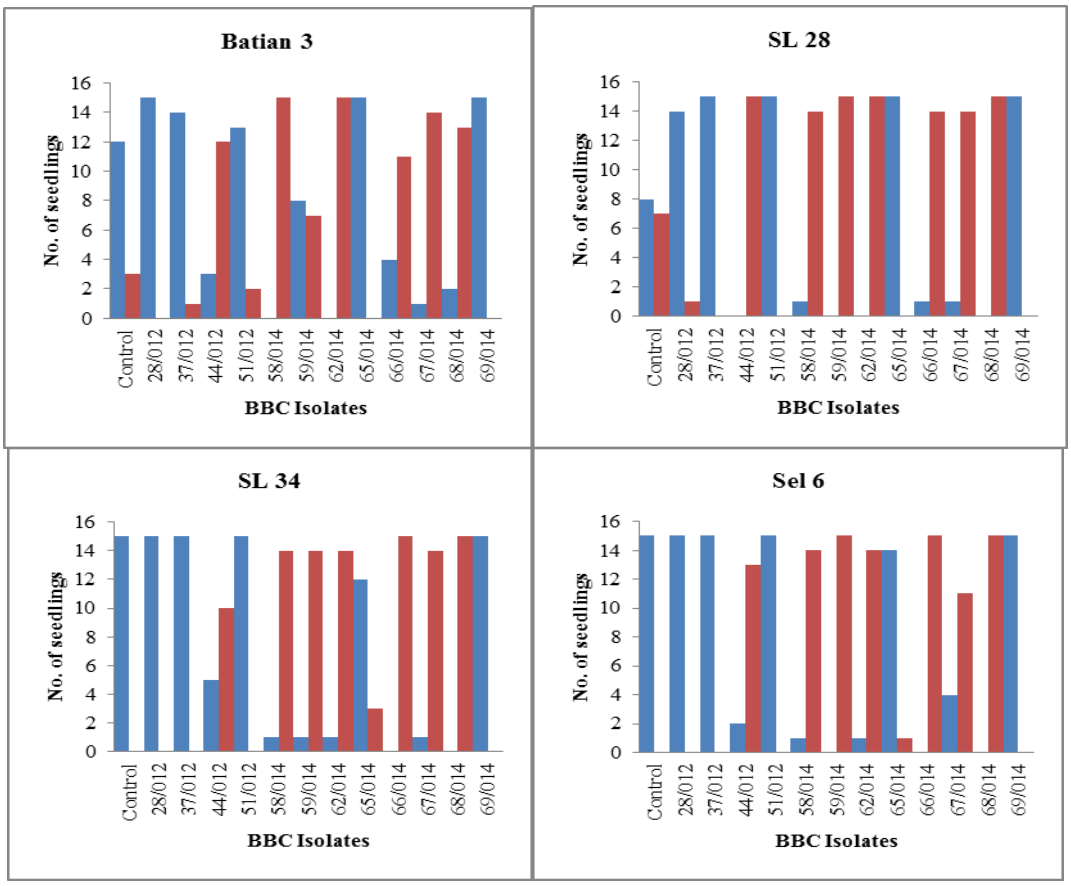

Figure 4.2: Distribution of resistant and susceptible seedlings of the 4 test coffee varieties. Resistant and susceptible

4.2 Characterization of the different *P. s. pv. garcae* isolates using 16S gene

All the isolates were amplified with the 16S rRNA primer (Plate 4.3) and the bands were estimated to be 1200 base pairs and therefore adequate for sequencing. There were a total of 1056 positions (neuclotides) in the final dataset using the forward primer (Figure 4.3) and 1066 using the reverse (Figure 4.4) primer. This confirmed that the estimation of the bands' (plate 4.3) weight after amplification was good.

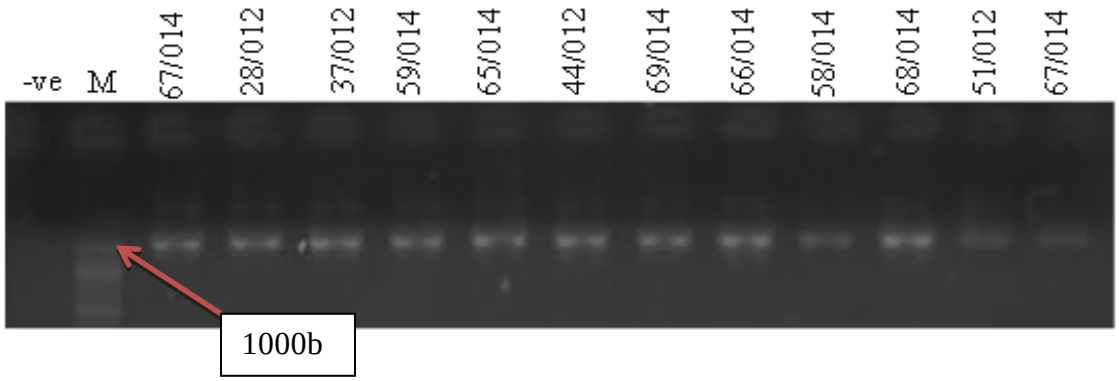

Plate 4.3: DNA Amplification of the 12 BBC isolates using the 16S rRNA primer

The sequence data revealed high diversity in evolutionary relationship among the 12 *P. s.* pv. *garcae* isolates. There was close similarity in the way the sequences from the 12 isolates were clustered by the 16S forward and reverse primers. The isolates Kisii-58/014 which had a disease severity of 4.60^{a}, Nakuru-44/012 with 3.82^{ab}, Mweiga with 66/014 (4.13^{ab}) and Kapsabet- 59/014 (3.48^{bc}) were clustered together in the phylogenetic tree. Two isolates with high disease severity namely Nakuru 62/2014 (4.08^{ab}) and Mweiga 67/2014 (3.62^{bc}) were however clustered in a different group. Isolate Kapsabet 28/2012 (1.82^{efg}), Nyeri 69/2014 (1.57^{fg}) and Kisii 37/2012 (1.85^{efg}) which caused the least disease severity were clustered together. The four isolates from Mweiga were separated with isolate Mweiga-66/014 which had a disease severity of 4.13 being clustered away from the others from that region. The two Isolates from Kisii [Kisii-37/012(1.85) and Kisii 58/014(4.60)] also appeared to be genetically separated. just as was the case of the Kapsabet isolates (Kap-28/012 and Kap-59/014).

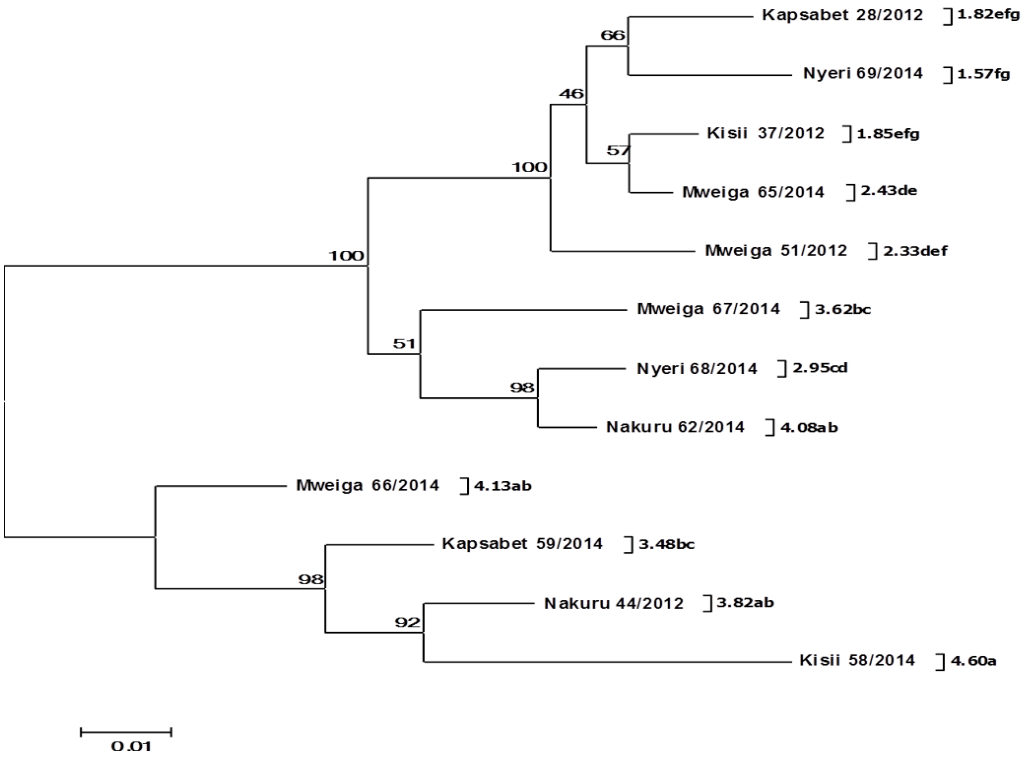

Figure 4.3: Molecular Phylogenetic tree using 16S rRNA Forward primer sequences generated by Maximum Likelihood method.

The figure following the label of the isolates represents the virulence mean of the isolates with the significant number according to Duncan's test. The tree with the highest log likelihood (-3648.2399) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown above the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved the 12 isolates of *P.syringae* pv. *garcae* nucleotide sequences.

