GENETIC DIVERSITY AMONG ISOLATES OF *Colletotrichum kahawae* CAUSING COFFEE BERRY DISEASE AND THEIR INTERACTIONS WITH VARIETIES AND BREEDING POPULATIONS OF ARABICA COFFEE.

BY

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DECLARATION BY THE CANDIDATE

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

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ABSTRACT

The objective of this study was to determine the nature and magnitude of genetic diversity of *Colletotrichum kahawae* Waller & Bridge (1993), the causal agent of coffee berry disease (CBD) and its implication on pathogen adaptation to resistant host varieties. Single conidia isolates of *C. kahawae* were subjected to genetic analysis using virulence tests, protein, isozyme and Random Amplified Polymorphic DNA (RAPD) markers. Two Benomyl tolerant strains and two non-pathogenic species, *C. acutatum* and *C. gloeosporioides* commonly found colonising the coffee plant were included in the analyses for comparison. Virulence tests revealed that a large proportion of the variation (64.81%) was due to main effects of varieties and isolates usually associated with differences in aggressiveness. The variety x isolate interaction effects, although significant ($p \leq 0.01$) was too small (14.60%) to suggest conclusively that races exists.

Buffer soluble proteins separated on a 10% polyacrylamide gel and stained with silver nitrate easily detected variation between *Colletotrichum* species in the region of 173 kDa, 45 kDa and 40 kDa. Small differences were also detected among isolates of *C. kahawae* with proteins of molecular weights of 123 kDa and 52 kDa but were neither diagnostic for virulence nor Benomyl tolerance.

A total of 12 enzyme systems were assayed and 28 putative loci detected. Isolates were classified into seven electrophoretic types (ETs) with *C. acutatum* and *C. gloeosporioides* forming single isolate phenotypes indicative of their separate taxonomic status. Isolates of *C. kahawae* were subdivided into five ETs. Esterase enzyme was the most variable, partitioning the isolates into three ETs. The

remaining two ETs were as a result of variability of the lactase dehydrogenase enzyme which was apparently linked to Benomyl-tolerance/susceptibility.

RAPD analysis further detected up to 80% polymorphic bands with 36 decamer oligonucleotide primers on the pathogenic and non-pathogenic species. Within the pathogenic species, only 7% of the bands were polymorphic arising from amplification by 3 primers. The variation however, detected no markers that could be traced back to differences in virulence (existence of races) or Benomyl tolerance/sensitivity. It was concluded that due to the large similarity of *C. kahawae* isolates it is unlikely that races exist.

Variation for resistance among genotypes of *Coffea arabica*, cultivar Ruiru 11 was mainly due to its heterogeneity and differences in aggressiveness of the pathogen. This conclusion was supported by the fact that the main effects of genotypes and isolates were significant ($p \leq 0.05$) while interaction effects were non-significant. Outstanding families combining field resistance to CBD with high productivity and fine quality were identified in an elite breeding population, "B22A". The unique features of these families distinguishing them from existing commercial varieties are discussed.

CHAPTER 1.

GENERAL INTRODUCTION.

1.1 Classification and centres of genetic diversity of coffee.

Coffee belongs to the Linnaean genus *Coffea*, a member of the large *Rubiaceae* family comprising of about 500 genera and over 6000 species (Wrigley, 1988). The genus *Coffea* has about 100 species, but only 4 are of commercial importance (Biratu, 1995). *Coffea arabica* L. (Arabica coffee) constitutes 80% of the world trade. *C. canephora* Pierre ex Froehner (Robusta coffee) contributes most of the remaining 20% while *C. liberica* Bull ex Hiern (Liberica coffee) and *C. dewevrei* De Wild (Excelsa coffee) contributes less than 1% (Wrigley, 1988; Biratu, 1995). The cultivated coffee species are evergreen shrubs with a natural height of 8 - 10 m depending on the species (Coste, 1992).

Arabica coffee which is by far the most important species has its primary centre of genetic diversity in the evergreen highlands of South West Ethiopia and the Boma Plateau in the Sudan, where it still grows wild as an undercanopy shrub (Sylvain, 1958; Meyer, 1969). Some indigenous populations have also been identified around Mount Imatong in Sudan and Marsabit in Kenya (Berthaud and Charrier, 1988). *C. arabica* is the only known self-compatible, allotetraploid species of the genus (2n = 4x = 44), the diploid progenitors of which are believed to be the East African species, *C. eugenioides* and one of the West African species such as *C. canephora* or *C. liberica* (Fernie, 1971).

The basic chromosome number of coffee is x = 11 (Bouharmont, 1963). *C. canephora* is an allogamous, self-incompatible diploid species (2n = 2x = 22). It is native to the Western and Central equatorial forests of Africa (Ferwerda, 1976). Being an outcrossing species, it occurs in a diversity of forms. The centre of greatest diversity is the Congo basin, in the Democratic Republic of Congo, formerly Zaire (Walyaro, 1983). *C. liberica* and *C. dewevrei* are indigenous to the dense forests of West Africa (Guinea, Liberia and Ivory Coast).

1.2 Distribution of the cultivated coffee.

The first cultivation of Arabica coffee was in the Yemen, where *C. arabica* was introduced from Ethiopia by Arab traders (Wrigley, 1988). From Yemen, coffee growing spread to the present producer regions of Asia, Latin America and Africa. The coffee trees from Yemen gave rise to two botanical types (Krug et al., 1939): *C. arabica* var. *typica* Crammer was introduced to Latin America and Asia. It has bronze-tipped young leaves and pendulous fruit bearing branches. *C. arabica* var. *bourbon* was introduced to South America and East Africa through the island of Reunion, formerly Bourbon. It has young green leaves and fruit bearing branches bent down only at the tips (Lashermes et al., 1996). In all these introductions, only a small number of plants were involved and therefore Arabica coffee cultivation rests on a very narrow genetic base (Ferwerda, 1976; Lashermes et al., 1996). Genetic variability of the species is further limited by its predominant self-fertilization nature.

Cultivation of Robusta coffee primarily started in its native lowland tropical regions of Western and Central Africa (Wrigley, 1988; Coste, 1992). It was introduced to Indonesia from Kwilu near Limbe in Cameroon where its production increased due to its resistance to leaf rust (*Hemileia vastatrix*) which was hindering production of Arabica coffee in Asia (Wrigley, 1988). Although most of Robusta coffee is produced in the West, Central and Eastern African countries of Ivory Coast, Angola, Uganda, Zaire and Cameroon, Indonesia is the largest single producer of Robusta coffee in the world followed by Ivory Coast. *C. liberica* and *C. dewevrei* are cultivated to a small extent in West Africa, Indonesia, French Guinea and Surinam (Walyaro, 1983).

In Kenya, growing of coffee dates back to 1893 when it was first introduced by Scottish missionaries and planted at Bura in Coast Province (Wrigley, 1988). The climate at Bura proved unsuitable and the cultivation of coffee was later transferred to Kibwezi in Makueni District, Eastern Province and Kikuyu in Central Province near Nairobi. Coffee Plantations were later established in the areas around Mount Kenya and Aberdare Ranges in Central Province, West of the Rift Valley and in Taita Hills in the Coast Province.

1.3 Coffee growing conditions.

Coffee growing conditions are found in a wide horizontal band on both sides of the equator between the tropics of Cancer and Capricorn known as the "Coffee belt" (Anon., 1994). An account of the soil and climatic requirements for the two important cultivated species, *C. arabica* and *C. canephora* have been reviewed by Wrigley (1988) and Coste

(1992). These authors have indicated that *C. arabica* requires a frost free subtropical climate. Rainfall should be between 1500-1800 mm per year and well distributed. A definite dry season preferably in the cooler part of the year is necessary to initiate dormancy. Ideal temperatures for growing *C. arabica* are around 20^0C but ranges of 17^0C to 30^0C characterize its growing regions provided the daily and seasonal fluctuations are minimum. Robusta coffee grows in the low altitude, humid conditions with a higher average temperature of 24 - 27 ^{0}C. However, both species require soils that are deep, well drained, loamy, slightly acidic (pH 5.0 - 6.0) and rich in humus and exchangeable bases, particularly potassium. Phosphorus is essential during flowering.

1.4 Economic importance of coffee.

Coffee is one of the most important agricultural commodities upon which the economy of more than 50 producer countries depends (Rodrigues et al., 1975). It is the world's second most traded commodity after fossil oil (Clarke, 1985). The world coffee production was estimated at 5.8 million tons of green beans in 1993 (Biratu 1995). Of the total production, Latin America contributed 66%, Africa 20%, Asia and Oceania 14%. The two largest producers, Brazil and Colombia accounted for 21% and 16% of the world's total exports respectively. The role of coffee as a foreign exchange earner varies from country to country. For example, in Uganda and Burundi, coffee exports contribute about 80% of the total hard currency revenue (Anon., 1994). For those countries where it contributes less to the overall balance of payment like Brazil (6%), Indonesia (2.7%) and Mexico (1.7%), it still plays its second key role of creating employment for a large

sector of the rural population. In Kenya where the economy largely depends on agriculture, coffee is the second most important agricultural product after tea contributing upto 25% of the total foreign exchange earnings (Opile', 1993). It is further estimated that out of the 70% of Kenya's workforce engaged in agriculture, 30% are employed by the coffee industry.

1.5 Constraints to coffee production in Kenya.

Kenya produces an average of 90,000-130,000 tonnes of clean coffee per year on an area of about 156,000 hactares. This represents about 2-3% of the world's coffee production (Njoroge, 1992). Coffee yields in Kenya have generally remained low with small holders recording an average of 534 kg of clean coffee per ha (2.8 kg of cherry per tree) and plantations recording an average of 1064 kg of clean coffee per ha (5.6 kg of cherry per tree) compared to yields of 3.5 tonnes per ha (18.4 kg of cherry per tree) which have been achieved in some estates (Karanja, 1996). Although Kenyan coffee fetches premium prices in the world market because of its fine quality, the total revenue would tremendously increase if production increased. The low coffee yields in Kenya are generally attributed to the high cost of inputs and losses due to pests and diseases. Attempts have been made in the past to address this problem by formulating research projects aimed at reducing the cost of production. On pest control, more emphasis is being placed on Intergrated Pest Management (IPM) which uses self-renewing forms of pest control such as biological control. Diseases attacking coffee in Kenya are mainly coffee berry disease (*Colletotrichum kahawae*, Waller et al., 1993), coffee leaf rust

(*Hemileia vastatrix* Berk. & Br.) and bacterial blight of coffee (*Pseudomonas syringae* pv. *garcae* van Hall). An Arabica coffee variety, Ruiru 11, developed at the Kenya's Coffee Research Station, Ruiru, and released to growers in 1985 is not only resistant to coffee berry disease (CBD) and coffee leaf rust but also combines high yield, good quality and compact growth for high density planting. By growing Ruiru 11, farmers save upto 30% of the production costs that go to CBD control alone (Nyoro and Sprey, 1986). The years following the release of the variety have been devoted to the improvement of the genetic base of resistance but faces the problem of possible pathogen variability. Virulence tests using isolates from Ethiopia (van der Graaff, 1978) and Kenya (Masaba and van der Vossen, 1980) revealed variation in aggressiveness (variety non-specific) but no races were detected. However, recently, it has been observed that races might be emerging. Rodrigues Jr. et al. (1991) found that Angolan and Malawian strains of *C. kahawae* posses characteristics different from the Kenyan isolates. The isolates were highly aggressive on the Catimor variety. Some lines of this variety are used in Kenya as the mother parents in the production of the disease resistant hybrid variety, Ruiru 11. It was concluded on the basis of virulence tests that physiological forms of the CBD pathogen might exist among the Angolan, Malawian and Kenyan isolates (Rodrigues Jr. et al ,1992). Within the Kenyan CBD pathogen population, certain strains were isolated from resistant coffee genotypes for this study which might be different from those normally attacking the susceptible varieties.

The objectives of this study were therefore:

1. to establish the nature of host x isolate interaction by performing virulence tests with isolates of *C. kahawae* on varieties of *C. arabica* varying in resistance.
2. to determine the level of genetic diversity in the pathogen population.
3. to determine the nature of interaction between the CBD resistant cultivar, Ruiru 11 and isolates of *C. kahawae* varying in virulence.
4. to identify families combining field resistance to CBD with high yield and good quality by evaluating an advanced breeding population of *C. arabica*, "B22A".

CHAPTER 2.

LITERATURE REVIEW.

2.1 *Colletotrichum* species on *Coffea arabica*.

A fungus causing leafspots and twig symptoms on *C. arabica* in Brazil was described by Noack (1901) as *C. coffeanum*. The name was also adopted by Mc Donald (1926) for the disease of immature fruits reported in coffee plantations in Western Kenya around Mt Elgon near the Kenya/Uganda border. Since the Brazilian strain was not pathogenic on green coffee berries, Rayner (1941) distinguished the East African strain causing coffee berry disease (CBD) as *C. coffeanum* var. *virulans*. The strain is believed to have arisen by mutation from a mild parasitic form like that causing brown blight (Nutman and Roberts, 1960 a) and was occurring on *C. eugenioides*, a diploid coffee species which is found in the high altitude forests of Western Kenya and Eastern Uganda (Robinson, 1976; Mogk, 1975).

Several *Colletotrichum* species colonize the coffee plant. Gibbs (1969), differentiated the species into four strains based on colony characteristics of single conidial cultures of isolates from host tissue grown on 2% Malt Extract Agar (MEA). The strains were: 1) CBD (var."*virulans*"), with slow growth, profuse greyish-black mycelia and conidia borne directly on hyphae, 2) ccp with slow growth, profuse pink areal mycelia, conidia borne directly on hyphae, 3) ccm with fast growth, profuse pale areal mycelia and conidia borne directly on hyphae and 4) cca with moderately fast growth, sparse pale areal mycelia, conidia produced in acervuli. Hindorf (1970) used discriminant analysis on a range of morphological characters and differentiated the

population of *Colletotrichum* from coffee into *C. acutatum* Simmonds (corresponding to ccp) and *C. gloeosporioides* Penz.; mycelial and acervuli forms (corresponding to ccm and cca. respectively). The author retained the name *C. coffeanum* for the CBD strain. *C. acutatum* and *C. gloeosporioides* are both saprophytic strains. *C. coffeanum* (Noack, 1901) and the earlier described *Gloeosporium coffeanum* (Delacroix, 1897) are probably synonymous with *C. gloeosporioides* (Waller et al., 1993). In a comparative study on a range of *Colletotrichum* isolates using morphological, pathological and biochemical criteria, Waller et al. (1993) differentiated the CBD pathogen from other similar *Colletotrichum* strains which occur on coffee and suggested that it belongs to a distinct species which was formally named *C. kahawae* sp.nov. Biratu (1995) observed that the CBD pathogen shares considerable similarities with *C. gloeosporioides* and therefore it should be classified at forma specialis level. The author suggested that the CBD causing pathogen being a host specific isolate should be called *C. gloeosporioides* f. sp. *coffeanum*. Sreenivasaprasad et al. (1993) analyzed both ribosomal and mitochondrial DNA of *C. kahawae* and *C. gloeosporioides* using Restriction Fragment Length Polymorphism (RFLP) and also found a close association between the two species. However, Hindorf et al. (1997) confirmed that *C. kahawae* correctly identifies the pathogen causing CBD and hence the name was used throughout this study. The genus *Colletotrichum* in which the species belongs is classified into *Eumycota*, a major subdivision of the *Deutromycotina*, in the class *Coelomycetes*, order *Melanconiales* and family *Melanconiaceae* (Biratu, 1995).

2.2 Coffee Berry Disease (CBD).

2.2.1 Symptoms.

Coffee berry disease attacks all stages of the developing crop including flowers and occasionally leaves (Mulinge, 1970). Symptoms on green berries appear as small, dark, sunken lesions typical of anthracnose which may spread to cover the whole berry (Figure 1). Under suitable conditions, the fungus readily sporulates forming a mass of pink conidia and also penetrates the interior of the berries destroying the beans. The resulting dry, black, mummified berries have no commercial value and sometimes are shed off the tree. Adverse post -infection conditions produce "scab" lesions which cover a restricted berry surface. Scab lesions are also observed when infections occur during the less susceptible stages of berry development and on resistant coffee varieties as a defence reaction (Mc Donald, 1932; Bock, 1956; Mulinge, 1970; Masaba and van der Vossen, 1982; Masaba, 1991).

2.2.2 Epidemiology.

Conidia produced in acervuli on developing bark of young twigs and on diseased berries provides the initial inoculum for a CBD epidemic (Nutman and Roberts, 1961; Wrigley, 1988). Figure 2 illustrates an acervulus section through a hypocotyl. Splashes of rain disperse the conidia to new infection sites. In the initial stages of the disease, infected bark may be the only source of inoculum, but as the disease

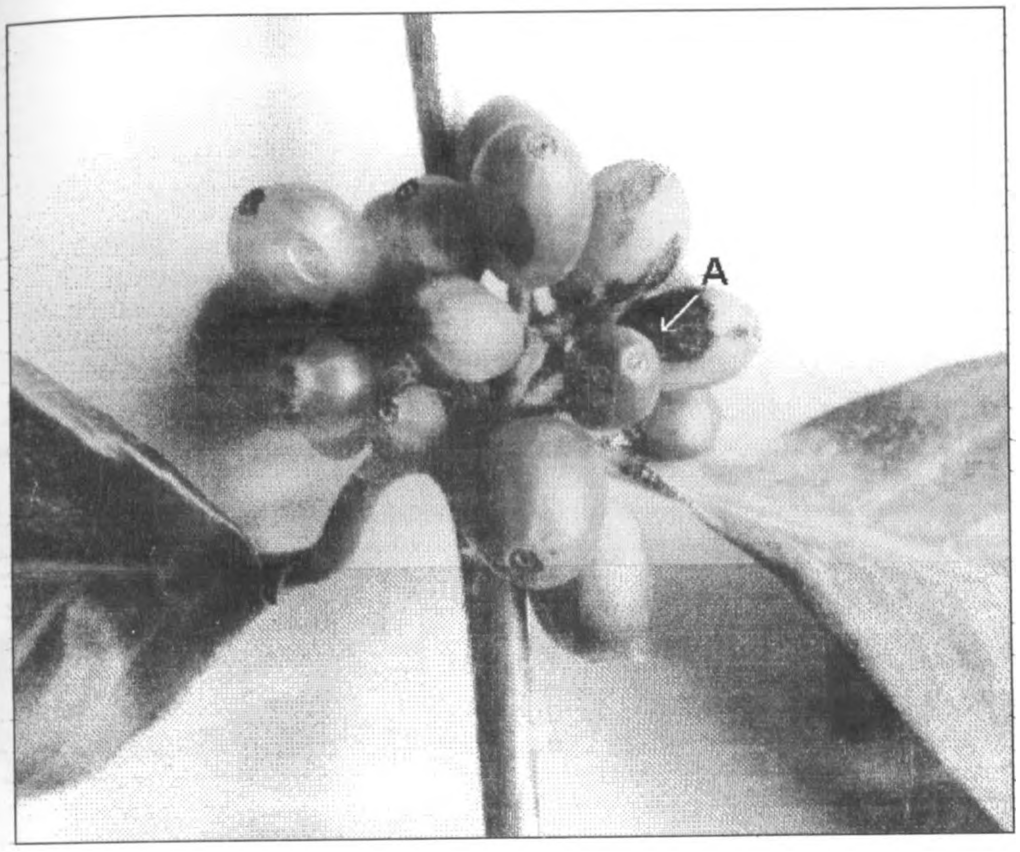


Fig. 1. Green coffee berries infected with *C. kahawae*.
A = A typical black sunken lesion.

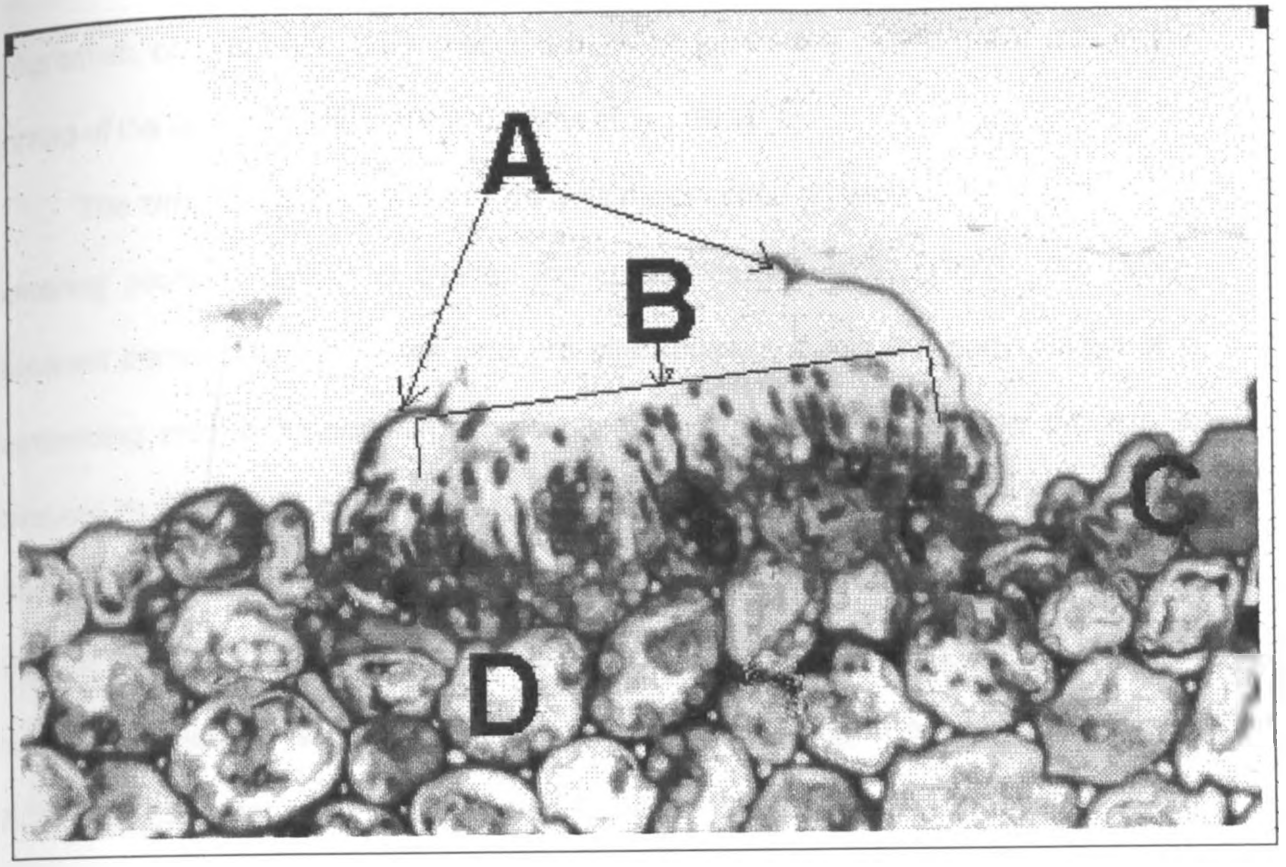


Fig. 2. An acervulus section on a hypocotyl stem.
A = Ruptured cuticle.
B = An acervulus producing mass of conidia.
C = Epidermal cells.
D = Collenchyma cells.

progresses, conidia from berries become more abundant and play a major role in the spread of the disease (Gibbs, 1969; Griffiths et al. ,1971).

The bimodal rainfall pattern in Kenya's major coffee growing areas results in two flowering seasons leading to overlapping crops (Masaba et al., 1982). Therefore, diseased berries from an earlier crop are often present during the early stages of a succeeding crop producing large amounts of inoculum. The infection process is favoured by availability of liquid water and suitable temperature. Nutman and Roberts (1960 b) reported that the minimum and maximum temperatures for spore germination were 15^0C and 30^0C, respectively, with an optimum of 22^0C. In the presence of nutrients, particularly sugars, germination increased resulting in a higher optimum of 27^0C and a broader range (Nutman and Roberts, 1960 b; Firman and Waller, 1977). However, in the field, infections were found to occur at temperatures below 15^0C (Waller, 1971; Cook, 1975) and even down to 10^0C (Bock, 1956).

It is generally observed that the incidence of CBD is high on coffee grown in the cooler high altitude zones than in the lower altitude areas. Mwang'ombe et al. (1991) investigated the effect of temperature on appressoria and lesion formation and found that the optimum temperature for appressoria formation was between 15-25^0C which coincided with the range of temperatures in the cool high altitude coffee growing zones and in the low altitude zone during the cold and wet months. At optimum temperature, Firman and Waller (1977) reported that spore germination occur within 4 hours. Germ-tubes and appressoria are produced, from which infection pegs develop to penetrate the host cuticle (Firman and Waller, 1977). Under field conditions, Bock (1956) observed that the typical black sunken lesions start to appear after one week but are fully developed after 2 weeks. Mulinge (1970) reported an incubation period of between 2-4

weeks. On older and more resistant berries, lesions take longer to appear. The disease has negligible effect on berries which are more than 5 months old until during ripening when the berries become susceptible to infection again (Masaba et al., 1982). The most susceptible stages coincide with the period of berry expansion, between 4-14 weeks after flowering (Mulinge, 1970).

2.3 Variability of *C. kahawae.*

C. kahawae is a host-specific pathogen of *C. arabica.* Soon after the disease was detected in Kenya in 1922 (Mc Donald, 1926) it spread rapidly to other African coffee growing countries such as Angola in 1930, Democratic Republic of Congo in 1937, Cameroon in 1957, Uganda in 1959, Tanzania in 1964, Ethiopia in 1971 (van der Graaf, 1981; van der Vossen, 1985) and Malawi in 1985 (Lutzeyer et al., 1993). The fact that the disease was not detected in Ethiopia, the primary centre of genetic diversity of *C. arabica* until 1971 suggests that the pathogen had not co-evolved with the host into a diversity of forms and therefore the rapid spread of the disease may have been due to narrow genetic base of the cultivated *C. arabica.* Pathogen diversity may have also been limited by lack of a perfect state which promotes sexual recombination. Morphological descriptive criteria such as conidial shape, conidial size, appearance in culture, rate of growth, temperature response and production of perithecia were therefore largely used for accurate classification of *Colletotrichum* species colonizing coffee (Gibbs, 1969; Hindorf, 1970; Firman and Waller ,1977). Virulence tests with isolates of *C. kahawae* (van der Graaf, 1978; Masaba and van der Vossen, 1980) also confirmed that the CBD pathogen exhibited variation for aggressiveness but no races

were detected. Although the narrow genetic base of the cultivated *C. arabica* and clonal nature of pathogen reproduction may have contributed to the earlier observed genetic uniformity, it has recently been reported that variation for virulence could be emerging (Rodrigues Jr. et al., 1992). The possible mechanisms which may be responsible for the apparent physiological specialization of the pathogen include, mutations, recombination and migration (Leung et al. ,1993; Burdon,1993).

In an asexually reproducing population, variants can emerge as a result of several mutations, one at a time which develop into co-adaptive gene complexes (Crow and Kimura, 1965). In wheat, Burdon (1993) reported that in a span of 10 years following an accidental introduction of a single race of *Puccinia striiformis* into Australia in 1979, eleven new races were detected differing from the pre-existing race at a single virulence locus. Luig and Watson (1970) also reported a proliferation of the Australian *P. graminis* f. sp. *tritici* in 15 years following the appearance of race 21-0 in 1954 which could be traced back to the process of spontaneous mutation. In Brazil, it was demonstrated that within 10 years of introduction of *Hemileia vastatrix* causing coffee leaf rust, 10 different races were detected in the breeding plots of the Instituto Agronomico in Campinas (Eskes, 1981). The interactions between the ploidy level of the pathogen, the rate of mutation and the size of the population determines how rapidly variants emerge (Burdon, 1993). In haploid pathogens, a single point mutation may lead to a change in pathogenicity whereas in diploid pathogens (assuming recessive virulence as is often the case), mutations will not be expressed unless the virulence gene is rendered into a homozygous state through a second mutation at the same locus or through a process of sexual or asexual recombination. High rate of mutation also accelerates the emergence of pathogen variants which are more easily detected in small

populations than in large populations. It is believed that the CBD pathogen is a product of mutation from a mild parasitic form of *Colletotrichum* species originally found in *C. eugenioides* (Nutman and Roberts, 1960a). Pathogen variants may also be generated by exchange of whole nuclei and/or cytoplasm (heterokaryosis). Sreenivasaprasad et al. (1995) distinguished different vegetative compatibility groups among isolates of *C. kahawae* and concluded that there is a teleomorph phase of the pathogen allowing sexual or parasexual recombination which is as yet undiscovered. Mwang'ombe et al. (1992) observed hyphal fusions in *C. kahawae* which might be responsible for exchange of genetic material. Leung et al. (1993) also reported considerable variation in *Rhynchosporium secalis* with apparently no sexual cycle and concluded that *R. secalis* exemplified the long unresolved question in plant pathogenic fungi, that some undetected forms of genetic recombination may be responsible for the high variability exhibited in presumably asexual populations.