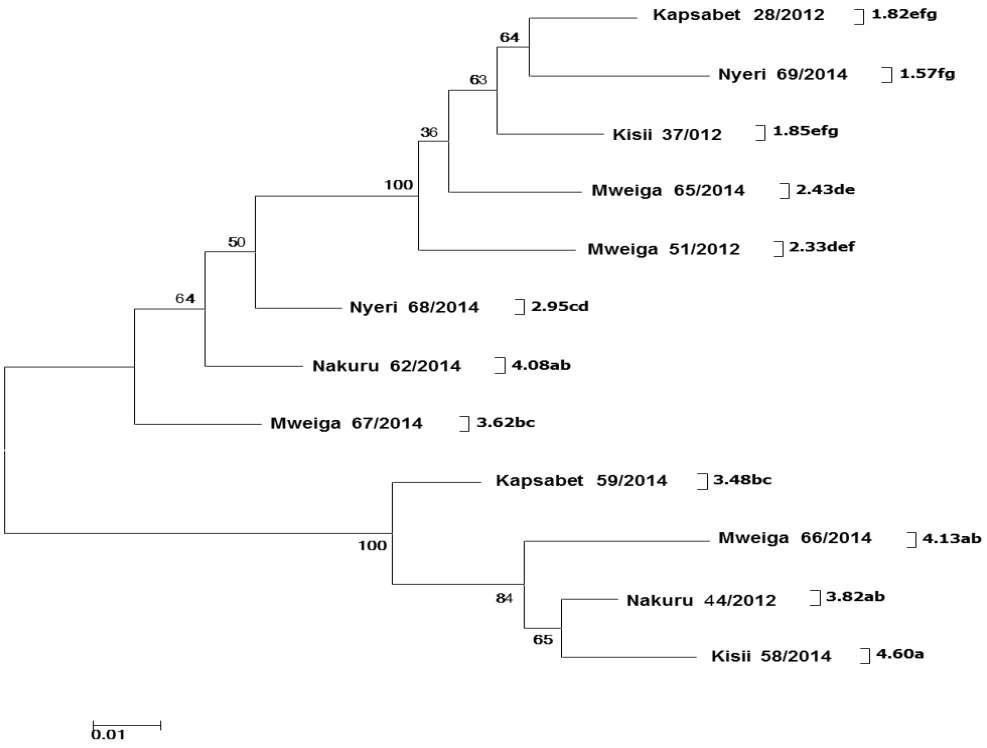

Figure 4.4: Molecular Phylogenetic analyses by Maximum Likelihood method using 16S rRNA reverse primer sequences.

The figure following the label of the isolates represents the virulence means of the isolates with the significant number according to Duncans test. The tree with the highest log likelihood (-3648.2399) is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown above the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved the 12 isolates of *P.syringae* pv. *garcae* nucleotide sequences.

Out of the 1263 nucleotides sequenced that translate to 421 amino acids and aligned, 357 were conserved sites, while 894 were the variable sites and 835 Parsim-informative sites. Variability between the most virulent and the less virulent isolates was observed at the codon site 247, 248 and 249 nucleotides as shown in Figure 4.5. The isolates which had a disease virulence means of 2.95 and above are designated with letter F while the

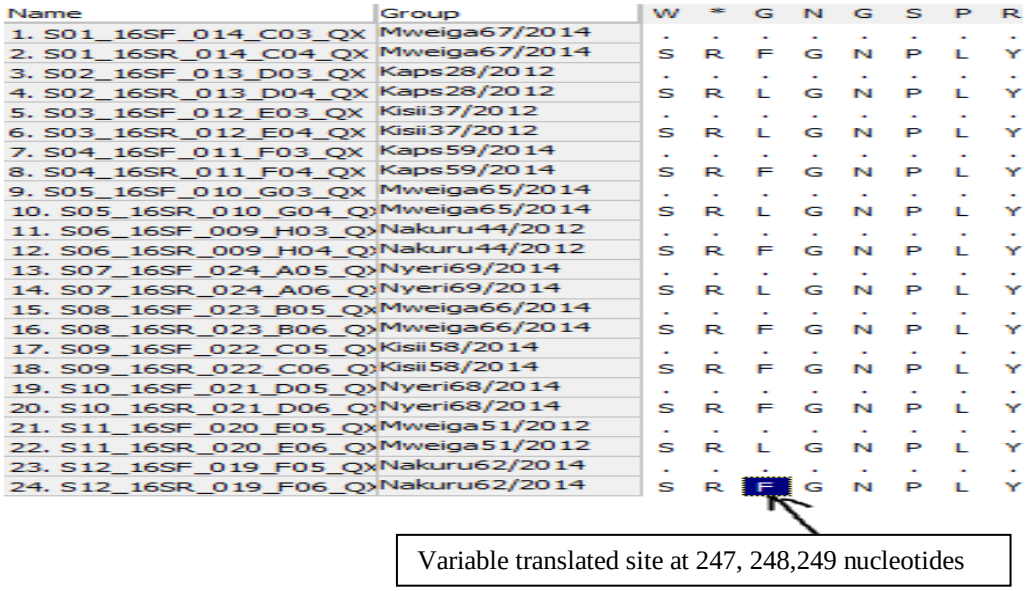

Figure 4.5: A region of the aligned BBC isolates translated sequence with high variability

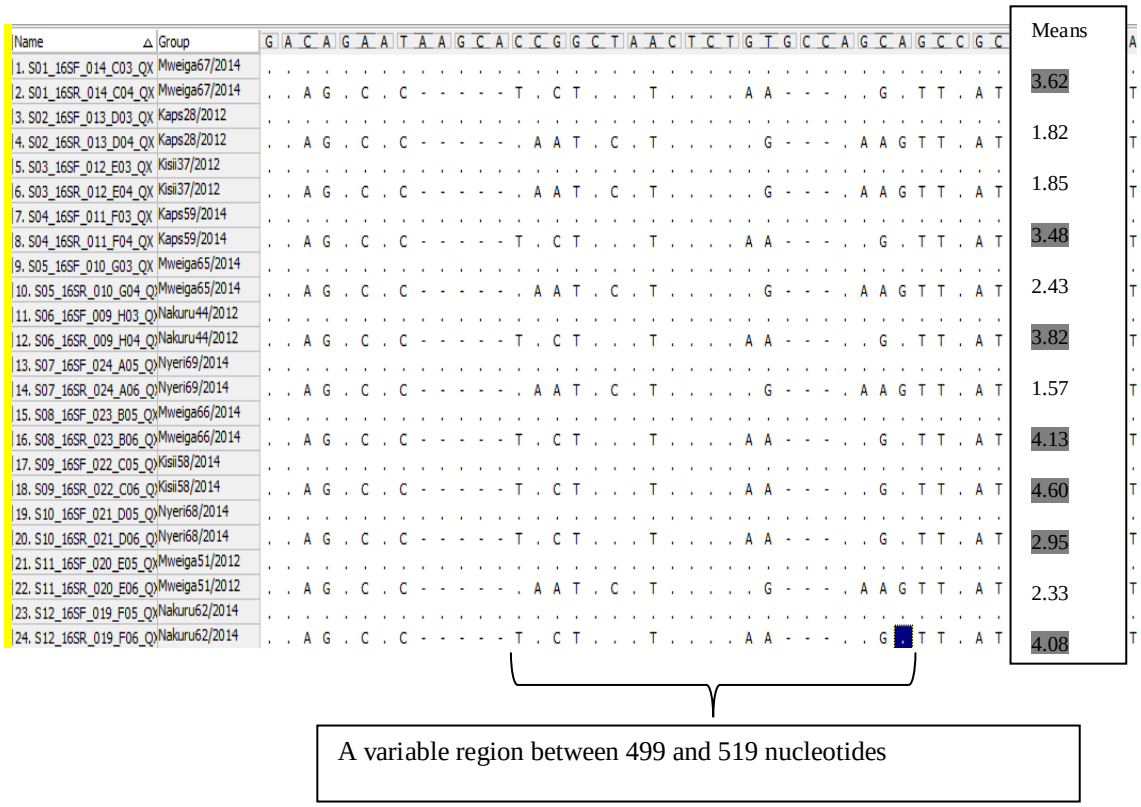

Figure 4.6: A region of high variable site closely linked to disease virulence means-

The figures on the right represent disease virulence means of the 12 isolates on the host. The highlighted means have similarities in their codons between 499 and 519 nucleotides and also have the highest disease means of 2.95 and above. The ones with a lower virulence are designated with letter L at site 247, 248 and 249 nucleotides in Figure 4.5. A similar pattern was observed at the site between 499 and 519 (Figure 4.6). All the sequences of the most virulent isolates with more than 2.95 disease means had T.CT…T….AA…..G. at the site between 499 to 519 nucleotides presenting a high variability from the least virulent isolates which had .AAT.C.T…..G….AAG on the same sites.

4.3: Identification of coffee genotypes with resistance to *P.s. pv. garcae* in Kenya

Meristematic tissues of the susceptible plants started showing signs of wilting and becoming water soaked after 4-5days and turning necrotic after 7 days (Kairu *et al*., 1997) although most seedlings showed no symptoms of infection by the end of the first week. Some seedlings shed the infected leaves at an advanced stage of the disease. Each seedling was scored independently for disease infection. The symptoms were scored using a scale of class 1 to 5, from the lowest to the highest, based on the degree of necrosis reached as described by Ito *et al*. (2008 with modification, where: 1 = absence of the dark necrotic lesions, with yellow halo (bacterial blight); 2 = small black lesion; 3 = black coalesing lesion 4 =black coalesed lesion over 50% and 5 = complete girdling around the meristem (Plate 4.4). The susceptible seedlings had black coalescing lesions that advanced to complete girdling. The data on disease symptoms reflected a steady increase (Figure 4.7) from the 14th day to 21st day.

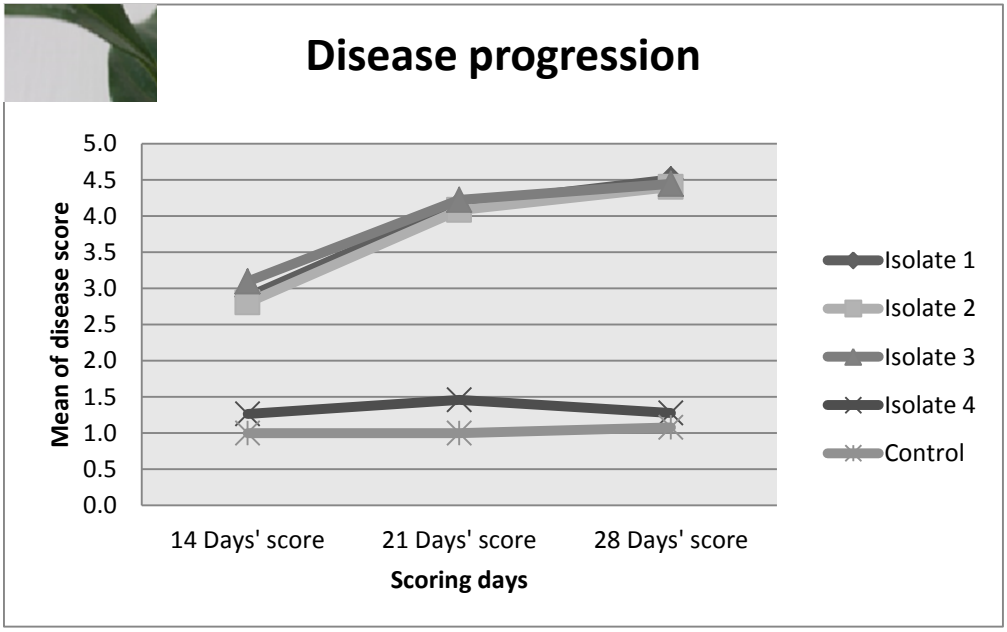

Plate 4.4 Classification of symptoms of BBC disease severity after inoculation.