Variation can also emerge as a result of migration or gene flow followed by mutation or recombination. The rapid spread of CBD from western Kenya where it was first detected to other parts of the country and eventually to other African coffee growing countries such as Angola, Democratic Republic of Congo, Cameroon, Uganda, Tanzania, Ethiopia and Malawi is a demonstration that there has been considerable migration. This migration followed by mutation or recombination has probably substructured the pathogen population. In our study, only Kenyan isolates were used to avoid risks of introducing new variants and considerable variation for virulence has been observed as well.

Host selection pressure may have only played a limited role in creating pathogen variants because the few CBD resistant varieties have not been widely grown to exert a

high selection pressure on the pathogen. For example, Catimor variety developed in Colombia with resistance to leaf rust is also resistant to CBD but its derivatives are widely grown in Latin America where CBD does not exist. Catimor derives its resistance from Hibrido de Timor, a spontaneous interspecific hybrid combining Robusta and Arabica genomes. Robusta coffee is not a host to *C. kahawae*. Some artificial interspecific hybrids between tetraploid Robusta and Arabica varieties were developed at the Coffee Research Station, Ruiru to introgress CBD resistance from Robusta into Arabica. However, in the course of this programme, CBD resistance was identified within Arabica varieties and therefore Robusta coffee was no longer important as a source of CBD resistance. To improve Robusta, the interspecific hybrids and their backrosses to Arabica varieties have continued to be evaluated for yield, rust resistance, quality and adaptability to the low lying Robusta growing Districts of Busia, Siaya and Kwale where CBD is not a serious problem (Omondi and Owuor, 1992). The Kenyan bred "Ruiru 11" occupies less than 5% of the total Kenyan coffee acreage and has not been propagated in enough quantities to be distributed to other coffee growing countries due to the limitations of hybrid seed production. The highly resistant varieties such as Rume Sudan, Pretoria and Padang used in this study are not commercially grown while K7 is recommended for the low altitude coffee zones where CBD is a minor problem due to its resistance to leaf rust.

Pathogen variation due to the use of Benzimidazole compounds in Kenya has also been reported (Cook and Pereira, 1976; Okioga, 1976; Javed, 1980; King'ori and Masaba, 1991, Mwang'ombe et al., 1992). Benzimidazoles were used to control CBD in the early 1970's but were withdrawn only after 2 years because resistant strains of the fungus developed.

2.3.1. Morphological characteristics.

C. kahawae is generally described as a slow growing fungus with profuse dark grey to greenish olivaceous mycelium in culture (Firman and Waller, 1977; Masaba, 1991; Waller et al., 1993). These typical characteristics are stable in young cultures, but older cultures become more variable, often pale and sterile (Masaba, 1991). On MEA, the fungus has an average growth rate of 2-4 mm/d at 25^{0}C (Waller et al., 1993) and conidia sizes average 13.1 x 3.8 µm, but may sometimes reach upto 20 µm long. They are produced on conidiophores in culture and in acervuli on infected tissue (Wrigley, 1988). Contrary to the previous observations that morphological and cultural characteristics of *C. kahawae* strains are generally similar, Rodrigues Jr. et al. (1991) compared strains from different countries and reported growth rates of 7.6 mm/d (Angolan strain), 6.5 mm/d (Malawian strain) and 1.9 mm/d (Kenyan strain) when grown on MEA at 20^{0}C. The average conidia sizes were 16.3 x 6.8 µm, 16.7 x 7.3 µm and 13.1 x 3.8 µm for Angolan, Malawian and Kenyan strains, respectively. Acervuli were present in high numbers in Angolan and Malawian strains, but absent in the Kenyan strain grown on MEA. Sporulating capacity was highest in Malawian isolates (1.6 x 10^7 conidia/ml), intermediate in Angolan strain (9.5 x 10^5 conidia/ml) and lowest in the Kenyan strain (2.7 x 10^3 conidia/ml). Biratu (1995) also reported a high growth rate of 7.5 mm/d in Ethiopian isolates.

On green berries inoculated with strains of *C. kahawae*, Hocking et al. (1967) reported that the fungus produced perithecia. However, subsequent studies failed to

confirm those results and therefore it is still generally believed that the CBD pathogen has no sexual stage (Firman and Waller, 1977).

2.3.2 Biochemical and molecular characteristics.

One of the difficulties of using morphological traits which are dependent on gene expression in characterizing fungal populations is that significant variation can occur among isolates of the same species under varying environmental conditions (Barrett, 1987). This has resulted in a dynamic system of fungal taxonomy with species and genus designations often existing in a state of flux (von Arx, 1957; Sutton, 1980). Therefore, recently, accurate identification of *C. kahawae* has been aided by both biochemical (protein and isozyme electrophoresis, substrate utilization and vegetative compatibility) and molecular (Restriction Fragment Length Polymorphism, RFLP and Random Amplified Polymorphism DNA, RAPD) methods (Waller et al., 1993; Sreenivasaprasad et al., 1993; Beynon et al., 1995; Biratu, 1995). These methods vary in their sensitivity to detect genetic differences existing in the fungal pathogens. Protein profiles are more reliable for the larger inter-specific differences than in detecting small intra-specific contrasts (Shipton and McDonald, 1970). However, profiles of total proteins are usually complex and often difficult to interpret (Nygaard et al. ,1989). While comparing protein bands of the pathogenic *C. kahawae* and the non-pathogenic *C. gloeosporioides* and *C. acutatum*, Biratu (1995) observed irregular band appearances and concluded that the technique was not sensitive in discriminating variation among the species. However, Blum et al. (1987) found the technique to be highly consistent, provided the conditions of electrophoresis were standardized. It was used successfully

to detect variation in *Ceratocystis fimbriata* f. sp. *platani* (Granata et al., 1992) and *Gaeumannomyces graminis* var. *tritici* and *Phialophora* sp. (Maas et al., 1990).

The development of isozyme electrophoresis provided a powerful tool for studying genetic variation (Market and Möller, 1959). The method derives its useful application from the fact that it can identify different molecular forms of an enzyme with the same substrate specificity that can be traced back to different alleles of a specific gene associated with a trait of interest. Two properties of isozyme electrophoresis that make it a useful technique for studying genetic variation are: 1) alleles of most isozyme markers are codominantly inherited thus allowing all possible genotypes to be distinguished in any segregating population unlike morphological markers which interact in a dominant-recessive manner limiting the number of phenotypes which can be observed (Steven, 1983). 2) isozyme analysis provide information about protein function unlike other electrophoretic procedures which only separate proteins on the basis of their physical properties (Nygaard et al., 1989). In a comparative study between *C. kahawae* on one hand and Mycelia and Acervuli forms of *C. gloeosporioides* on the other hand, Biratu (1995) assayed 7 enzyme systems. Esterase (EST), malate dehydrogenase (MDH) and 6 phosphoglucose dehydrogenase (6-GPD) detected polymorphism while isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP) and glucose phosphate isomerase (GPI) were monomorphic and shikimic acid dehydrogenase (SKDH) lacked activity. Lack of variation for IDH, LAP, GPI and SKDH was believed to be a reflection of the high level of relatedness of *C. kahawae* and *C. gloeosporioides*. Waller et al. (1993) used the property of substrate metabolism to distinguish *C. kahawae* from other *Colletotrichum* species occurring on coffee and to demonstrate the similarity among isolates of *C. kahawae*. The authors reported that none of the isolates of the CBD

pathogen was able to metabolize citrate or tartrate as a sole carbon source and concluded that this property may be directly related to the reduced saprophytic capability of the pathogen. Beynon et al. (1995) performed complimentation tests with mutants of *C. kahawae* in the nitrate assimilation pathway and reported at least five vegetative compatibility groups (VCGs). The authors concluded that since compatibility systems are genetically controlled, there could be a teleormorph phase of the pathogen which has not yet been discovered allowing sexual crossing between different VCGs. Fungal species comprising large numbers of VCGs often have a sexual phase (Leslie, 1993). There is evidence that hyphal fusions occur in *C. kahawae* which could be responsible for exchange of genetic material (Mwang'ombe et al, 1992).

There is greater potential in studying genetic variation using molecular techniques which analyse the DNA directly. For instance, RFLP generates a large number of genetic markers that are often multi-allelic, codominant, lack deleterious or strong epistatic effects and can be determined from any tissue (Michelmore and Hulbert, 1987). The distinctness of *C. kahawae* and its close relationship with *C. gloeosporioides* was confirmed by molecular analysis of RFLP and RAPD profiles (Sreenivasaprasad at al., 1993). Further RFLP analysis distinguished *C. kahawae* from *C. gloeosporioides* and *C. acutatum* but failed to detect polymorphism among isolates of *C. kahawae* (Beynon et al., 1995). RAPD analysis has attracted widespread interest because it can compare individuals at a large number of loci. The method does not discriminate between coding and non coding regions hence the large number of bands which makes it more accurate in estimating natural diversity.

2.4 Control of coffee berry disease.

2.4.1 Host-plant resistance.

The first evidence for the genetic control of resistance was provided by Biffen (1905). The author described the monogenic recessive resistance of wheat (*Triticum aestivum*) to *Puccinia striiformis*. Later a gene-for-gene hypothesis was postulated by Flor (1946) when working on flax resistance to *Melampsora lini*. It was demonstrated that flax is resistant to a particular race of *M. lini* only, if a cultivar carries a dominant resistance gene corresponding to a dominant avirulence gene of the pathogen. This was later found to be the basis for a gene-for-gene relationship, not only for plant-fungus interactions but also for other host-parasite interactions including viruses, bacteria, nematodes and insects (Day, 1974).

In a comparison of host-plant resistance to other methods of controlling pests and diseases, Hogenboom (1993) listed the following advantages: 1) it is a means of controlling diseases and pests that is relatively inexpensive, biologically safe and therefore self-sustaining; 2) it is a cummulative process where resistant cultivars form the basis for later cultivars with multiple resistance ; 3) it can be used as a component of integrated disease and pest management programmes; 4) in some cases like the viral diseases, resistance is the best, if not the only means of control. In most host -pathogen interactions, dominant resistance genes are more frequently encountered probably because they are easily detected in the F1 populations which tends to favour their selection unlike recessive genes which show up later in the breeding programme (de Wit

and van Kan, 1993). Similarly, in most pathogens, dominant avirulence has been described more frequently than recessive avirulence. Resistance can be categorised into two types (van der Plank, 1969): 1) vertical resistance based on a few major genes and 2) horizontal resistance based on minor genes each with a small additive effect.

2.4.2 Resistance of *C. arabica* to coffee berry disease (*C. Kahawae*).

The French Mission coffee, one of the first Arabica coffee types to be grown in Kenya was generally observed to be less susceptible to CBD than the later selections such as SL 28 and SL 34 (van der Vossen et al., 1977). Selection for true resistance within the French Mission coffee was considered unrewarding because of its apparent lack of genetic variability. *C. arabica* germplasm planted at the Coffee Research Station (CRS), Ruiru, comprising of 250 botanical and agricultural varieties, introductions from Ethiopia, the primary centre of genetic diversity of *C. arabica* and a number of compact cultivars introduced from South and Central America such as Caturra, Catuai, San Bernardo, Turrialba compact and Catimor provided the source of genetic variation on which to select for CBD resistance. A number of these accessions showed a high degree of field resistance to CBD and leaf rust, but none of them possessed all the desired traits of disease resistance, high yield, good quality and growth habit (van der Vossen and Walyaro, 1981).

The biggest contribution to the success of the breeding programme in Kenya was the development of a pre-selection test for CBD resistance on 5-6 weeks old hypocotyl seedlings (van der Vossen et al., 1977). This tremendously accelerated the breeding work which could have been otherwise too long, if the disease assessment was done

only on mature coffee trees. The hypocotyl test was also found to be highly correlated with field resistance of mature trees. Inheritance studies using a diallel analysis of 11 *C. arabica* varieties varying in CBD resistance revealed three genes of resistance on separate loci (van der Vossen and Walyaro, 1980). The highly resistant variety Rume Sudan carries the dominant R- and the recessive resistance k- genes. The R- locus has multiple alleles with R_1R_1 alleles in Rume Sudan and R_2R_2 alleles in Pretoria, which also carries the k- gene. The moderately resistant variety K7 carries only the recessive k- gene. The variety Hibrido de Timor and its hybrid derivative Catimor carries one gene for CBD resistance on the T-locus with intermediate gene action. However, in the genetically heterogenous natural populations of Arabica coffee from Ethiopia, van der Graaff (1978) reported continuous variation for resistance to CBD and concluded that the resistance was polygenically controlled. Waller, (1971) and Cook, (1975) observed that such variation could be caused by various macro- and micro- climatic factors which bias genetic interpretations of inheritance based on field observations alone. van der Graaff (1982) further criticized the use of mean grade of seedling distribution instead of modal grade as selection criteria for resistance. However, Owuor and Agwanda (1990) compared the two criteria on the basis of their sensitivity, severity and correlation and concluded that the two statistics yielded similar results and there were no practical gains in adopting the modal grade over the mean grade criterion.The mechanism of resistance to CBD was found to be based on formation of cork barriers which were reported to be stable against changes in pathogen populations (Masaba and van der Vossen, 1982). High levels of caffein content has also been associated with resistance to CBD (Biratu et al. 1995).