Key: 1 –no infection (class 1), 2- signs of water soaking with small scab or tiny brown lesions (class 2), 3- small black lesions (early class 3), 4- black lesion becoming wider and starting to coalesce (class 3), 5- black coalesced lesion over 50% (class 4), 6- complete girdling around the meristem (class 5).

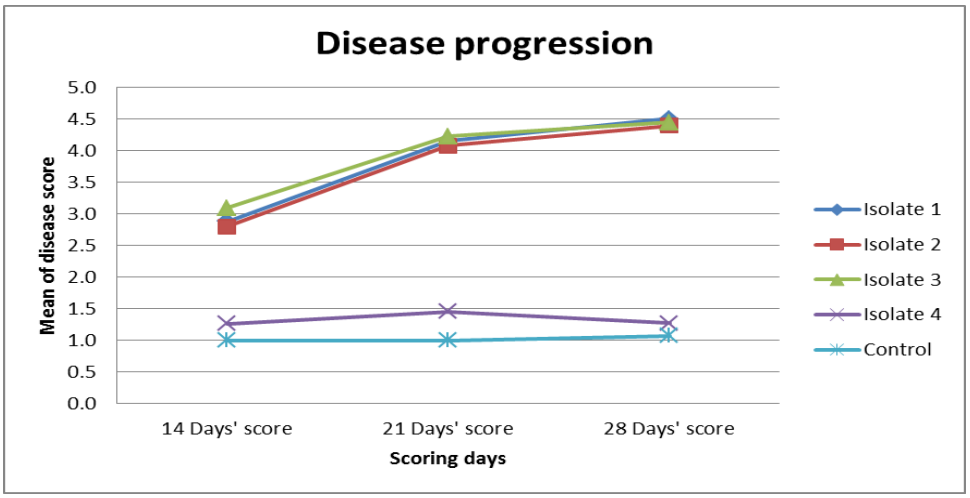

Figure 4.7: Disease progression tread on the 24 coffee genotypes inoculated with the selected *P.s.* pv. *garcae* isolates.

Isolate 1:Kisii58/014, Isolate 2: Nakuru-62/014, Isolate 3: Mweiga-66/014, Isolate 4: Nyeri-69/014 and Isolate 5: Control-ddH_2O.

However, the rate of disease progression appeared to slow down towards day 28 showing that day 21 could be the most appropriate to evaluate the disease severity. Most of the seedlings were in class four and five on the 28th day in most of the genotypes.

Isolate 4, Nyeri-69/014 was the least virulent with a mean score of 1.5 on 21st day post inoculation and 1.3 on the 28th day. The three most virulent isolates had a mean of approximately 4.2 on the 21st day and 4.4 on the 28th day. There was no significant variation ($P<0.05$) between the three most virulent BBC isolates (Table 4.3). Isolate 3 Mweiga-66/2014 had the highest disease mean (4.22^{a}) followed by Isolate 1 Kisii-58/2014 (4.16^{a}) and Isolate 2 Nakuru-62/2014 (4.08^{a}) respectively. Isolate 4: Nyeri-/69/2014 had a disease mean of 1.46^{b}. However, the disease means score for Caturra, Mokka 5, Selection 5a, Selection 6 and SL 28 on day 21st was almost similar for the three most virulent isolates (Mweiga-66/2014, Kisii-58/2014 and Nakuru-62/2014).

Table 4.3: Virulence of the four BBC isolates against the 24 coffee genotypes

Isolate	**Disease severity**	**Rank**
Isol 3:Mweiga-66/2014	4.22^{a}	1
Isol 1:Kisii-58/2014	4.16^{a}	2
Isol 2:Nakuru-62/2014	4.08^{a}	3
Isol 4:Nyeri-69/2014	1.46^{b}	4
Control	1^{c}	5
LSD (0.05)	0.33	
CV	19.34%	

N= 72 Means marked with the same letter(s) are not significantly different at $P<0.05$

Inoculation results revealed significant ($P<0.05$) differences among the genotypes (Table 4.4) with Rume Sudan (2.852^{i}) having the lowest disease severity and Mokka 5 (4.359^{a})

being the most susceptible variety. Rume Sudan was the most resistant with a disease severity mean of 2.9 followed by Robusta and HDT each with 3.4 and Selection 5a with 3.5. SL 28 was the most susceptible (4.233^{ab}) among the commercial varieties and Batian 3 (3.715^{fgh}) and K7 (3.707^{fgh}) being among the most resistant in that category. There was no significant difference between Bourbon (3.915^{cdef}), Catimor (3.907^{cdef}), Dilla alghe (3.904^{cdef}), Geisha (3.889^{cdef}), Batian 1 (3.889^{cdef}), Ruiru 11 (3.870^{cdef}) and Caturra (3.830^{cdef}) which had moderate resistance to BBC. The coffee species Robusta (*C. canephora*) which has T (C*k*-1) gene and exhibits significant variability in resistance to CBD (Gichimu *et al.*, 2014) was one of the most resistant genotype with a mean of 3.422^{h} which compared well with HDT (3.426^{h}).

Table 4.4 Disease severity of the 3 most virulent P. s. pv. garcae isolates against the coffee genotypes

S/no.	Genotype	Disease severity	S/no.	Genotype	Disease severity
1	**Mokka 5**	4.359^{a}	13	**Batian 1**	3.889^{cdef}
2	**Dilla**	4.256^{ab}	14	**Ruiru 11**	3.870^{cdef}
3	**SL 28**	4.233^{ab}	15	**Caturra**	3.830^{cdef}
4	***Coffea eugenioides***	4.122^{abc}	16	**Sarchimor**	3.826^{def}
5	**Selection 6**	4.093^{abcd}	17	**Batian 2**	3.804^{defg}
6	**SL 34**	4.052^{bcde}	18	**West Pokot**	3.785^{efg}
7	**Harar**	4.019^{bcde}	19	**Batian 3**	3.715^{fgh}
8	**San Ramon**	3.970^{bcdef}	20	**K7**	3.707^{fgh}
9	**Bourbon**	3.915^{cdef}	21	**Selection 5a**	3.511^{gh}
10	**Catimor**	3.907^{cdef}	22	**HDT**	3.426^{h}
11	**Dilla alghe**	3.904^{cdef}	23	**Robusta**	3.422^{h}
12	**Geisha**	3.889^{cdef}	24	**Rume Sudan**	2.852^{i}
LSD (5%)		**0.296**			
Means marked with the same letter(s) are not significantly different at P<0.05					

Coffea eugenioides had a disease mean of 4.122^{abc} which was among the most susceptible genotypes. Most genotypes reacted differently to the four isolates. Isolate 1 caused the highest disease score in Batian 1 than isolates 2 & 3. However, the same isolates registered lower disease score in Batian 2 & 3. Isolate 2 also had lowest disease score in Batian 1 but had the highest in Batian 2 & 3. This variation could be associated to the factors related to host pathogen interaction. However, the reaction of HDT and Robusta to the isolates appeared to be consistent with both scoring a mean of 3.4 against the 3 most virulent isolates as illustrated in Figure 9. Batian 3 had a wide range of disease scores against the four BBC isolates scoring a mean of 3.2 against isolate 1 Kisii-58/2014; 4.2 against isolate 3 Mweiga-66/2014 and 4.8 against isolate 2 Nakuru-62/2014 on 21^{st} day after inoculation. The reaction of the three Batian lines was different, with Batian 1 having no significant difference with Ruiru 11 (3.889^{cdef} and 3.870^{cdef}, respectively) while the others had 3.804^{defg} (Batian 2) and 3.715^{fgh} (Batian 3).

Ruiru 11 which is known for its resistance to both CLR and CBD had a high mean score of above 4 against isolate Nakuru-62/2014 and Mweiga-66/2014 although the mean was lower against isolate Kisii-58/2014. K7 was moderate in resistance against isolate Mweiga-66/2014 and Kisii-58/2014 but was quite susceptible to isolate Nakuru-62/2014 and had no overall significant difference with Batian 3. Isolate 2 (62/014 from Nakuru-Little farm Bahati and isolate 1(58/014) from Kisii had the highest disease score in seven genotypes while isolate 3 (66/014) from Mweiga was most virulent in nine genotypes (Figure 4.8). The change in disease infection from day 14 to day 21 among all the genotypes was not uniform among the genotypes.

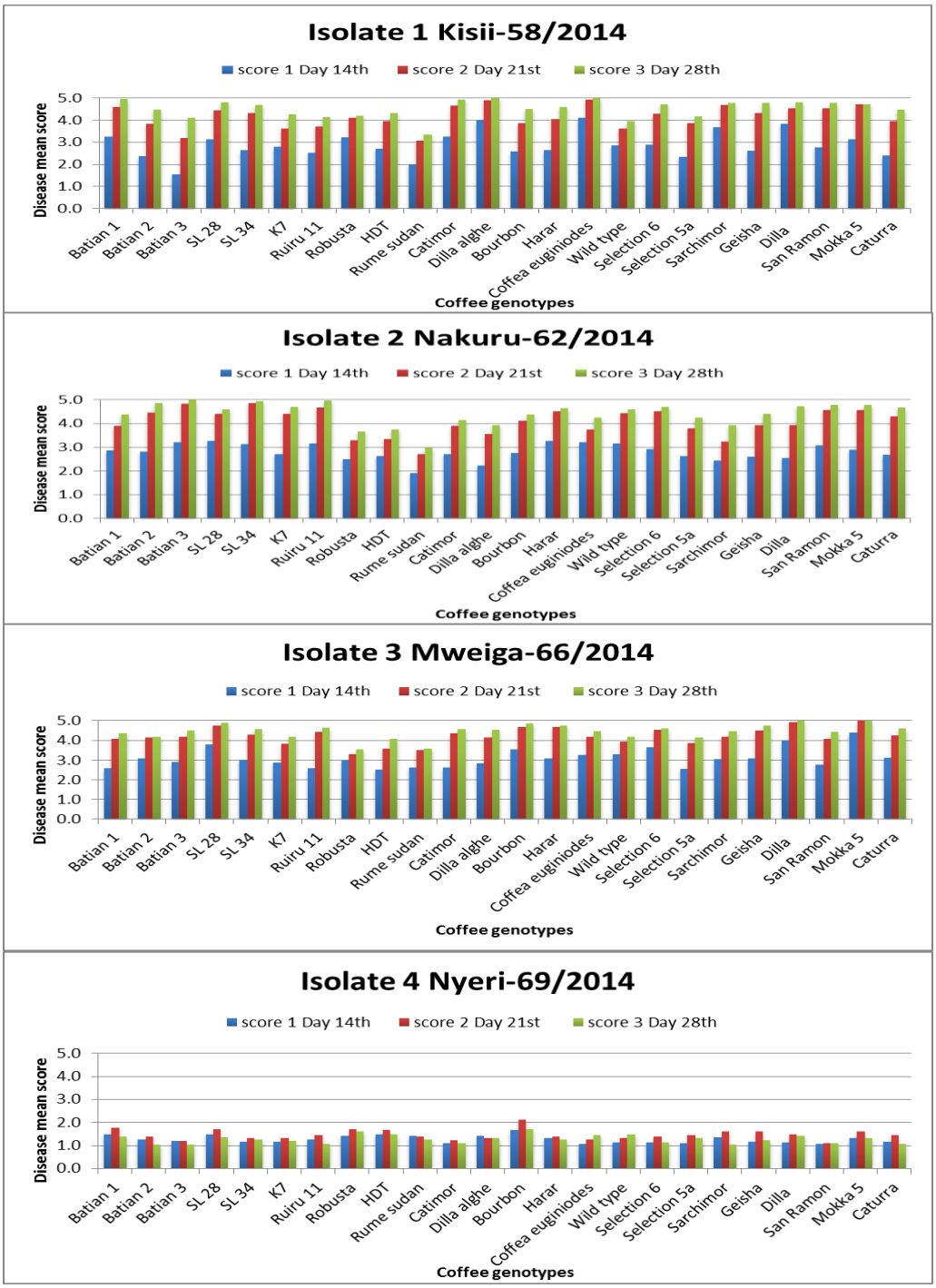

Figure 4.8: Disease progression on coffee genotypes against four BBC isolates

Coffea eugeniodes had the lowest change (19.5%) followed by Robusta (*Coffea canephora* 20.7%) and Wild type coffee (24.7%) (Figure 4.9). Geisha had the highest increase in disease score (47.1%) followed by Batian 3(46.4%) and Selection 5a (45.0%).

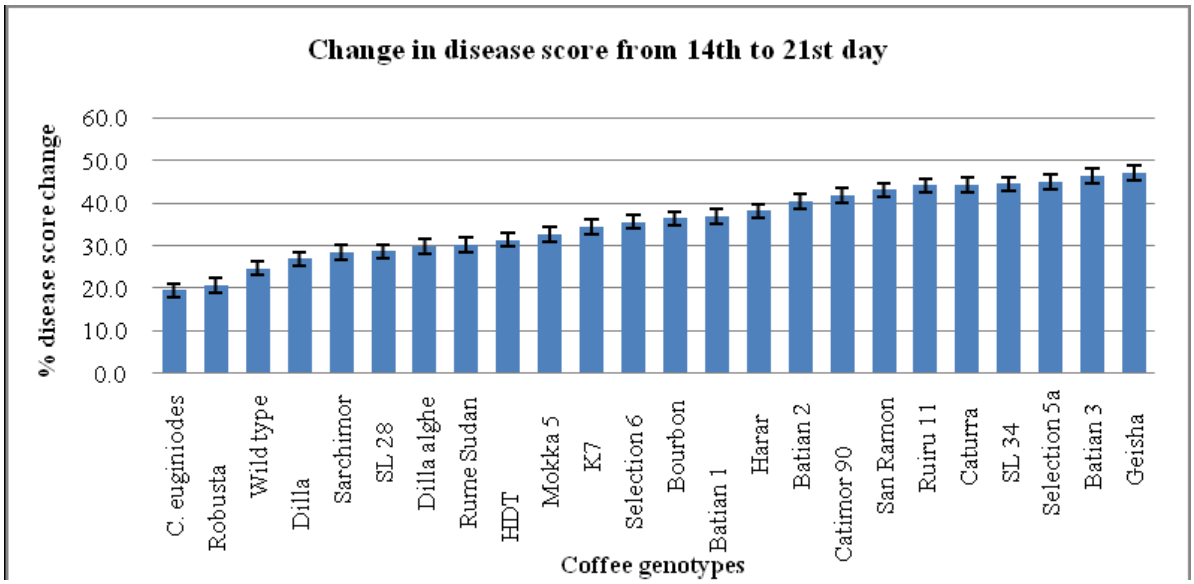

Figure 4.9: Change in disease infection on coffee genotypes from day 14th to 21st

Nine genotypes had disease severity increase of over 40% from day 14 to 21. Although Rume Sudan had a disease infection severity of 30.2%, it was still the most resistant compared with the other genotypes. Mokka 5 had a disease severity change of 32.7% to reach the maximum of 5. SL 28 had the smallest change of disease severity (28.7%) compared to the other commercially grown varieties while SL 34 had the highest change (44.5%). Only four varieties scored a mean of 3.5 and below using the average disease means of the three most virulent isolates (Figure 4.10) with Rume sudan scoring the lowest disease mean of 2.9 followed by Robusta and HDT each with 3.4 and selection 5a with 3.5. The disease severity appeared severe on all the varieties possibly due to the high concentration of inoculum (2×10^9) used in the inoculation. Varieties Mokka5 (4.4), Dilla (4.3), SL 28(4.2), SL 34(4.1), *C. euginiodes* (4.1) and Selection 6 (4.1) in that order were the most susceptible with mean scores above 4.0.

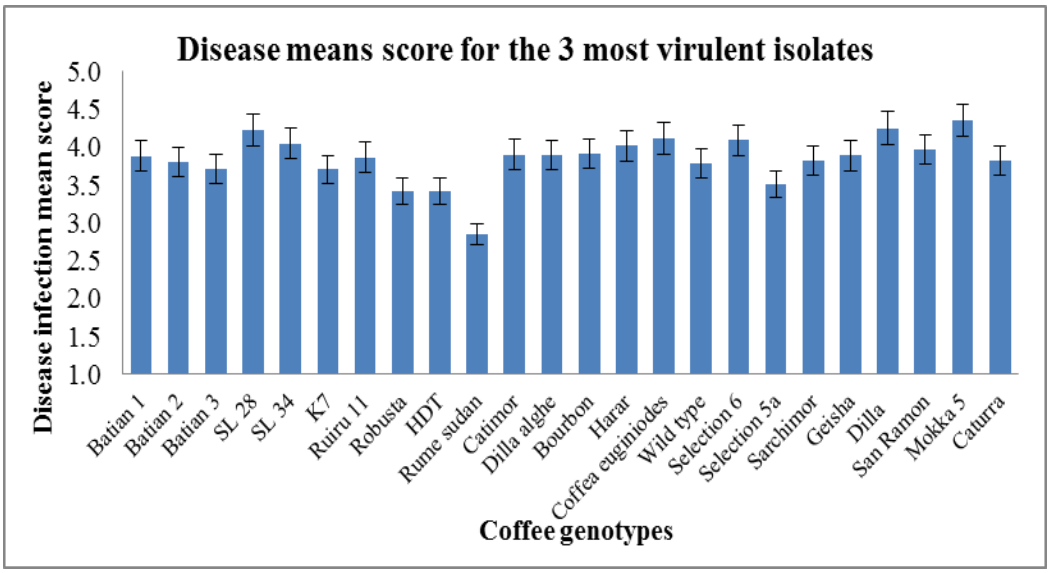

Figure 4.10: BBC infection on coffee genotypes against the 3 most virulent isolates.

SL 28 and SL 34, the Kenyan traditional commercial varieties which are known for their high yields, adaptability and good quality appeared susceptible to BBC just as they are susceptible to both CBD and CLR with a mean score of 4 against the three most virulent isolates. Variety K7 and Batian 3 had the lowest disease score (3.7) among the Kenya commercial varieties.

4.4. Characterization of selected coffee genotypes for BBC resistance using SSR primers

The amplification of the 11 SSR primers was successful (Plate 13and 14) although some primers could not amplify all the DNA samples. A maximum of two bands were observed among the genotypes. Geisha was amplified by all the primers except Sat 207 while Sarchimor was amplified with all primers other than Sat 207 and Sat 255. The bands observed were about 200bp (plate 4.6 and 4.7).