The breeding programme was aimed at combining disease resistance, high yield, fine quality and compact growth ammenable to high density planting. It was initiated at the Coffee Research Station, Ruiru, Kenya in 1971. To achieve this objective, a hybridization programme was designed to produce single crosses between the progenitors of CBD resistance and some of the Kenyan selections such as SL 28 , SL34 and SL 4 including introductions from other programmes such as N39 from Tanzania and Bourbon. The single crosses were combined into multiple crosses to have resistance from different sources in a single plant. This was followed by backcrossing to restore the yield and quality of the traditional varieties. The outstanding backcross progenies are being used as the male parents in the production of hybrid variety Ruiru 11 released in 1985. The female parents are advanced generations, F_3, F_4, and F_5 of Catimor variety introduced from Colombia. Catimor, apart from having CBD resistance, (although narrow based), imparts compact growth character in Ruiru 11 and special coffee leaf-rust resistance genes SH^6 and SH^9 that are effective against all known races of *Hemileia vastatrix*. Ruiru 11 is therefore a genetically heterogeneous population of hybrids that are morphologically similar, especially for compact growth and disease resistance. Further improvement of the variety has focused on selection for hybrids within the population with good bean and cup quality comparable to the traditional varieties. Omondi (1994) reported that resistance to CBD in the hybrid population exhibits low heritability and is predominantly controlled by non-additive genetic effects (dominance and epistasis). Since some strains of *C. kahawae* have been isolated from Ruiru 11, investigation into the possible physiological specialization of the fungus and pyramiding of resistance genes is necessary.

2.4.3 Chemical Control of CBD.

Rayner (1952) observed that some degree of control of CBD in Kenya could be achieved by spraying fungicides. Several investigations were carried out not only to screen fungicides which gave good results, but also to relate fungicide application with major epidemiological factors such as weather conditions and inoculum sources (Bock 1963, Nutman and Roberts 1960, a & b). Originally, it was believed that inoculum from the bark was the key factor in epidemiology of CBD. This led to the recommendation of early season spray programmes aimed at reducing the amount of inoculum load produced by the bark. In the subsequent years, early season sprays proved partially or totally ineffective against CBD and in some cases increased the disease level (Wallis and Firman, 1967). In a reassessment of the work, Gibbs (1969) found that the bark was an important primary source of inoculum in the absence of diseased berries, but as soon as diseased berries appeared, they were of greater significance in spreading the disease than the primary infection. Griffiths et al. (1971), therefore recommended that, if the climatic and cropping conditions are favourable for CBD infection, fungicide sprays should be aimed at continuously protecting the developing crop. This intensive spray programme was found to be very expensive accounting for upto 30% of the total cost of production in the Estate sector (Nyoro and Sprey 1986). Majority of the small scale farmers being unable to carry out the recommended intensive spray programmes ended up applying one or two fungicide sprays per year. Such occasional sprays were found to induce higher levels of CBD than what would occur in their total absence (Griffiths, 1972). In addition, the CBD fungus was reported to have developed resistance to some of the recommended systemic fungicides only after 2-3 years of continuous spraying

(Cook, 1975; Okioga, 1976). These strains are still persisting in the pathogen population despite the chemicals having been withdrawn almost immediately the phenomenon was detected (King'ori and Masaba, 1991; Mwang'ombe et al., 1992).

2.4.4 Biological Control of CBD.

It has been observed that CBD is less severe on coffee farms which have never been sprayed with fungicides (Masaba, 1991). Inadequate fungicide sprays or improper timing of sprays also result in higher levels of disease than where the sprays have not been applied (Bock, 1963). An investigation to detect interactions between the pathogen, disease and saprophytic microflora in vitro, confirmed that fungicides used to control CBD have substantial quantitative and qualitative effect on the non-target microflora, some of which are antagonistic to the CBD pathogen (Masaba, 1991). The author observed that *Fusarium stilboides,* and to some extent *Cladosporium tenuissima*, *Penicillium glaborum*, *Epicoccum purpurescence*, *Nigrospora sphaerica* and *Alternaria alternata* showed promising inhibitory effects with *C. kahawae* on detarched berries. It was indicated that if biological control of CBD in the field is investigated further, it may be possible to integrate it with other methods of CBD control such as host plant resistance.

CHAPTER 3.

MATERIALS AND METHODS.

3.1 Sampling isolates of *C. kahawae*.

Green infected berries were obtained from 10 locations in the coffee growing districts of Kenya namely, Gatanga and Coffee Research Station, Jacaranda in Thika District, Karatina in Nyeri District, Kiriaini and Kangema in Muranga District, Yara Estate in Kiambu District, Kitale in Trans-Nzoia District, Coffee Research Substation, Koru in Kericho District, Kisii Coffee Research Substation, in Kisii District and Bahati in Nakuru District. The locations were representative of the agroecological zones where Arabica coffee is grown in Kenya (Figure 3). A list of the isolates, the distribution and altitudes of the sampling sites (Jaetzold and Schmidt 1983 (a) and (b)) are presented in Table 1. At each location, 9 diseased berries were randomly sampled from which 9 single conidia isolates were derived (one isolate per berry) making a total of 90 isolates for the 10 locations. In locations where both resistant and susceptible varieties were grown, a larger proportion of the berries were obtained from the resistant varieties to increase the chances of obtaining different pathotypes. The sample size was determined by the computer aided design of experiments and modelling (CADEMO) statistical programme (BIORAT, GmbH, Rostock) at the following specified precision requirements: α = 5%, β = 20% and d = 25% where α = risk of the first kind, β = risk of the second kind and d = minimum difference of practical interest. CADEMO was also used in all experiments to determine the minimum number of replications required.

Fig.3. The main coffee growing areas in Kenya and localities from which test isolates of *C. kahawae* were obtained.

ble 1. A list of *C. kahawae* isolates, spatial distribution and altitudes of the sampling locations, parenthesis) and phenotypes of the host variety.

olates	Sampling location	Host variety phenotype	Isolates	Sampling location	Host variety phenotype
1	Gatanga -Thika District	Resistant	R6.1	Kitale -Trans-Nzoia District	Resistant
2	"(0.92°S, 36.93°E,1670 m)	"	R6.2	"(0.99°N, 34.99°E, 1890 m)	"
3	"	"	R6.3	"	"
4	"	"	R6.4	"	"
5	"	"	R6.5	"	"
6	"	"	R6.6	"	"
7	"	Susceptible	R6.8	"	Susceptible
8	"	"	R6.9	"	"
9	Karatina-Nyeri District	Resistant	S9.1	Koru-Kericho District	"
1	"(0.49°S, 37.11°E, 1981 m)	"	S9.2	"(0.16°S, 35.22°E, 1615 m)	"
2	"	"	S9.3	"	"
3	"	"	S9.4	"	"
4	"	"	S9.5	"	"
5	"	"	S9.6	"	"
6	"	"	S9.7	"	"
7	"	Susceptible	S9.8	"	"
8	"	"	S9.9	"	"
9	Kiriaini-Muranga District	Resistant	R10.1	Kisii-Kisii District	Resistant
1	"(0.67°S, 36.86°E,1760 m)	"	R10.2	"(0.68°S, 34.76°E, 1680 m)	"
2	"	"	R10.3	"	"
3	"	"	R10.4	"	"
4	"	"	R10.5	"	"
5	"	"	R10.6	"	"
6	"	"	R10.7	"	"
7	"	Susceptible	R10.8	"	"
8	"	"	R10.9	"	"
9	Kangema-Muranga District	Resistant	S11.1	Bahati-Nakuru District	Susceptible
1	"(0.68°S, 36.97°E, 1700 m)	"	S11.2	"(0°, 36.13°E, 1830 m)	"
2	"	"	S11.3	"	"
3	"	"	S11.4	"	"
4	"	"	S11.5	"	"
5	"	"	S11.6	"	"
6	"	"	S11.7	"	"
7	"	Susceptible	S11.8	"	"
8	"	"	S11.9	"	"
9	Yara Estate-Kiambu District	"	S12.1	Jacaranda-Thika District	"
0	"(1.12°S, 36.77°E, 1767 m)	"	S12.2	"(1.06°S, 36.45°E, 1603 m)	"
1	"	"	S12.3	"	"
2	"	"	S12.4	"	"
3	"	"	S12.5	"	"
4	"	"	S12.6	"	"
5	"	"	S12.7	"	"
6	"	"	S12.8	"	"
7	"	"	S12.9	"	"

2 Isolation of *C. kahawae.*

To obtain monoconidial isolates, berries were washed individually in running stilled water and incubated at 24°C for 48 hours in petri dishes containing moist llulose wadding to promote sporulation. Pure cultures from each berry were obtained inoculating malt extract agar (MEA) and subsequently sub-culturing from regions here culture characteristics corresponded with those documented for *C. kahawae*. The sulting cultures were suspended in distilled water and standardized to a concentration 2 x 10^6 conidia/ml by haemocytometer counting and serial dilution. The suspensions re spread on pure agar in thin lines for conidia to germinate. After 24 hours, single rminating conidia were re-isolated and subcultured on 3.4% MEA (Oxoid) containing 04% streptomycin. After growth for 7 days at room temperature, the isolates were ored in 3 replicates at 4°C until required.

3 Virulence tests on hypocotyl seedlings of *C. arabica* varieties.

virulence tests were performed to determine the nature of host x isolate teraction and the proportion of pathogen variation attributable to physiological ecialization and differences in aggressiveness. Eleven varieties of *C. arabica*, which cluded Rume Sudan, Pretoria, Hibrido de Timor, K7, Padang, SL28, SL34, Caturra, recta, Mokka and Laurina were selfed to generate experimental seeds. Some haracteristics of the varieties as described by Millot (1969) and Walyaro (1983) are resented in Table 2. For each of the 90 isolates, 100 seeds of each variety were sown 3 replications in moist sterilized sand in plastic boxes with closely fitting transparent

Table 2. Some characteristics of *C. arabica* varieties used for virulence tests.

Variety	Description
Rume Sudan	Introduced into Kenya from Boma plateau in Sudan. Resistant to CBD at the R- and k- loci.
Pretoria	Ex-Guatemala variety introduced to Kenya from Lyamungu, Tanzania. Resistant to CBD at the R- and k- loci.
Hibrido de Timor	Introduced to Kenya from the Rust Research Centre (CIFC) at Oeiras, Portugal in 1960. It is a spontaneous hybrid combining CBD resistance at the T- locus and rust resistance that is effective against most races.
K7	A selection from the French Mission coffee used as a commercial cultivar in Kenya. It is resistant to CBD at the k- locus and to the most prevelant race II of *Hemileia vastatrix*
Padang	Introduced from Guatemala and shows partial resistance to CBD.
SL28	A single tree selection from the former Scot Laboratories, Nairobi. A commercial variety with fine quality but susceptible to both CBD and Leaf rust.
SL34	Another selection from the former Scot Laboratories used as a commercial variety in Kenya. It has excellent quality but susceptible to CBD and leaf rust.
Caturra	Introduced into Kenya from Kivu, Zaire. It is a compact variety originally from Brazil.
Erecta	Ex-Puerto Rico variety with erect branching habit introduced to Kenya from Lyamungu, Tanzania.
Mokka	Of Arabian origin. A small conical shaped tree with small leaves and small round beans of excellent liqour.
Laurina	Ex-Guatemala, a conical shaped tree, with long fruits having a thick pericarp and low caffein content.

Source: Millot (1969) and Walyaro (1983)

lids. The boxes were kept at room temperature in the laboratory and watered regularly at intervals of 2 days. The seedlings were ready for inoculation after six weeks when their hypocotyl stems were 3-5 cm long and the cotyledons still enclosed in the testae. Inoculation procedures described by van der Vossen et al. (1977) were followed. Conidia suspensions were prepared from 10 days old monoconidial cultures by flooding the petri dishes with 5 ml of distilled water and scrapping the conidia bearing mycelia off the agar medium while gently mixing. The suspension was filtered using a Hessian cloth and standardized to 2×10^6 conidia/ml by haemocytometer counting followed by serial dilution. Viability of conidia was tested by placing a drop of inoculum on a cavity slide and counting from 100 conidia, the proportion that germinated after 4 hours. Inocula with conidial germination in excess of 80% were used for virulence tests.

The hypocotyl seedlings were sprayed with enough inoculum to cover the whole surface of each seedling using a hand sprayer fitted with an atomizing nozzle. For each replicate, control plants were sprayed with distilled water. Infection was induced by maintaining the lids of the boxes closed to increase humidity and covering the boxes with black polythene sheets to provide darkness. A double inoculation was performed after 48 hours. The same conditions were maintained for a period of 4 days followed by an incubation period at a lower temperature of 19-20°C with polythene sheets and lids removed from the boxes to allow for normal humidity and light. The boxes were arranged in the incubation room in a completely randomized design. At the end of the incubation period which lasted about 3 weeks and determined by the full expression of the disease on susceptible SL28 variety, the seedlings were individually scored for disease symptoms developed on the hypocotyl stem using the scale of van der Vossen et al.

(1977). The disease scale comprised of 12 grades based on symptoms presented in Table 3. A mean grade of infection (G) was computed for each box as follows:

$$G = 1/N \sum_{i=1}^{12} i\, n_i$$

Where, i is the disease grade, n_i is number of seedlings in grade i and N is the total number of seedlings scored. The mean grade data was used to perform an analysis of variance according to the following random effects model (Steel and Torrie, 1981):

$Y_{ijk} = \mu + a_i + b_j + ab_{ij} + e_{i(jk)}$ where:

Y_{ijk} = mean grade of variety i, isolate j and replication k.

μ = the overall mean,

a_i = the effect of variety i,

b_j = the effect of isolate j

ab_{ij} = the interaction effect of variety i and isolate j,

$e_{i(jk)}$ = the experimental error.

The data was analysed as an 11 x 90 factorial design using the MSTAT version 4.0, statistical programme (Michigan State University). The form of analysis of variance is presented in Table 4. The relative proportion of each source of variation was computed according to the method of Roumen (1993). The variety x isolate interaction component was considered an important indicator for the presence/absence of races.