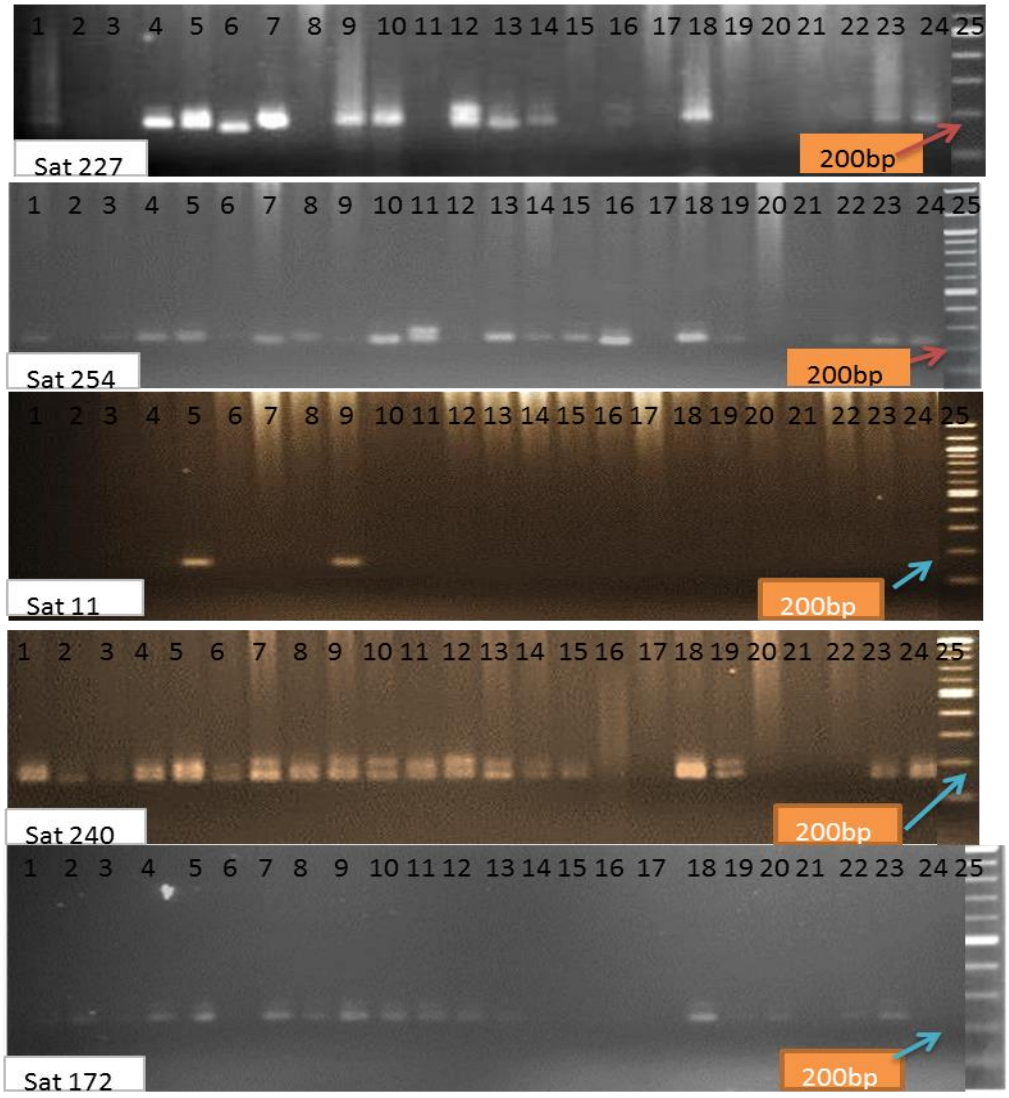

Plate 4.5: Gel pictures showing amplification of 24 coffee genotypes with Sat 227, 254, 11,240 and 172 SSR primers.

(1. Harar, 2. West Pokot, 3. Batian 2, 4. Batian 1, 5. Geisha, 6. Ruiru 11, 7. Bourbon, 8. San Ramon, 9. Sarchimor, 10. K7, 11. Caturra, 12. Selection 5a, 13. Cat 90, 14. Batian 3, 15. Dilla Alghe, 16. HDT, 17. Robusta, 18. SL34, 19. Selection 6, 20. SL 28, 21. *C.euginiodes*, 22. Dilla, 23. Rume.Sudan 24. Mokka5 and 100 base pair DNA ladder)

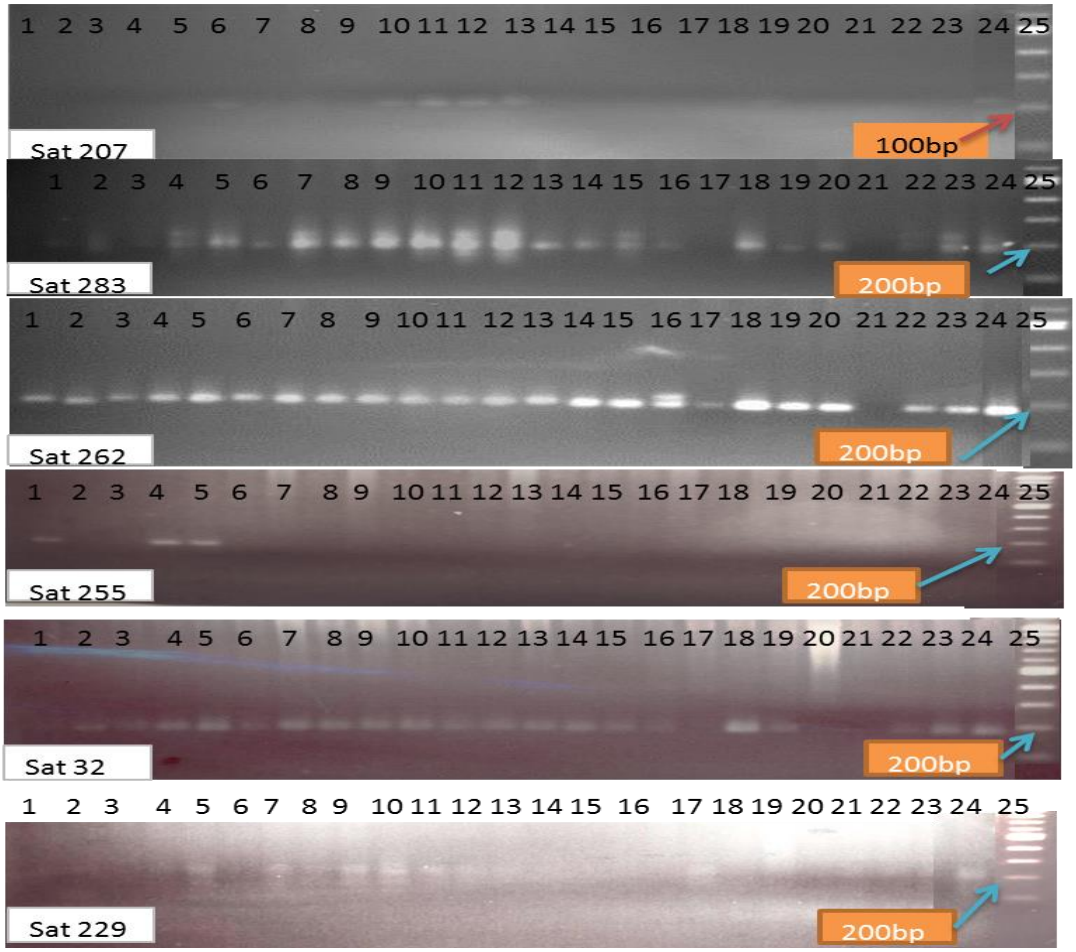

Plate 4.6 Gel pictures showing amplification of 24 coffee genotypes with Sat 207, 283, 262, 255, 32 and SSR primers.

(1.Harar, 2.West Pokot, 3.Batian 2, 4.Batian 1, 5.Geisha, 6.Ruiru 11, 7.Bourbon, 8.San Ramon, 9.Sarchimor, 10.K7, 11.Caturra, 12. Selection 5a, 13.Cat 90, 14.Batian 3, 15.Dilla Alghe, 16.HDT, 17.Robusta, 18.SL34, 19. Selection 6, 20.SL 28, 21. *C.euginiodes*, 22.Dilla, 23.Rume Sudan 24.Mokka5 and 100 base pair DNA ladder)

The analysis of the DNA fragment revealed existing polymorphism among the genotypes. Sat 227, 240, 254 and 293 produced 3 bands while Sat 172, 262 and 229 produced 2 bands (Table 4.5). The other primers had one band. *C. euginiodes* did not

amplify with any of the primers while *C. canephora* (Robusta) amplified with only Sat 262.

Table 4.5: DNA fragments score from 11 SSR primers on coffee genotypes

		SSR primers and band produced																					
Lab No.	Genotype	S227-B1	S227-B2	S227-B3	S11-B1	S240-B1	S240-B2	S240-B3	S254-B1	S254-B2	S254-B3	S172-B1	S172-B2	S207-B1	S283-B1	S283-B2	S283-B3	S262-B1	S262-B2	S255-B1	S32-B1	S229-B1	S229-B2
1	Harar	1	1	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	1	1	1	0
2	West Pokot	0	0	0	0	0	0	0	0	0	0	0	1	0	1	1	0	0	1	0	1	1	0
3	Batian 2	0	0	0	0	0	0	1	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
4	Batian 1	1	1	1	0	0	1	1	0	1	0	1	1	0	1	1	0	0	1	1	1	1	0
5	Geisha	0	1	1	1	1	1	1	0	1	0	1	1	1	0	1	0	0	1	1	1	1	1
6	Ruiru 11	0	0	1	0	1	0	1	0	1	0	0	0	0	0	1	0	0	1	0	1	0	0
7	Bourbon	0	1	1	0	1	0	1	0	1	0	1	1	0	1	1	0	0	1	0	1	1	1
8	San Ramon	0	0	0	0	0	1	1	0	1	0	1	1	0	1	1	0	0	1	0	1	1	1
9	Sarchimor	0	1	1	1	1	0	1	0	1	0	1	1	1	1	1	0	0	1	0	1	1	1
10	K7	0	1	1	0	1	0	1	0	1	0	1	1	1	1	1	0	0	1	0	1	1	1
11	Caturra	0	0	0	0	1	0	1	1	1	0	1	1	1	1	1	1	0	1	0	1	1	0
12	Selection 5a	1	1	1	0	1	0	1	0	1	0	0	1	0	1	1	1	0	1	0	1	1	0
13	Catimor 90	0	0	1	0	1	0	1	1	1	0	0	1	0	0	1	0	0	1	0	1	1	0
14	Batian 3	0	0	1	0	1	0	1	1	1	0	0	0	0	0	1	0	0	1	0	1	1	0
15	Dilla Alghe	0	0	0	0	1	1	1	1	1	0	0	0	0	1	1	0	0	1	0	1	0	0
16	HDT	1	0	1	0	0	0	0	1	1	0	0	0	0	0	1	0	1	1	0	1	0	0
17	Robusta	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0
18	SL 34	0	1	0	0	1	1	1	1	1	0	1	1	1	1	1	0	0	1	0	1	1	1
19	Selection 6	0	0	1	0	0	0	1	0	1	0	0	1	0	0	1	0	0	1	0	1	0	0
20	SL 28	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0	1	0	0	1	0
21	*C. euginiodes*	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	Dilla	0	1	0	0	0	0	0	0	1	0	1	1	0	1	1	0	0	1	0	1	1	0
23	Rume Sudan	0	1	0	0	1	0	1	1	1	0	1	1	0	1	1	0	0	1	0	1	1	0
24	Mokka 5	0	1	0	0	1	0	1	0	0	1	0	0	1	1	1	1	0	1	0	1	1	0
	Total bands	**4**	**11**	**11**	**2**	**13**	**5**	**17**	**7**	**19**	**1**	**10**	**17**	**6**	**13**	**22**	**3**	**1**	**23**	**3**	**21**	**17**	**6**

Key; S- Sat, B-band

The similarity analysis of the fragments score using dendrogram clustered the genotypes into 5 major classes (Figure 4.11). Mokka 5 which was the most susceptible was classified alone. Rume Sudan which was the most resistant genotype against BBC was clustered together with HDT, Dilla Alghe, Catimor 90, Ruiru 11, Batian 3, Batian 2 and Selection 6. SL 28 was grouped alongside Robusta (*C. canephora*), *C euginiodes* and West Pokot (wild coffee). The other major group had Geisha, Selection 5a, Caturra,

Sarchimor, Bourbon, K7, Batian 1, Dilla, San Ramon and SL 34. The genotypes in that group had also a close similarity in disease severity.

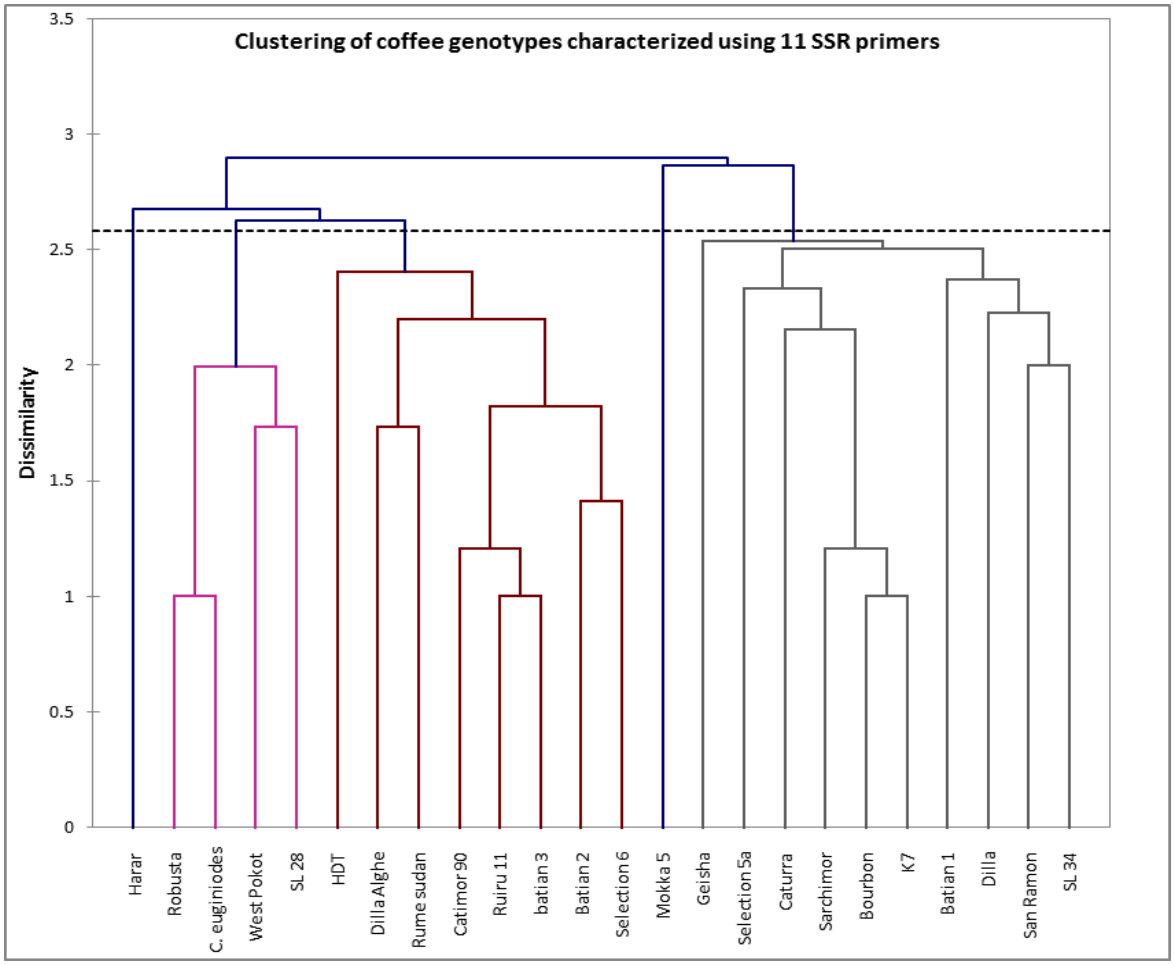

Figure 4.11: Clustering of the coffee genotypes using the 11 SSR primers

Batian 3 and Batian 2 which were better in disease resistance than Batian 1 were clustered together different from the less resistant one. Polymorphism was evident amongst the varieties with Sat 254 producing the highest polymorphism among the selected genotypes. All the 24 genotypes were amplified with sat 254 other than Rume sudan, *C. euginiodes*, *C. canephora*, SL 28 and Wild type. Eight varieties (Caturra, Catimor 90, Batian 3, Dilla Aghe, HDT, SL 34 and Rume Sudan 1 & 2) had 2 bands each via Sat 254.

CHAPTER FIVE

DISCUSSION

5.1 Determination of *P.s. pv. garcae* isolates

The disease symptoms started to be observed on day five (5) from inoculation as had earlier been reported by Ithiru *et al*. (2013). There was significant difference amongst the isolates at 95% level of confidence. This could largely be contributed to virulence factors in the different isolates used in the study. However all the isolates were found to be pathogenic. It has been reported that the outcome of host–pathogen interactions is controlled by compatibility factors, which consist of virulence factors in the pathogen and susceptibility factors of the host (Bing *et al*., 2006). Further, it was observed that isolates from the same region performed differently indicating possible existence of diversity amongst them as in the case of isolates from Kisii: 58/014(4.6) and 37/012(1.85) and Kapsabet: 59/014(3.42) and 28/012 (1.82). Jackson *et al.*(1999) reported that varietal resistance to halo-blight disease of bean (*Phaseolus vulgaris* L.) caused by *Pseudomonas syringae* pv. *phaseolicola* (Pph) is determined by gene-for-gene interactions involving five resistance (R) genes in the host and five matching avirulence (avr) genes in the pathogen. Depending on the presence or absence of functional avr genes, nine races of Pph were distinguished. There appears to be several avirulence genes controlling virulence of the *P. s.* pv. *garcae* which may be due to their distinguished races.

5.2 Characterisation of different *P. s. pv. garcae* isolates using 16S

The results revealed existance of genetic divergence among the *P.s.* pv. *garcae* found in Kenya. The difference of isolates collected from the same region was also evidently observed with the example of isolates from Kisii, Kapsabet and Nyeri which had significant difference. This could be attributed to the possible existance of different strains of *P.s.*pv. *garcae* in Kenya. Ribosomal RNA (16S rRNA) used in

characterisation of the isolates is an universal tool for the phylogenetic analysis and interrelationship determination among organisms using PCR and gene sequencer (Clarridge, 2004). The gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification (Kolbert *et al.*, 1999). The pattern of the sequences of the isolates were clustered in the phylogenetic tree according to their intensity of disease severity. The 16S sequencing is reported to be capable of reclassifying bacteria into completely new species, or even genera (Weisburg *et al.*, 1991). It has also been used to describe new species that have never been successfully cultured.

Apart from being prevalent in medical microbiology as a rapid and cheap alternative to phenotypic methods of bacterial identification (Clarridge, 2004), the use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used because of its presence in almost all bacteria, its often existance as a multigene family, or operons and its unchanged function suggesting that random sequence changes are a more accurate measure of time (Janda & Abbott, 2007). The characterisation of the isolates revealed great diversity among the existing *P.s* pv. *garcae* species in Kenya and possible existance of different strains of the pathover *garcae*. Differences in Kenyan isolates of *P. s.* pv. *garcae* have also been reported by Kairu (1997) and Mugiira *et al*. (2011). However, there was high sequence homology among all the isolates evaluated at 61% at 100% level as expected since they belong to the same pathover. As was reported by Michael *et al*., 2005, the core genome of *P. syringae* is less prone to horizontal gene transfer and therefore provides the best indication of the clonal evolutionary history of a bacterial species. The variation found within the 12 isolates of *P. s.* pv. *garcae* is therefore thought to be due to the flexible genome which largely evolves through horizontal genetic exchange (through gene acquisition and loss).

5.3 Identification of coffee genotypes with resistance to *P. s. pv. garcae* in Kenya

The coffee genotypes conserved ex-situ at CRI, Ruiru include many *C. arabica* accessions from Ethiopia, Sudan, Kenya, Tanzania, Angola, India, Re Union, Portugal, South and Central America (Kathurima *et al.*,2011). Characterisation using SSR primers revealed low to moderate allelic/genetic diversity amongst coffee varieties which was comparable to other previous work (Hendre *et al.*, 2008). However, the existing differences revealed clustered Mokka which was the most susceptible genotype to BBC in its own class. The varietal differences could not be directly associated to BBC resistance although some genotypes that had shown close relatedness in the disease resistance like Rume Sudan and HDT were also clustered together. The results also confirmed report by Agwanda *et al.*(1997) that HDT, Catimor and Rume Sudan genetical constitution is diverse from most of the commercial cultivars (K7, SL 34 and SL 28). Genotypes (Batian 3, Batian 2, Catimor 90, Ruiru 11 and HDT) with a close genetic relatedness were also clustered together, association that could largely be due to the presence of *T* gene.