Table 3. Coffee berry disease scoring scale.

Grade	Symptom	Grade	Symptom
1	No visible symptoms.	7	Narrow black lesions, some more than 1 cm long.
2	A few scab lesions.	8	Black lesions becoming wider and starting to coalesce.
3	Small scab or tiny brown lesions.	9	Large coalescing black lesions but not complete girdling.
4	Scab and brown lesions.	10	Large coalescing black lesions and complete girdling of the stem.
5	Scab and brown lesions and a few small black lesions.	11	Most of the stem affected, more than 1/3 of the stem shrivelled and seedling dead.
6	Brown and narrow black lesions.	12	Whole stem affected and shrivelled, seedling dead.

Source: van der Vossen et al. (1977)

Table 4. Analysis of variance for mean grade of infection on varieties of *C. arabica* inoculated with isolates of *C. kahawae*.

Source	df*	MS	Relativé proportion of total variation**
Varieties	g-1	M4	SSV/SST
Isolates	s-1	M3	SSS/SST
Varieties x Isolates	(g-1)(s-1)	M2	SS(V x S)/SST
Error	gs(r-1)	M1	SSE/SST

* g = No. of varieties = 11
s = No. of isolates = 90
r = No. of replicates = 3

** SSV = Sums of squares of variety effects.
SSS = Sums of squares of isolate effects.
SS(V x S) = Sums of squares of variety x isolate effects.
SSE = Sums of squares of experimental error.
SST = Total sums of squares.

The relative contribution of individual varieties (W_i) and isolates (W_j) to the total interaction component were computed using a method adopted from ecovalence analysis of Wricke (1962) cited by Baradat (1989) (used in genotype x environment interaction analyses to partition the contribution of individual genotypes and environments) as follows:

$$W_i = r\sum_{j=1}^{B}(\bar{Y}_{ij.} - \bar{Y}_{i..} - \bar{Y}_{.j.} + \mu) \text{ and}$$

$$W_j = r\sum_{i=1}^{A}(\bar{Y}_{ij.} - \bar{Y}_{j.} - \bar{Y}_{i..} + \mu) \text{ where,}$$

A = number of varieties,

B = number of isolates,

r = number of replication

$\bar{Y}_{ij.}$ = mean of variety i x isolate j combination across all replications,

$\bar{Y}_{i..}$ = mean of variety i across all isolates and replications,

$\bar{Y}_{.j.}$ = mean of isolate j across all varieties and replications

μ = overall mean.

The interaction components due to variety i (W'_i) and isolate j (W'_j) were respectively computed as follows:

$$W'_i = 100\ W_i / \sum_{i=1}^{A} W_i$$

$$W'_j = 100\ W_j / \sum_{J=1}^{B} W_j$$

3.4 Analysis of total proteins, isozymes and Random Amplified Polymorphic DNA.

Profiles of total buffer soluble proteins, isozymes and Random Amplified Polymorphic DNA (RAPD) were analysed not only to compare the methods as criteria for delimiting *Colletotrichum* species and characterizing isolates of *C. kahawae* but also to screen for markers closely associated with virulence and Benomyl tolerance and to estimate the level of genetic diversity among isolates of *C. kahawae* and between the pathogenic and non-pathogenic species.

3.4.1 The SDS-polyacrylamide gel electrophoresis of total buffer soluble proteins.

Twelve isolates (R1.6, S12.8, S9.1, R2.3, R3.4, R10.9, R4.5, S2.8, R6.8, S9.9, S11.9 and S12.5) varying in virulence, two Benomyl tolerant strains of *C. kahawae* and two non-pathogenic *Colletrotrichum* spp. (*C. gloeosporioides* and *C. acutatum*) were

plated on MEA. The isolates were designated according to the phenotype of the host variety, where, R-isolates were from resistant varieties and S-isolates were from susceptible varieties. This was followed by a numerical code for the locality of origin separated from the serial number of the isolate by a point. Two mycelial plugs obtained from the actively growing margins of the plates were used to inoculate 100 ml of nutrient broth in 250 ml flasks. The nutrient broth consisted of 0.1% meat extract, 0.2% yeast extract, 0.5% peptone and 1% glucose (Maas et al., 1990). Each isolate was inoculated in 3 replicates and incubated at room temperature in a rotary shaker for 10 days. The resulting mycelial mats were collected on a mesh sieve and washed in deionized distilled water. The inoculum plug was removed and the mats were blotted dry between several layers of towelling paper. The dry mycelia were lyophilized for 2 days at -20°C and stored at the same temperature until required .

Seventy mg of mycelia were ground with a pestle in a mortar containing 1.0 g pure sea sand to ease maceration. The powder was suspended in 1.4 ml of extraction buffer consisting of 1.06 M Tris, 10% (v/v) glycerol and 5% (v/v) 2-mercaptoethanol forming a slurry. The slurry was transferred to reaction tubes and emersed in a boiling waterbath for 3 minutes to dissociate the proteins. The tubes were centrifuged for 15 minutes at 12000 g. Two hundred µl of the supernatant was transferred to new reaction tubes containing 10 µl of 0.1% (w/v) bromophenol blue indicater.

Buffer soluble proteins were separated by a 10% sodium dedocyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). For gel preparation, glass plates measuring 16 x 18 cm^2 were washed with detergents, 70% ethanol, rinsed with deionized distilled water and then clean dried. Placing one of the glass plates on a clean dry surface, plastic spacers (0.75 mm) were cleaned, dried and fixed along the edge of

the laid glass plate. A second glass plate was placed on the spacers to form a sandwich. The sandwich was tightened with improved clamps on a gel casting stand (LKB). The whole set was transferred to a freezer at -20°C for 20 minutes. Electrophoresis solutions were prepared according to the recommendations of Laemli (1970). All solutions and buffers were prepared with deionized distilled water as follows:

- Separation gel solution - Acrylamide (30.0g) and methylene bis acrylamide (0.8g) were added to 100 ml of distilled water. The solution was filtered and stored at 4°C.
- Separation gel buffer - 3 M Tris adjusted to pH 8.8 with conc. HCl.
- Stacking gel solution - Acrylamide (16.8 g) and methylene bis acrylamide (4.2 g) added to 100 ml distilled water.
- Stacking gel buffer - 0.5 M Tris adjusted to pH 6.8 with conc. HCl.
- Ammonium peroxide solution - 20% (w/v) ammonium peroxide solution (APS) was prepared fresh for every experiment.
- Sodium dedocyl sulphate - 10% (w/v) sodium dedocyl sulphate (SDS) was prepared and stored at room temperature.

A 10% separating gel was prepared by mixing 12 ml separating gel stock solution, 3.84 ml separating gel buffer, 4 ml of glycerol and 17.4 ml double distilled water. Dissolved air in the solution was removed by degassing in a lyophilizer (LYOVAC GT2, LEYBOLD-HERAEUS) for 10 minutes. To the degassed solution, 144 µl, SDS, 20 µl, TEMED and 20 µl, APS was added and without delay pipetted into the 2 cold glass sandwiches. To avoid drying up of the gels and disturbance of the acrylamide solution, a layer of about 2 mm butanol was slowly added to the top of the gels and left for an hour. The butanol layer was then removed and the gels washed twice with 3 M Tris buffer,

refilled with the same buffer and left overnight at room temperature to polymerise. The next day, the overlaying buffer was removed and the gels washed twice with deionized distilled water. Sample well forming combs with 10 or 20 teeth were placed on every sandwich. A 4% stacking gel was pipetted on top of each sandwich. The gel was prepared from 2.5 ml stacking gel solution, 1.25 ml stacking gel buffer, 2.3 ml glycerol, 3.8 ml deionized distilled water, 100 µl SDS, 20 µl TEMED and 20 µl APS. Care was taken to avoid the formation of air bubbles by slowly adding the solutions on one corner. After an hour of polymerisation, the combs were removed, the wells washed with electrode buffer and the gel was ready for electrophoresis. The electrode buffer was prepared from 0.03 M Tris, 0.2 M glycine, 0.1% SDS and pH adjusted to 8.6. Each well was filled with the electrode buffer and 20 µl denatured protein samples. Marker extracts (5 µl) instead of protein samples were loaded on the first and last wells. The marker proteins used were albumine, bovine serum (MW 67000); ovalbumin, chicken egg (MW 45000); glyceraldehyde-3-phosphate dehydrogenase, rabbit muscle (MW 36000); bovine erythrocytes (MW 29000); trypsinogen, bovine pancrease (MW 24000); trypsin inhibitor, soybean (MW 20,000); α-lactalbumin, bovine milk (MW 14000) and aprotinin, bovine lung (MW 6500). The vertical electrophoresis chamber was filled with electrode buffer upto the 3.5 l mark. For cooling, water was circulated at 10°C through the unit during the run. The cathode buffer container was fixed to the glass plates and the rubber gaskets greased with silicon to avoid leakage. The unit was transferred to the electrophoresis chamber, filled with 0.5 l of electrode buffer and connected to the voltage electrode supplied by LKB 2197 power supply. Electrophoresis was run with a constant current of 1000v and 60 mA for two gels or 1000 v and 30 mA for a single gel. Each run

took 120-150 minutes for the bromophenol blue dye to reach the 1 cm mark above the bottom of the gel.

After electrophoresis, protein bands were made visible by using silver staining method of Blum et al. (1987) outlined in Table 5. Before drying, gels were shaken in a solution of 30% (v/v) methanol for 30 minutes then transferred to a solution of 3% (v/v) glycerol for another 30 minutes. Drying was done under a vacuum between cellophane sheets in a gel drier at 80°C for 1 hour. The method described by Cole et al., (1991) was adopted for statistical analysis. Protein bands were recorded as presence/absence matrix, with a column for each isolate and a row for each band position.

In order to compare protein bands from different gels, one arbitrarily chosen isolate, S11.9 was used as a reference and was included in all electrophoretic runs. The bands were assigned a value corresponding to their mobilities (Rf) relative to the distinct S11.9 band of molecular weight 49 KDa that also commonly occurred in other *C. kahawae* isolates. Bands with similar Rf values were considered the same proteins and isolates were assigned a value "1" when present or "0" when absent. Statistical analysis of protein electrophoresis data were performed using the SPSS version 6.1 statistical package. For each pair of isolates, the simple matching coefficient, (S), was computed according to Maclean et al. (1995) as follows: $S = 2\, m_{xy} / [m_x + m_y]$, where,

m_{xy} = the number of shared bands,

m_x = the number of bands in isolate x,

m_y = the number of bands in isolate y.

The dissimilarity coefficient (D) was obtained by subtracting the similarity coefficient from unity. The dissimilarity matrix was used to construct a Euclidian Distance Matrix as a

Table 5. The Procedure for silver staining of proteins

Steps	Solutions[a]	Time of treatments[b]
1. Fix	50% MeOH, 12% AcOH, 0.5 ml 37% HCOH/l	1 hour
2. Wash	50% EtOH	3 x 20 minutes
3. Pretreat	$Na_2S_2O_3.5H_2O$(0.2g/l)	1 minute [c]
4. Rinse	H_2O	3 x 20 seconds[c]
5. Impregnate	$AgNO_3$ (2 g/l)	
	0.75 ml 37% HCOH/l	20 minutes
6. Rinse	H_20	2 x 20 seconds[c]
7. Develop	Na_2CO_3 (60g/l):	
	0.5 ml 37% HCOH/l $Na_2S_2O_3.5H2O$ (4mg/l)	10 minutes
8. Wash	H_2O	2 x 2 minutes
9. Stop	50% MeOH: 12% AcOH	10 minutes
10. Wash	50% MeOH	20 minutes[d]

(a) MeOH = Methanol, AcOH = Glacial acetic acid, HCOH = Formaldehyde, EtOH = Ethanol, $Na_2S_2O_3.5H_2O$ = Sodium thiosulphate, $AgNO_3$ = Silver nitrate and Na_2CO_3 = Sodium Carbonate.

(b) Steps 1-10 were carried out on a shaker at room temperature (20-25°C).

(c) The times indicated were observed exactly to ensure reproducibility of image development.

(d) After step 10, the gels were transferred to 4°C for storage and subsequent drying

Source: Blum et al. (1987)

measure of genetic distance between pairs of isolates. The Euclidian distance between two individuals is equivalent to their total number of observed differences. A dendrogram based on the Euclidian Distance Matrix was constructed by the Unweighted Paired Group Method of Arithmetic Avarages (UPGMA) of Sneath and Sokal (1973). UPGMA forms groups in a stepwise manner by pairing similar isolates according to the magnitude of their observed distances.

3.4.2 Isozyme electrophoresis.

A 10% polyacrylamide gel without SDS was used to assay 12 enzyme systems including esterase (EST EC 3.1.1.1), malate dehydrogenase (MDH, EC 1.1.1.37), glucose-6-phosphate dehydrogenase (GPDH, EC 1.1.1.49), isocitric dehydrogenase (IDH, EC 1.1.1.42), lactase dehydrogenase (LDH, EC 1.1.1.27), malic enzyme (ME, EC 1.1.1.40), adenylate kinase (AK, EC 2.7.4.3), hexokinase (HEX, EC 2.7.1.1), phosphoglucose isomerase (PGI, EC 5.3.1.9), mannose-6-phosphate isomerase (MPI, EC 5.3.1.8), fructose-1-6-diphosphate (FDP, EC 3.1.3.11) and hexose phosphate isomerase (PHI, EC 5.3.1.9.). Isolates, culture methods and lyophilization of mycelia were similar as in protein electrophoresis. Enzymes were extracted from 0.035 g lyophilized mycelia in 3 ml of extraction buffer (0.05 M sodium phosphate, pH 7.2, 10% (w/v) polypyrolidone (PVP), 20% (v/v) glycerol, 0.1% (w/v) egg albumin powder, 0.05% (v/v) triton x 100 and 14 mM mercaptoethanol). The extracts were centrifuged at 12000 g for 15 minutes at 0°C. The supernatant (300 µl) was recovered, mixed with 10 µl of 0.5% (w/v) bromophenol blue and stored at -20°C in aliquotes of 40 µl until required. Each

aliquot was loaded in sample holding wells and electrophoresis was run with voltage and current similar to protein electrophoresis until the bromophenol blue dye migrated to the 1 cm mark above the bottom of the gel. The enzyme staining methodologies were as described by Oudemans and Coffey (1991) and are outlined in Table 6. The incubation of all the enzymes took place at 37°C in the dark until the bands could be observed. The genetic basis of isozyme banding patterns could not be infered from this study hence each band was considered a different locus and loci were denoted with enzyme abbreviation and a prefix B for band followed by the relative migration distance (Welz et al. 1994). Based on differences in band mobility, isozyme phenotypes or electrophoretic types (ETs) were distinguished. For every ET, the frequency of isolates were recorded.