Plants consistently resist certain pathogens but succumb to others; resistance is usually pathogen species- or pathogen strain-specific. The screening results revealed that most of the Kenyan commercially grown coffee varieties are susceptible or partially resistant to BBC which is a great threat to the industry. The three Batian lines which are the most recently released commercial true breeding coffee varieties in Kenya with tall stature and resistant to the other two major fungal diseases (Gichimu *et al.*, 2010) presented a variation in their reaction against the four isolates. The varieties had earlier been reported by Ithiru *et al.* (2013) to have a moderate resistance to BBC and are also high yielding with good bean and liquor quality that compares to Ruiru 11 and SL28. The differences observed in their reaction to the four isolates indicate a possibility of existing different strains of *P. s.* pv. *garcae*. Vertical resistance is specific to certain races or strains of a pathogen species and is often controlled by single *R* gene and can be less durable. Horizontal or broad-spectrum resistance against an entire pathogen species is

often only incompletely effective, but more durable, and is often controlled by many genes that segregate in breeding populations. Rume Sudan which is a genotype widely used in Kenya as a donor for resistance to other major coffee diseases was the most promising candidate as a source of resistance to *P. s.* pv. *garcae* scoring the lowest disease severity mean against isolate Kisii-58/2014 and Nakuru-62/2014 and was second after Robusta against Mweiga-66/2014, presenting a wide range of disease resistance. That consistence in resistance is a desirable trait towards breeding for resistance from a genotype which is highly resistant to CBD conferred by the dominant *R*- and the recessive *k*-genes (Agwanda *et al*., 1997; Gichimu *et al*., 2014).

Resistance to CBD in Ruiru 11 is known to be controlled by among others, the *T* (*Ck-1*) gene from Robusta coffee presenting significant variability in resistance to CBD (Gichimu *et al.,* 2014). Ruiru 11 is a composite F1 hybrid of variety Catimor (as the female) and breeders lines that yielded Batian most of which have HDT in their pedigree as the male selections (Kathurima *et al.*, 2011). The breeding programmes to develop the male parents involved backcrossing and selfing at various selection stages which affected the amount of Robusta genome passed on to the next generation. This can explain the wide range of diversity observed between HDT and its derivatives (Catimor line 90, Ruiru11, Batian 1, Batian 2 and Batian 3) confirming the report by Kathurima *et al.*(2011). HDT, a spontaneous interspecific hybrid of *C. arabica* and *C. canephora* also appeared to be similar to Robusta (*C. canephora*) in its reaction to BBC and it is reported that clone 1349/269 of the variety HDT which was introduced to Kenya in 1960 from Portugal and its hybrid derivative Catimor carry one gene for CBD resistance on the *T*-locus with intermediate gene action (Agwanda *et al*., 1997; Gichimu *et al*., 2014). Progenies of HDT and advanced inbred lines of its cross to *C. arabica* cv. Caturra (referred to as cv. Catimor), are used as donor parents for resistance to the major fungal diseases in Kenya. The results also confirmed the report by Ito *et al*. (2008) that HDT could be a source of resistance to BBC. Different lines of HDT have been used worldwide to breed coffee varieties that are resistant to different pathogens (Kathurima *et al*., 2012) including Ruiru 11 and Batian lines. The similarity in resistance between

Robusta and HDT against the BBC isolates could be associated to their genetic relatedness. Batian 3 appeared to be more prospectus than the other Batian lines with respect to the search for BBC resistance. The difference among the three Batian lines could be due to their pedigree where Batian 3 has the three major genes associated with CBD resistance namely *R* from Rume Sudan, *T* from HDT and *k* from K7. It could also be suggested that the reason there was no significant difference between Batian 3 and K7 was due to occurrence of common *k* gene that effected resistance to BBC in the two genotypes. The other two Batian lines had no K7 in their pedigree.

The study revealed that most of the Kenyan commercially grown coffee is susceptible or partially resistant to BBC which is a great threat to the coffee industry. While K7 is a Kent type commercial variety moderately resistant to CBD, other resistance donors (HDT & Rume Sudan) correspond to exotic germplasm where the valuable resistant genes are associated with undesirable traits (Agwanda *et al.,* 1997). Utilization of commercial cultivars with desirable traits like Batian 3 in breeding for resistance to major coffee diseases is important. Batian 3 and K7 which is moderately resistant to CBD and is reported to have the recessive *k*-genes (Agwanda *et al.,* 1997) were the most resistant genotypes among the commercial coffee varieties in Kenya. There was no significant difference observed between Ruiru 11 and one of its parent namely Catimor. They were rated moderately susceptible confirming report that Ruiru 11 is infected by BBC under field conditions (Ithiru *et al.,* 2013). The study confirmed report by Masaba (1998) that BBC infection was lowest on Catimor, intermediate on SL34 and highest on SL28. *C. euginioides* which does not have desirable attributes of a commercial variety was among the most susceptible genotypes indicating no possible source of BBC resistance. The wild type genotype from West Pokot which closely resembles *C. euginiodes* in its morphological attributes was rated among the moderately resistant genotypes behind Batian 3. Though it had earlier been reported that SH1 gene found in the Arabica coffee genotypes Harar, Dilla & Alghe, S12 Kaffa and Geisha confers simultaneous resistance to some races of *H. vastatrix* and to *P. s.* pv. *garcae* (Ito *et al.*,2008) these genotypes recorded a relatively high disease score with no significance

difference (Harar 3.11, Dilla 3.17, Dilla alghe 2.98, and Geisha 3.08). The results indicate possible existence of diverse strains of *P. s.* pv. *garcae* as was observed in the virulence test of the BBC isolates and their molecular characterization.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

6.1 Conclusions

There was evidence of difference in virulence of the *P.s.*pv. *garcae* isolates collected from different areas reflecting the high level of diversity of the pathogen. The results indicate possible existence of diverse strains of *P.s.*pv. *garcae* that could be associated to virulence. Rume sudan was the most resistant genotype although it is not a commercial variety while K7 and Batian 3 appeared to be the most promising commercial varieties resistant to BBC. The SSR markers used could not be linked to BBC resistance

6.2 Recommendations

The existance of diverse strains of the *Ps*.pv.*garcae* poses a threat to coffee farming and therefore a breeding programme to introgres resistance genes into the commercial varieties through pyramiding of resistance genes could offer a durable resistance to the coffee. The commercial cultivar Batian 3 which is resistant to both CBD and CLR should be utilized as a recurrent parent in the breeding programme. Developing a nomenclature of the possible different strains of *P. s.* pv. *garcae* in Kenya is highly recommended. The methodology used in screening for resistance to BBC is recommedded for its efficiency and reliability.

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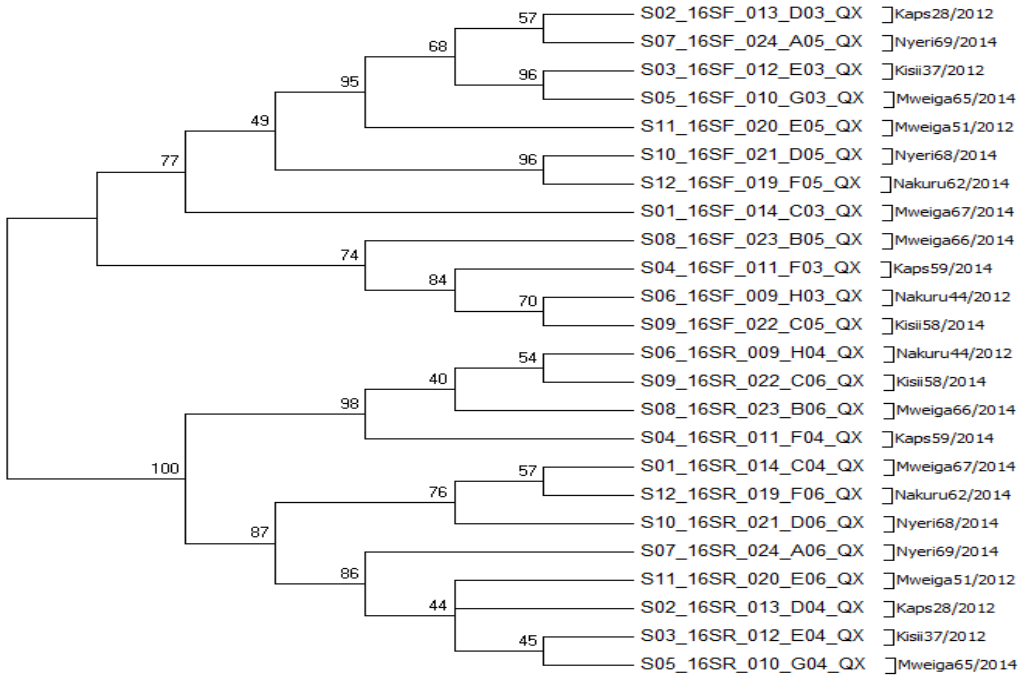
APPENDICES

Appendix 1: An evolutionary relationship of taxa with both primers (F-forward, R-reverse).



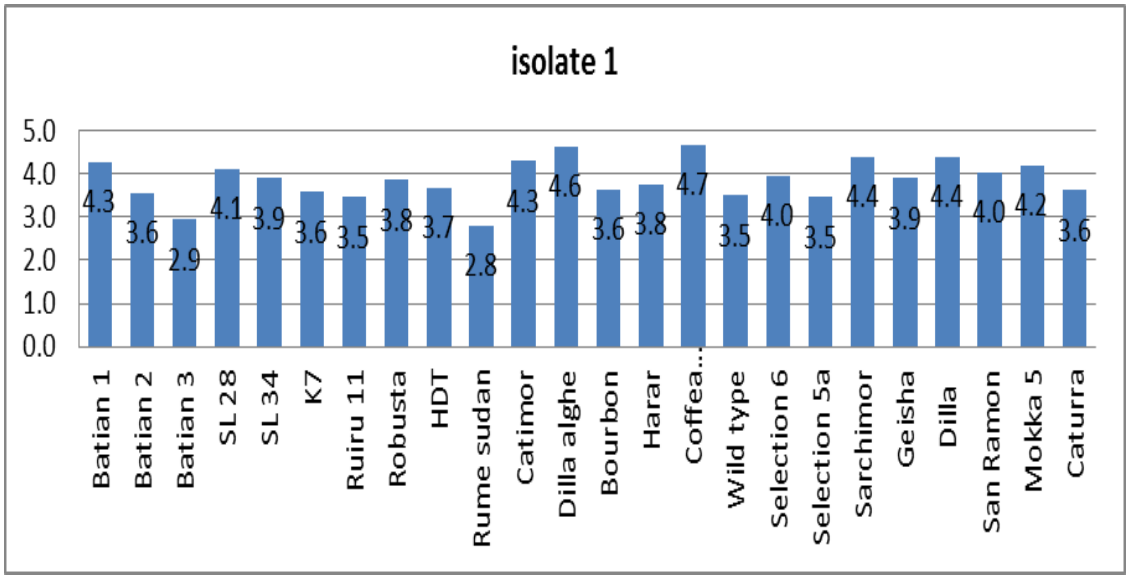
The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 1.61116563 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown above the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method [3] and are in the units of the number of base substitutions per site. The analysis involved 24 nucleotide sequences (12 with Reverse

primer 12 Forward primer).Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 956 positions in the final dataset. Evolutionary analyses were conducted in MEGA6.