Table 6. Enzyme staining procedure.

Enzyme	Staining buffer (pH, Conc.)	Substrate (mg)	Salt (mg)	Cofactor (mg)*	Linking enzyme (units)§	Dye (mg)¶	Catalyst (mg)#	Other+
EST	Phosphate sol'n A‡ 50 ml	0.1%α-naphtyl-acetate sol'n	-	-	-	1% FBRR	-	-
	Phosphate sol'n B‡ 10 ml	(in acetone) 2 ml						
GPDH	0.1 M Tris (pH 8)	Glucose-6-phosphate	$MgCl_2$	NADP	-	NBT	PMS	-
	50 ml	50 mg	200 mg	20 mg		5 mg	1 mg	
IDH	0.1 M Tris (pH 8)	Isocitric acid	$MgCl_2$	NADP	-	NBT	PMS	-
	45 ml	25 mg	100 mg	20 mg		5 mg	1 mg	
LDH	0.03 M Tris (pH 8)	1 M Lithium lactate	-	NAD	-	MTT	PMS	-
	70 ml	5 ml		20 mg		6 mg	1 mg	
MDH	0.1 M Tris (pH 8)	Malic acid	-	NAD	-	NBT	PMS	-
	45 ml	700 mg		20 mg		5 mg	1 mg	
ME	0.05 M Tris (pH 8)	Malic acid	-	NADP	-	NBT	PMS	-
	80 ml	1400 mg		20 mg		5 mg	1 mg	
AK	0.07M Tris (pH 7)	Glucose	$MgCl_2$	NADP	G6PDH	NBT	PMS	HEX,
	45 ml	50 mg	200 mg	30 mg	20 units	5 mg	1 mg	50units & ADP 150 mg
HEX	0.1 M Tris (pH 8)	Glucose	$MgCl_2$	NADP	G6PDH	NBT	PMS	ATP
	45 ml	2000 mg	200 mg	20 mg	10 units	5 mg	1 mg	250 mg
PGI	0.15 M Tris (pH 8)	Fructose-6-phosphate	$MgCl_2$	NADP	G6PDH	NBT	PMS	-
	45 ml	20 mg	200 mg	100 mg	10 units	5 mg	1 mg	
MPI	0.1 M Tris (pH 7)	Mannose-6-phosphate	$MgCl_2$	NADP	G6PDH	MTT	PMS	PGI
	45 ml	10 mg	100 mg	10 mg	10 units	5 mg	1 mg	20 units
FDP	0.1M Tris (pH 7)	Fructose-1,6-diphosphate	$MgSO_4$	NADP	G6PDH	NBT	PMS	PGI
	50 ml	30 mg	250 mg	20 mg	20 units	5 mg	1 mg	20 units
PHI**	0.1 M Tris/HCl (pH8)	Fructose-6-phosphate	$MgCl_2$	NADP	G6PDH	NBT	PMS	-
	10 ml	40 mg	30 mg	15 mg	60 units	10 mg	4 mg	

* NADP = β-nicotinamide adenine dinucleotide phosphate; NAD = β-nicotinamide adenine dinucleotide.

§ G6PDH = glucose-6-phosphate dehydrogenase.

¶ FBRR = Fast blue RR salt; MTT = 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NBT = nitro blue tetrazolium.

\# PMS = phenazine methane sulphonate.

\+ Additional linking enzymes include hexokinase (HEX) and phosphoglucose isomerase (PGI). ADP = adenosine 5'-diphosphate, ATP = adenosine 5'-triphosphate.

** PHI was stained as an agar overlay: 40 mg of agar was dissolved in 10 ml of the stain solution, poured over the gel and allowed to solidify.

‡ Phospate solution A = 27.8 g of monobasic sodium phoshate/litre H_2O; phosphate solution B = 53.6 g of dibasic sodium phosphate/litre H_2O.

Source: Oudeman and Coffey (1991)

3.4.3 RAPD analysis.

DNA was extracted from lyophilized mycelia using the CTAB (cetyltrimethylammonium bromide) protocol of Möller et al., (1992). The mycelia was ground in sterile sea sand and suspended in 700 µl warm TES (100 mM Tris, pH 8.0, 10 mM EDTA, 2 % SDS) in a 2 ml reaction tube. Associated proteins were digested by adding 4.5 µl proteinase K and incubated at 55-60°C for 1 hour with slight shaking several times at intervals of 10 minutes. The salt concentration was raised by adding 1.96 µl of 5 M NaCl and 91 µl of 10% warm CTAB then incubated at 65°C for 10 minutes. While mixing gently, 700 µl SEVAG (chloroform : isoamylalcohol, 24:1 v/v) was added and incubated at 0°C, then centrifuged for 10 min at 4°C. The supernatant (660 µl) was transferred to 1.5 ml reaction tube and 255 µl of 5 M NH_4AC added and mixed gently before incubating in ice for 30 min and centrifuging at 4°C. The supernatant (850 µl) was again transferred to a fresh 2 ml reaction tube and 800 µl isopropanol was added and carefully mixed to precipitate the DNA followed by immediate centrifugation for 5 min. The DNA pellets were washed twice in cold 70% ethanol by shortly centrifuging and dried in a speed vacuum for 10 minutes. Dry pellets were resolubilized in 500 µl TE (Tris-EDTA buffer), shortly warmed in a 50°C waterbath (maximum 30 minutes) and centrifuged at room temperature. Five µl DNAse-free RNAse was added and incubated at 37°C for 30-60 minutes. The DNA was precipitated in 400 µl isopropanol, centrifuged for 3 minutes at room temperature and the pellets resolubilized in 100 µl warm TE (50° C). DNA concentration in the preparation was determined by taking photometric readings at $OD_{260/280}$ (assuming 1 OD_{260} = 50 mg DNA / ml). Equal amounts of DNA

from each sample were run on 0.8% agarose gel and stained with ethidium bromide to detect the presence of proteins (phenolic compounds) and DNAse. Pure samples were distinguished from contaminated samples by their equal staining intensities. When incubated with a restriction enzyme ECOR I, pure DNA without DNAse produced a smear in the entire lane. The polymerase chain reaction (PCR) was conducted in 25 µl volume containing a final concentration of 10 mM Tris, 50 M KCl, 2.5 mM $MgCl_2$, 0.01% gelatine, 100 mM dNTP-mix (Pharmacia), 0.2 µM decanucleotide primer (University of British Columbia, Vancouver, Canada), 0.75 units Taq-polymerase (Pharmacia), 1 µl template DNA and overlaid with a drop of mineral oil. Reactions were performed in a programmable thermocycler (MJ Research, Inc. Watertown MA, model PTC-100-96). Amplification conditions were set with an initial denaturation step at 94°C for 5 minutes followed by 45 cycles of annealing for 1 min. at 35°C, extension for 3 min. at 72°C and denaturation for 1 min. at 94°C. The final step consisted of 1 minute at annealing temperature followed by 6 minutes at 72°C to produce fully double stranded fragments. The PCR products were separated on a 1.5% agarose gel in TAE (Tris-ammonium-EDTA buffer). The gel was stained in ethidium bromide visualized and photographed under UV light 260 nm. The DNA markers used were λ-Bst E 11-digest and 100 bp ladder. RAPDs generated by single primer PCR were used to compare relationship among isolates. For each isolate, a data record was constructed in which each band of a particular molecular weight, as generated by each primer, was represented as either being present, "1" or absent, "0". A binary matrix was constructed combining all the data records for each isolate-primer combination which yielded reproducible bands. The

SPSS programme was used to construct a Euclidian Distance matrix and to perform an Avarage Linkage Heirarchical Cluster Analysis.

3.5 Interactions between isolates of *C. kahawae* varying in virulence and genotypes of the hybrid Ruiru 11 population.

Ruiru 11 hybrid progenies from 66 crosses were inoculated with 8 isolates of *C. kahawae* varying in virulence to determine the nature of genotype x isolate interaction and its implications on pathogen adaptation to the resistant cultivar. The Ruiru 11 variety is a composite of hybrids obtained by crossing two parent populations established at the seed garden of the Coffee Research Foundation, Ruiru, in June 1981. The male parent population comprises of outstanding selections from a multiple cross programme involving CBD resistant donor parents such as Rume Sudan (RS), Hibrido de Timor (H T) and K7 with the high yielding, good quality but susceptible cultivars such as SL28, SL34, Bourbon (B) and drought resistant selections from Tanzania (DR1) among others. The mother parent population comprises of F3, F4 and F5 generations of the Catimor variety (CAT) introduced from Colombia. Colombia together with Kenya produce the mild Arabica coffee. Catimor is a cross between Caturra and Hibrido de Timor. It imparts into Ruiru 11 the dominant compact growth character, resistance to leaf rust that is effective against the most common races of *Hemileia vastatrix* and good quality particularly large bean size. Its resistance to CBD is monogenic and therefore narrow based. The 66 Ruiru 11 hybrid crosses used in this study were made during the October 1995 - March 1996 seed production season. The parentage of the progenies is presented in Table 7.

Table 7. Ruiru 11 progeny numbers and their pedigrees.

Male parents(Complex crosses)	Catimor mother parents										
	CAT.86	CAT.88	CAT.90	CAT.119	CAT.124	CAT.127	CAT.128	CAT.129	CAT.130	CAT132	CAT.134
SL28 x B3.96 = (RS x SL28)(B x HT)	1	-	3	4	5	-	7	8	9	-	11
SL28 x B3.97 = (RS x SL28)(B x HT)	-	-	-	15	16	-	18	-	-	21	22
SL28 x B3.97 = (RS x SL28)(B x HT)	23	24	25	-	-	-	29	-	31	-	-
SL28 x B3.116 = (RS x SL28)(B x HT)	34	35	36	-	38	39	40	41	42	43	44
SL28 x B3.185 = (RS x K7)(HT x SL34)	45	46	-	48	-	50	-	52	-	54	-
SL28 x B3.863 = (SL34 x RS)HT	56	-	-	59	60	61	62	63	64	65	66
SL28 x B3.866 = (SL34 x RS)HT	67	-	69	-	71	72	-	74	75	76	77
SL28 x B3.886 = (SL34 x RS)HT	-	79	80	81	82	-	84	85	86	-	-
SL28 x B3.887 = (SL34 x RS)HT	89	-	91	-	-	-	-	-	97	98	-
SL28 x B3.879 = (SL34 x RS)HT	100	-	-	-	-	-	-	-	-	-	-
SL28 x B4.691 = (DR1 x HT)SL28	-	-	-	-	-	-	139	-	-	-	-
SL28 x B4.406 = (RS x SL28)SL28	144	-	-	-	148	-	-	-	-	-	-

Before making the crosses, the male parents were subjected to at least 4 weeks of water stress to induce dormancy. Flowering was initiated by rain showers or irrigation following the dormancy period. On the day of anthesis, flowers were collected and taken to the laboratory where pollen was brushed off. The pollen was dessicated and stored under a vaccum at -20°C. Flowering was also initiated on the mother Catimor parents. The flowers were emasculated at the "candle light" stage starting approximately 4 days before anthesis. The emasculated flowers were isolated immediately using grease proof paper bags or isolation tents. On the day of anthesis, the stored pollen from male parents was thawed at room temperature and tested for viability before pollinating the stigmas of the emasculated mother parents. Viability was tested by germinating pollen in a drop of 2% sucrose solution on a glass slide and counting the proportion of germinating pollen after 4 hours. Pollen with proven viability of more than 80% was used for making crosses. Pollinated trees were immediately isolated for a period of two weeks until the stigmas were completely withered and rendered non-receptive to pollen. The isolation materials were removed and the seeds allowed to develop until maturity usually lasting about 8 months from the date of pollination. At maturity, the seeds were harvested , processed and sown in the laboratory for inoculation. The experiment was arranged in a two replicate completely randomized design in the laboratory with 100 hypocotyl seedlings per replication. Isolates R1.6, S12.8, S9.1, R2.3, R3.4, R10.2, R4.5 and S2.8 were used for inoculation according to the method of van der Vossen et al. (1977) described in section 3.2 above. The mean grade of infection was recorded and subjected to analysis of variance of the form presented in Table 8.

Table 8. Analysis of variance for mean grade of infection on Ruiru 11 hybrid population inoculated with isolates of *C. kahawae*.

Source	df*	MS	EMS**
Genotypes	g-1	M4	$\sigma^2_e + r\sigma^2_{gs} + sr\sigma^2_g$
Isolates	s-1	M3	$\sigma^2_e + r\sigma^2_{gs} + gr\sigma^2_s$
Varieties x Isolates	(g-1)(s-1)	M2	$\sigma^2_e + r\sigma^2_{gs}$
Error	gs(r-1)	M1	σ^2_e

* g = No. of genotypes = 66
s = No. of isolates = 7
r = No. of replicates = 2

** σ^2_g = variance due to variety effects.
σ^2_s = variance due to isolate effects.
σ^2_{gs} = variance due to variety x isolate interaction effect.
σ^2_e = Error variance

Components of variance were estimated by equating observed mean squares to their expectations as follows:

σ^2_g = (M4 - M2) / sr,
σ^2_s = (M3 - M2) / gr,
σ^2_{gs} = (M2 - M1) / r,
σ^2_e = M1.