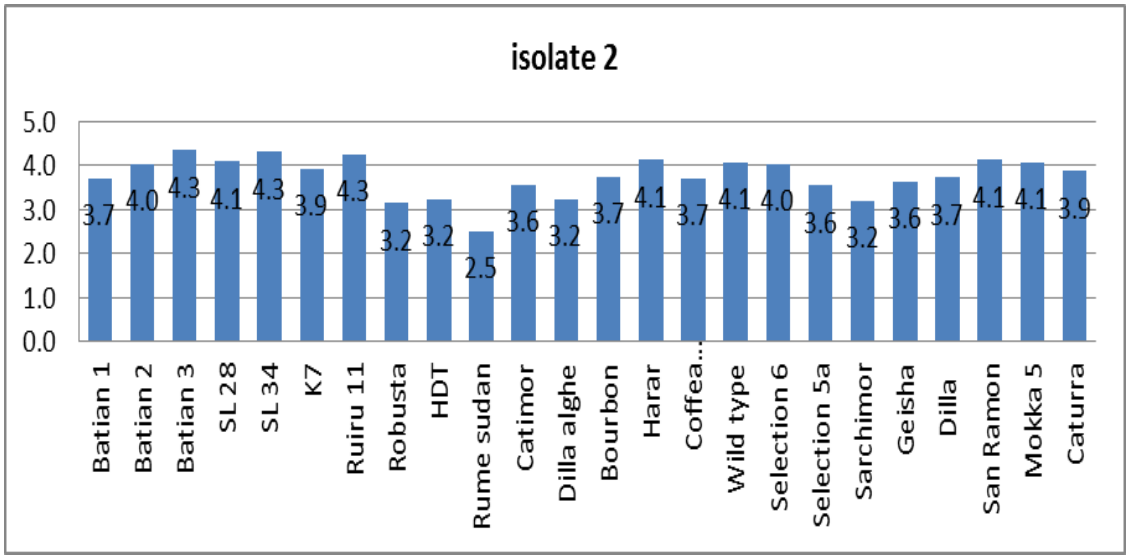
Appendix 2: ANOVA on reaction of the genotypes inoculated with 4 selected isolates

Source	df	Type I SS	MS	F	P
Blocks	2	0.610094	0.305047	1.422013	.2433 ns
Main Effects					
Isolate	4	284.2259	71.05648	331.2382	.0000 ***
Genotype	23	12.94061	0.562635	2.622791	.0001 ***
Interaction					
Isolate x Genotype	92	39.563	0.430033	2.004648	.0000 ***
Error	238	51	0.05523	0.2145178<-	
Total	359	388.3949			

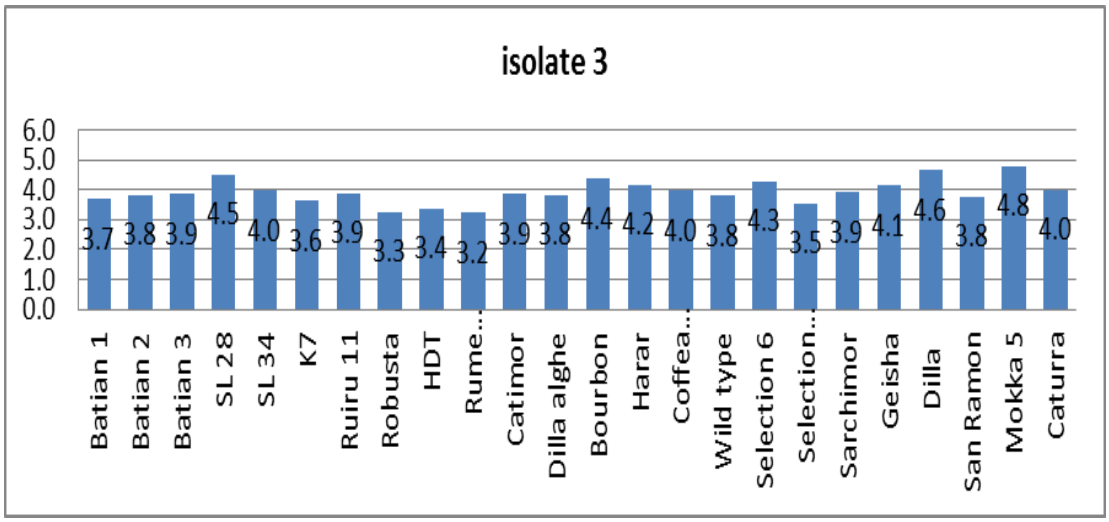
Appendix 3: Disease infection means with isolate 1



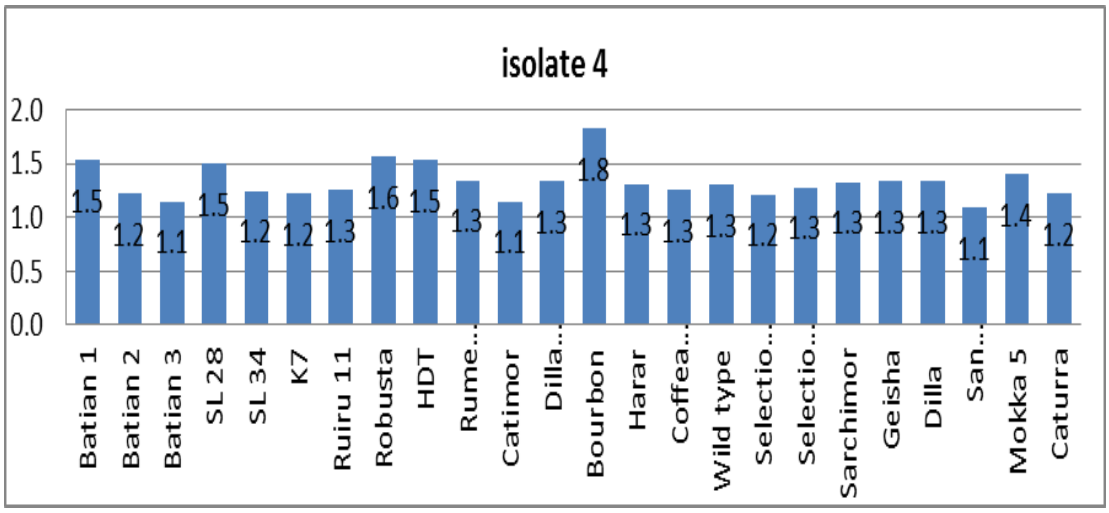
Appendix 4: Disease infection means with isolate 2



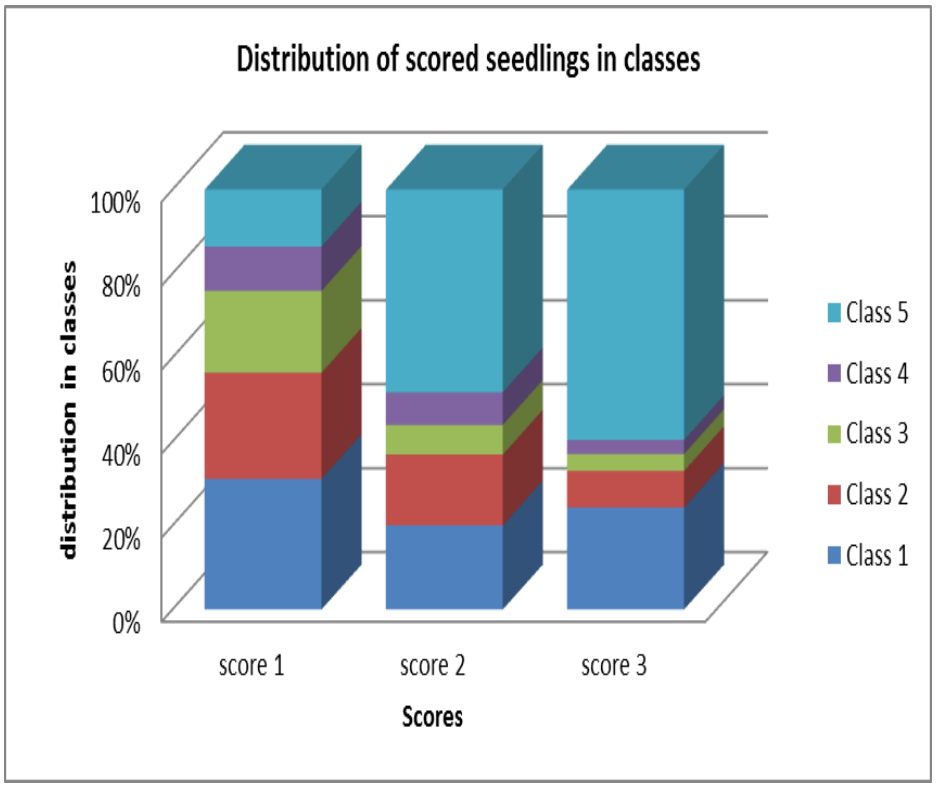
Appendix 5: Disease infection means with isolate 3



Appendix 6: Disease infection means with isolate 4



Appendix 7: Distribution of seedlings in disease infection classes



Score 1-day 14th, score 2- day 21st and score 3- day 28th.