Estimates of phenotypic variance (σ^2_p) and broad sense heritability (H^2) for resistance were computed according to Falconer (1989) as follows:

$\sigma^2_p = \sigma^2_g + \sigma^2_{gs} + \sigma^2_e$

$H^2 = \sigma^2_g / \sigma^2_p$

3.6 Evaluation for yield, quality and field resistance to CBD in an advanced breeding population "B22A".

Variation analysis was performed on an advanced breeding population designated "B22A" to identify genotypes combining field resistance to CBD with high yield and good quality. The selection field was planted with 32 entries of multiple cross families involving the CBD resistant donor parents such as Rume Sudan (RS), Hibrido de Timor (HT), and K7 with the high yielding, good quality and adaptable varieties such as SL 28, SL 34, SL4, Bourbon (B) and N39 at Oaklands Breeding Station, Ruiru, in 1988. The pollen supply parents in the production of the hybrid Ruiru 11 described in Section 3.5 are cloned from outstanding selections of this programme. The planting materials were generated and established in the field according to the following standard procedures:

3.6.1 Planting materials.

The multiple cross families listed in Table 9 were generated according to the procedure described in section 3.5. The seeds were harvested at maturity, processed and sown in plastic boxes in the laboratory for inoculation and screening using the method of van der Vossen et al. (1977) described in section 3.2. Highly resistant seedlings in disease classes 1-3 were transplanted in polybags containing top soil, river sand and farm yard manure in a ratio of 3:2:1. Inorganic fertilizer (Triple Super Phosphate) and Furadan were also included in the mixture at a rate of 250 g and 140 g for every 30 units of a 20-litre measure respectively. The seedlings were raised in the nursery for a period of 12 months. The shade was gradually thinned to harden the seedlings in preparation for field planting.

3.6.2 Field establishment and experimental design.

The trial was planted in a three replicate completely randomized design with 8 seedlings per plot. The plots comprised of two rows with seedlings planted in a staggered form. Three months prior to field planting, holes measuring 60 cm x 60 cm x 60 cm were dug keeping the top soil separate. The holes were filled with a mixture of top soil, one unit of a 20-litre measure of cattle manure and 100 g of Double Super Phosphate (45% P_2O_5). During planting, holes were opened up in the mounds that were sufficiently big to accomodate the roots.The seedlings were planted after carefully removing the polythene

Table 9. Families of population "B22A" and their pedigrees

Family No.	Tree No.	Pedigree	Family No.	Tree No.	Pedigree
1	B15.895	SL28 x (SL34 x RS)HT	18	B15.1533	SL28 x (N39 x HT)(SL4 x RS)
2	B15.727	SL28 x (SL34 x RS)HT	19	B15.931	SL28 x (SL34 x RS)HT
3	B15.81	SL28 x (SL34 x RS)HT	20	B15.1070	SL28 x (N39 x HT)(SL4 x RS)
4	B15.89	SL28 x (SL34 x RS)HT	21	B15.113	SL28 x (N39 x HT)(SL4 x RS)
5	B15.92	SL28 x (SL34 x RS)HT	22	B15.1559	SL28 x (N39 x HT)(SL4 x RS)
6	B15.262	SL34 x (SL34 x RS)HT	23	B15.1525	SL28 x (N39 x HT)(SL4 xRS)
7	B15.675	SL28 x (SL34 x RS)HT	24	B15.1093	SL28 x (N39 x HT)(SL4 x RS)
8	B15.239	SL34 x (SL34 x RS)HT	25	B15.1292	SL34 x (SL34 x RS)HT
9	B15.283	SL34 x (SL34 x RS)HT	26	B15.1077	SL28 x (N39 x HT)(SL4 x RS)
10	B15.726	SL28 x (SL34 x RS)HT	27	B15.1534	SL28 x (N39 x HT)(SL4 x RS)
11	B15.638	SL28 x (SL34 x RS)HT	28	B15.97	SL28 x (RS x K7)(HT x SL34)
12	B15.728	SL28 x (SL34 x RS)HT	29	B15.98	SL28 x (RS x K7)(HT x SL34)
13	B15.624	SL28 x (SL34 x RS)HT	30	B15.96	SL28 x (RS x K7)(HT x SL34)
14	B15.1400	SL28 x (RS x SL28)(B x HT)	31	B15.136	SL28 x (RS x K7)(HT x SL34)
15	B15.1365	SL28 x (RS x SL28)(B x HT)	32	B15.1533	SL28 x (N39 x HT)(SL4 xRS)
16	B15.928	SL28 x (SL34 x RS)HT	33	-	SL28
17	B15.902	SL28 x (SL34 x RS)HT	34	-	Catimor progeny 88

bags and grass mulching provided. The spacing between seedlings was 2.74 m x 2.74 m.

3.6.3 Cultural practices.

Cultural practices were performed according to the coffee production recommendations (Anon 1987). Weeds were controlled by hand. The trees were capped at 1.83 m and maintained as single stems with regular handling to remove excess growth. Nitrogen fertilizers were applied at a rate of 200 kg N/ha split into two applications per year during the the long and short rains. Compound fertilizer (20:20:20:) was applied 6 months before flowering each year to boost production.

3.6.4 Data collection.

Data were recorded over a period of five years on plot mean basis using four trees per plot chosen from the two middle trees per row. The plot mean data was used to calculate family means. The following characters were measured:

3.6.4.1. Cherry yield per tree - Cherry yield was obtained on plot mean basis and expressed in kg/tree.

3.6.4.2. Bean grade characters:- A bean grader was used to determine the fraction of various bean sizes which were then expressed as percentage by weight as described by Walyaro (1983) and Njoroge (1992).

3.6.4.2.1 PB (Peaberries) - determined as the fraction of beans retained by a piano wire screen with 4.43 mm spaces. Peaberries result from abnormal fruit development.

3.6.4.2.2 AA - the fraction of heavy beans retained by number 18 (5.95 mm) screen.

3.6.4.2.3 AB - the fraction of heavy beans retained by number 15 (5.95 mm) screen.

3.6.4.2.4 TT - light beans separated from AA and AB by a pneumatic separator.

3.6.4.2.5 C - the fraction of beans retained by a piano wire screen with 2.90 mm spaces.

3.6.4.2.6 T - the smallest beans which go through the piano wire screen with 2.90 mm spaces.

3.6.4.3 Bean and Liqour quality characters - Bean grades AA and AB from each family were combined and submitted to Mild Coffee Trade Association (MCTA) panel of liquores in samples of 250 g. Sensory evaluation was used for the assessment of liquor quality. The following characters were evaluated:

3.6.4.3.1 Quality of raw beans - size and colour of beans with scores of 0 - 7 where 0 = fine and 7 = poor. (Details in Appendix 1)

3.6.4.3.2 Quality of roast beans - the general appearance and centre cut of roast coffee on a scale of 0 - 5 where, 0 = fine and 5 = poor.

3.6.4.3.3 Liqour quality attributes included:

3.6.4.3.3.1 Acidity - with scores of 0 - 4 where, 0 = pointed and 4 = lacking.

3.6.4.3.3.2 Body - with scores of 0 - 4 where, 0 = full and 4 = lacking.

3.6.4.3.3.3 Flavour - with scores of 0 - 7 where 0 = fine and 7 = poor.

3.6.4.3.4 Overall standard - this was the overall evaluation of liquor quality based on attributes in 3.6.4.3.1 - 3.6.4 3.3. with scores of 0 - 7 where , 0 = fine and 7 = very poor.

3.6.4.4 Field evaluation of CBD - Number of berries with active CBD lesions (not scabs) expressed as a percentage of total berries on two most infected branches.

Data available for Ruiru 11 for the same period was also used to make comparisons with the genotypes of population "B22A".

3.6.5 Data analysis.

An analysis of variance was performed according to the following random effects model:

$Y_{ij} = \mu + a_i + b_j + e_{ij}$ where:

Y_{ij} = observation on ith family in the jth year,

μ = the overall mean,

a_i = effect of the ith family,

b_j = effect of the jth year,

e_{ij} = experimental error.

The form of analysis of variance with expected mean squares is shown in Table 10. Phenotypic correlations were computed separately for pairs of bean grade characters and liquor quality characters. The analyses were performed using the MSTAT statistical computer programme. The correlation coeficients were subjected to statistical tests at $P \leq 0.05$. Broad sense heritability estimate was computed for all characters as follows: $H^2 = \sigma^2_g / \sigma^2_p$ where, the phenotypic variance, $\sigma^2_p = \sigma^2_g + \sigma^2_e$. and σ^2_g was obtained from the analysis of variance as (M3 - M1)/y while σ^2_e = M1.

Table 10. Analysis of variance for yield, quality and disease incidence on population "B22A"

Source of variation	df	Mean square	Expected mean square
Families	f-1	M3	$\sigma^2_e + y\sigma^2_f$
Years	y-1	M2	$\sigma^2_e + f\sigma^2_y$
Error	(f-1)(y-1)	M1	σ^2_e

where g and y are number of families and years respectively.
σ^2_f, σ^2_y and σ^2_e are variance estimates due to families, years and error respectively

CHAPTER 4.

RESULTS.

4.1 Virulence of *C. kahawae* isolates on *C. arabica* varieties.

Virulence tests with 90 isolates of *C. kahawae* and 11 varieties of *C. arabica* revealed highly significant ($P \leq 0.01$) effects due to varieties and isolates as well as variety x isolate interaction (Table 11). Of the total variation, variety effects accounted for 49.83%, isolates accounted for 14.89% and variety x isolate interaction effects contributed 14.60%. The singe largest variation was due to varieties which was probably an indication that they had not been previously subjected to selection for CBD thus reflecting the natural diversity for CBD resistance. The main effects of isolates and the interaction effects of varieties x isolates were almost equal in magnitude with respect to the relative contribution towards overall variation. The significant effects of varieties and isolates could be attributed to differences in aggressiveness among isolates. Difference in aggressiveness is manifested as an increase or a decrease in virulence of an isolate on the entire range of host differentials. On the contrary, differential interaction between isolates and varieties is an indication that strains might exist which only cause disease on specific compatible host varieties. This is usually manifested by shifts in ranks when resistant varieties are subjected to different pathogen strains. Based on the relative proportion of the main effects (64.72%) and the interaction effects (14.60%), it can be

Table 11. Analysis of variance for mean grade of infection on 11 varieties of *C arabica* with 90 isolates of *C. kahawae*.

Source of variation	df	MS	% of total variation
Varieties	10	836.30**	49.83
Isolates	89	28.08**	14.89
Variety x isolates	890	2.75**	14.60
Error	1980	1.75	20.68

** Significant at $P \leq 0.01$,

concluded that variation in the pathogen population is predominantly due to aggressiveness but with small differential effects.

The mean grade of infection for varieties averaged over isolates are presented in Table 12. Resistance was only partial in varieties Rume Sudan, Pretoria, K7 and Padang while varieties Hibrido de Timor, SL28, SL34, Caturra, Erecta, Mokka and Laurina were susceptible. Since the effects of isolate x variety interaction were significant, the mean grade of infection for each variety was expressed relative to the most susceptible variety (100%). The varieties were ranked starting with the most resistant to the most susceptible for each isolate. The ranks were used to compute the Euclidian Distance Matrix and to perform a hierarchical cluster analysis (Figure 4). Grouping varieties based on ranks is expected to reflect the effects of variety x isolate interaction which is the main cause for shifts in ranks when varieties are subjected to different isolates.

Table 12. Mean grade of infection on *C. arabica* varieties with 90 isolates of *C. kahawae.*

Variety	Mean grade *
Rume Sudan	7.59 F
Pretoria	7.13 H
Hibrido de Timor	10.25 D
K7	7.36 G
Padang	8,04 E
SL 28	11.20 AB
SL 34	10.81 C
Caturra	11.21 AB
Erecta	11.04 B
Mokka	11.41 A
Laurina	10.67 C

* Means followed by the same letter are not significantly different according to Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$.

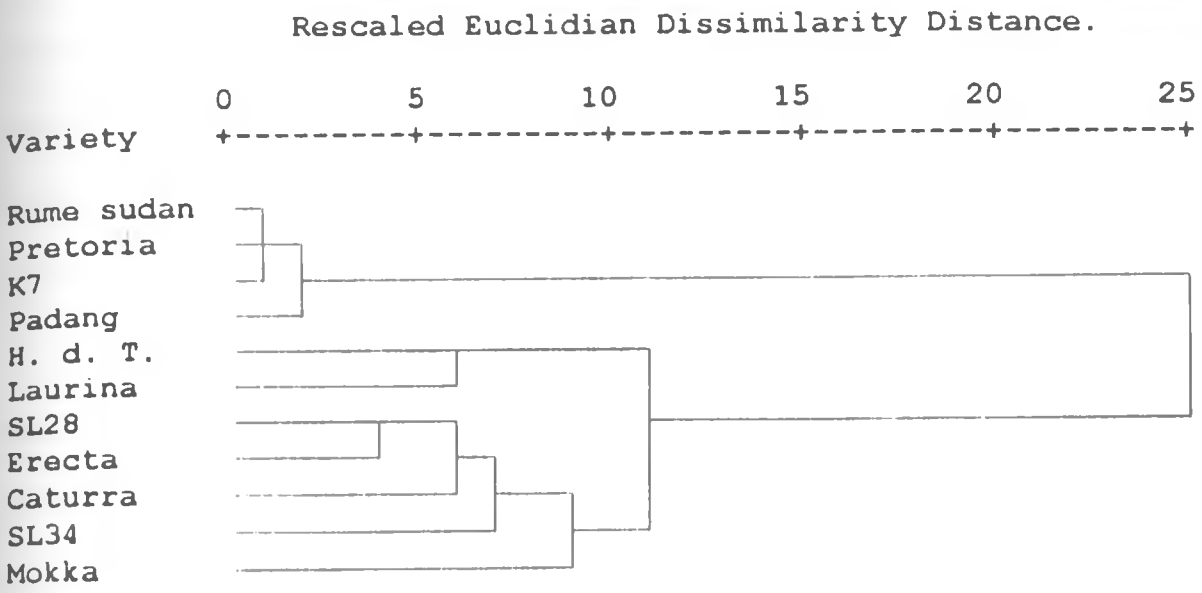


Fig. 4: Dendrogram derived from the Euclidian Distance Matrix based on resistance of *C. arabica* varieties to isolates of *C kahawae*.

The cluster analysis categorised the varieties into two main groups. The group comprising of Rume Sudan, Pretoria, K7 and Padang formed one tight cluster at a rescaled distance of 2 out of 25. The second group comprised of Hibrido de Timor, Laurina, SL28, SL34, Caturra, Erecta, and Mokka. They were grouped at a rescaled cluster distance of 11 out of 25. From these results, it can be observed that cluster analysis based on ranks divided the varieties into the same groups as virulence analysis based on mean grade of infection.

The hierarchical cluster analysis was also used to group the isolates. Twelve major virulence groups were distinguished (Figure 5). The groups were denoted with Roman numerals, I to XII. Isolates were neither grouped according to their locality nor host variety of origin, implying that within a location or a diseased plant in the field, pathogen population was highly variable. Most important was to further investigate the relative contribution of individual varieties and isolates to the total interaction component. The partial interaction effects due to varieties (W_i) and isolates (W_j) including their relative contributions to the variety x isolate interaction (%) are presented in Tables 13 and 14 respectively.

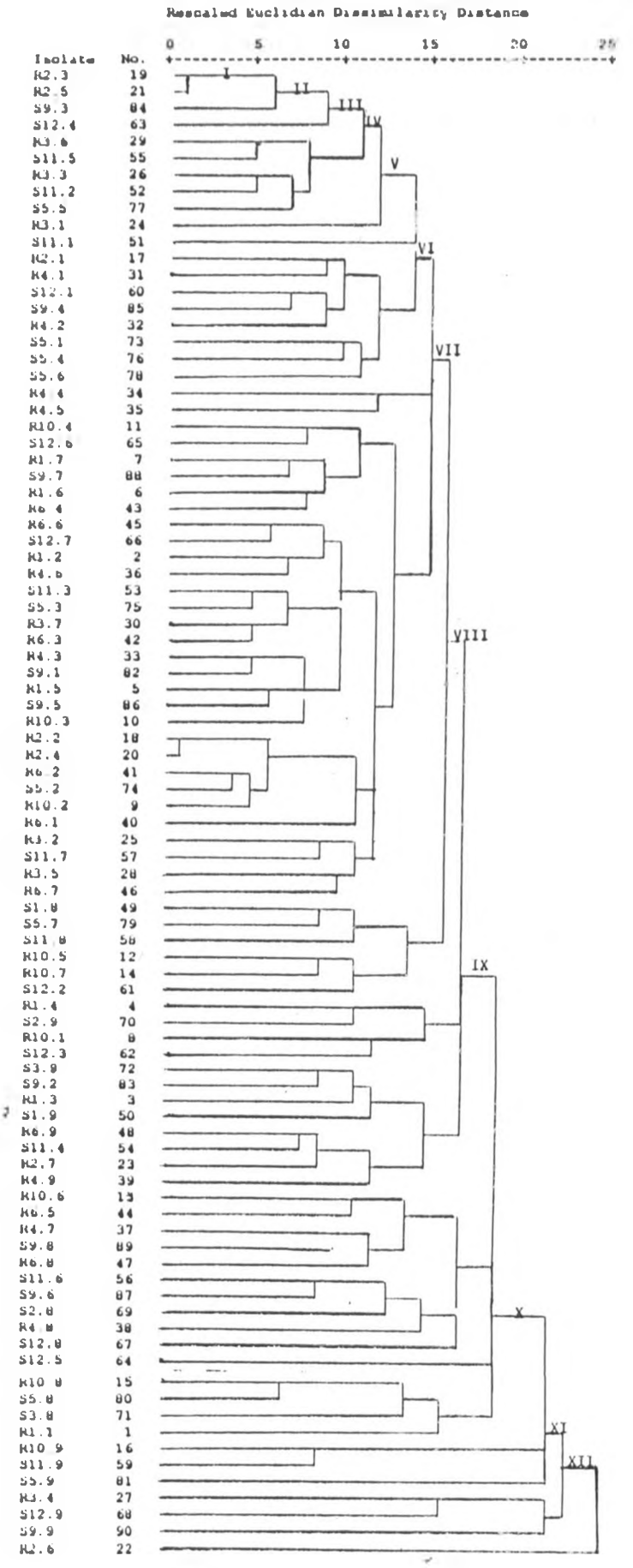


Fig. 5: Dendrogram derived from the Euclidian Distance matrix based on virulence of *C. kahawae* isolates on varieties of *C. arabica*.

Table 13 The partial interaction effects due to varieties (W_i) and their relative contributions to the total variety x isolate interaction

Varieties	Partial interactin effects of varieties (W_i)	Relative contribution of individual varieties to total variety x isolate interaction(%)
Rume Sudan	413.94	16.18
Pretoria	365.31	14.28
Hibrido de Timor	132.81	5.19
K7	409.83	16.02
Padang	440.79	17.23
SL28	97.95	3.83
SL34	110.13	4.31
Caturra	131.40	5.14
Erecta	137.16	5.36
Mokka	163.56	6.39
Launna	154.89	5.98

Table 14 The partial interaction effects due to isolates (W_i) and their relative contributions to the total variety x isolate interaction

Isolates	Partial interaction effects of isolates (W_i)	Relative contribution of individual isolates to total variety x isolate interaction(%)	Isolates	Partial interaction effects of isolates (W_i)	Relative contribution of individual isolates to total variety x isolate interaction(%)
R1.1	10.78	0.41	R6.1	14.20	0.54
R1.2	11.30	0.43	R6.2	33.92	1.29
R1.3	11.83	0.45	R6.3	7.10	0.27
R1.4	26.82	1.01	R6.4	10.52	0.40
R1.5	67.05	2.55	R6.5	32.08	1.22
R1.6	48.64	1.85	R6.6	48.91	1.86
R1.7	8.41	0.32	R6.7	20.77	0.79
S1.8	65.21	2.48	S6.8	52.58	2.00
S1.9	43.12	1.64	S6.9	31.29	1.19
R2.1	13.67	0.52	S9.1	40.23	1.53
R2.2	18.14	0.69	S9.2	13.67	0.52
R2.3	19.98	0.76	S9.3	7.88	0.30
R2.4	7.88	0.30	S9.4	46.54	1.77
R2.5	8.94	0.34	S9.5	15.51	0.59
R2.6	31.55	1.20	S9.6	11.83	0.45
R2.7	9.46	0.36	S9.7	39.44	1.50
S2.8	31.55	1.20	S9.8	59.16	2.25
S2.9	58.37	2.22	S9.9	81.25	3.09
R3.1	18.41	0.70	R10.1	9.47	0.36
R3.2	15.25	0.58	R10.2	34.71	1.32
R3.3	19.46	0.74	R10.3	19.72	0.75
R3.4	21.56	0.82	R10.4	24.72	0.94
R3.5	9.20	0.35	R10.5	39.44	1.50
R3.6	38.92	1.48	R10.6	34.18	1.30
R3.7	22.88	0.87	R10.7	27.87	1.06
S3.8	48.91	1.86	R10.8	47.85	1.82
S3.9	27.08	1.03	R10.9	38.92	1.48
R4.1	9.47	0.36	S11.1	20.77	0.79
R4.2	14.20	0.54	S11.2	6.57	0.25
R4.3	15.25	0.58	S11.3	18.14	0.69
R4.4	10.52	0.40	S11.4	24.45	0.93
R4.5	27.61	1.05	S11.5	28.14	1.07
R4.6	29.98	1.14	S11.6	128.58	4.89
R4.7	36.02	1.37	S11.7	19.98	0.76
S4.8	33.13	1.26	S11.8	46.54	1.77
S4.9	26.03	0.99	S11.9	80.99	3.08
S5.1	6.31	0.24	S12.1	8.94	0.34
S5.2	16.83	0.64	S12.2	8.15	0.31
S5.3	20.25	0.77	S12.3	13.67	0.52
S5.4	24.72	0.94	S12.4	29.71	1.13
S5.5	16.57	0.63	S12.5	89.66	3.41
S5.6	13.94	0.53	S12.6	33.66	1.28
S5.7	31.55	1.20	S12.7	15.51	0.59
S5.8	39.97	1.52	S12.8	37.08	1.41
S5.9	69.42	2.64	S12.9	18.14	0.69

Results in Table 13 indicates that the partial contribution of individual varieties to the total variety x isolate interactions were high for Rume Sudan (16.18%), Pretoria (14.28%), K7 (16.02%) and Padang (17.23%). Individual contributions of the remaining varieties were less than 10%. Table 14 indicates that the partial contributions of most isolates to the total variety x isolate interactions were less than 3% except S9.9 (3.09%), S11.6 (4.89%), S11.9 (3.08%) and S12.5 (3.41%). The pattern of virulence of these isolates on varieties with high relative contribution to the total variety x isolate interactions is illustrated in Figure 6. It was observed that with the exception of S9.9 the pattern of virulence was generally high for isolate S11.9 but low for isolates S11.6 and S12.5. Isolate S9.9 was highly aggressive on K7 and Pretoria but less aggressive on Rume Sudan and Padang. Varietal differences changed in both magnitude and direction when inoculated with different isolates. The latter is manifested by interactions of cross-over type and shifts in ranks.

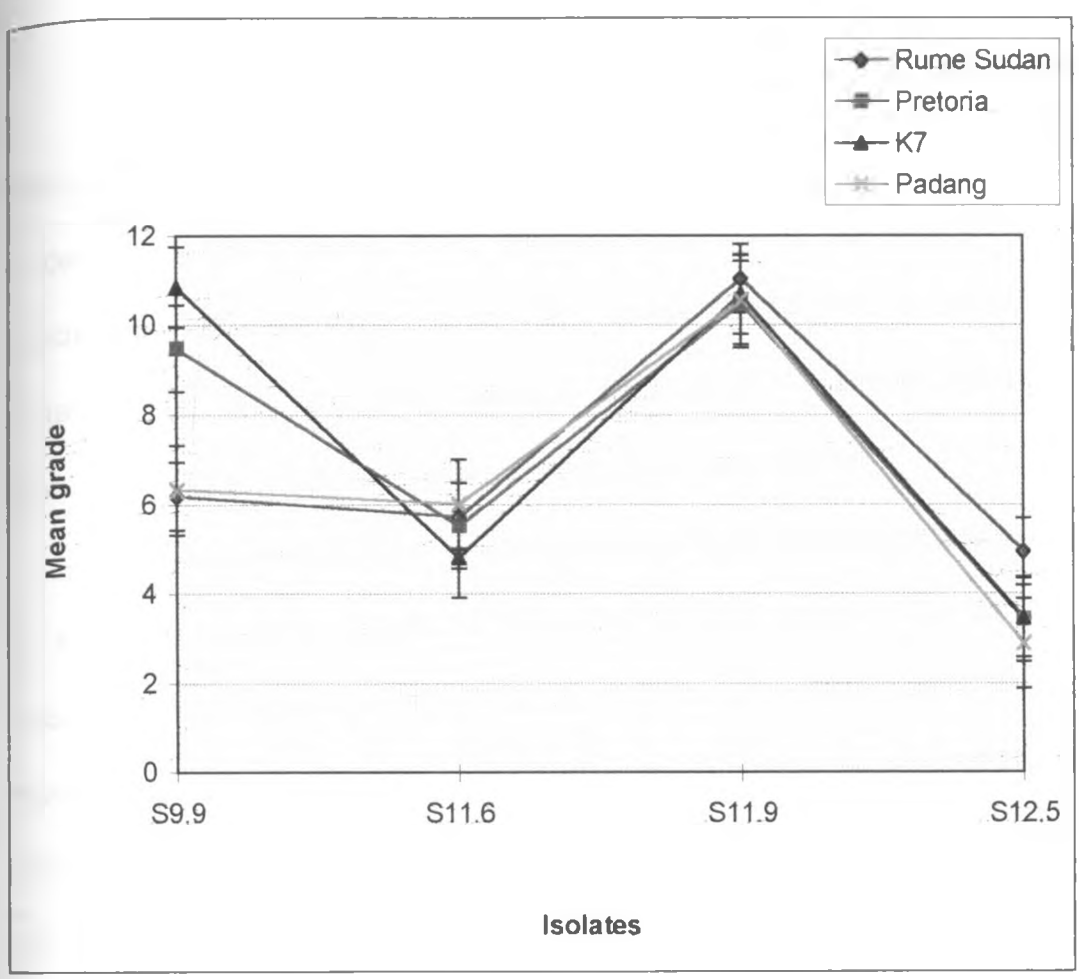


Fig. 6. Virulence of selected *C. kahawae* isolates on some *C. arabica* varieties.

4.2 Protein electrophoresis.

Most isolates produced protein profiles with a large number of bands ranging between 20 and 30 (Figure 7). Using the earlier described protein extraction procedure, bands produced by *C. gloeosporioides* were not clearly resolved. The species was therefore excluded from any further comparative analysis using protein patterns. For the remaining isolates, only clearly resolved bands that were reproducible in at least two electrophoretic runs were scored.

The non-pathogenic *C. acutatum* had protein profiles distinctly different from *C. kahawae* isolates. Bands of 173 kDa, 45 kDa and 40 kDa distinguished the species from *C. kahawae*. Most isolates of *C. kahawae* had similar protein profiles. However, bands of 123 kDa and 52 kDa differentiated strains within the species. Using Average Linkage Hierarchical Cluster Analysis, the Benomyl tolerant strain BR.1 was grouped together with the Benomyl sensitive strains (R4.5 and S9.1) at a rescaled cluster distance of 1 out of 25 (Figure 8). The two Benomyl sensitive isolates belong to the same virulence group (VI) in Figure 5. Single isolate clusters were formed by isolates S9.9 and BR.4 at cluster distances of 2 and 3 out of 25 respectively. Another divergent cluster at a distance of 4 out of 25 comprised of isolates R10.9, S12.8 and S12.5. These isolates were grouped into cluster numbers IX (R10.9 and S12.8) and X (S12.5) by virulence analysis (Figure 5). Isolates R6.8, R1.6 and R2.3 were grouped at a cluster distance of 6 out of 25. Grouping based on virulence categorized the isolates into three separate groups namely, IX, VI and I respectively. Other clusters comprised of isolates R3.4 at 8 out of 25 and isolates S11.9 and S2.8 at 10 out of 25.

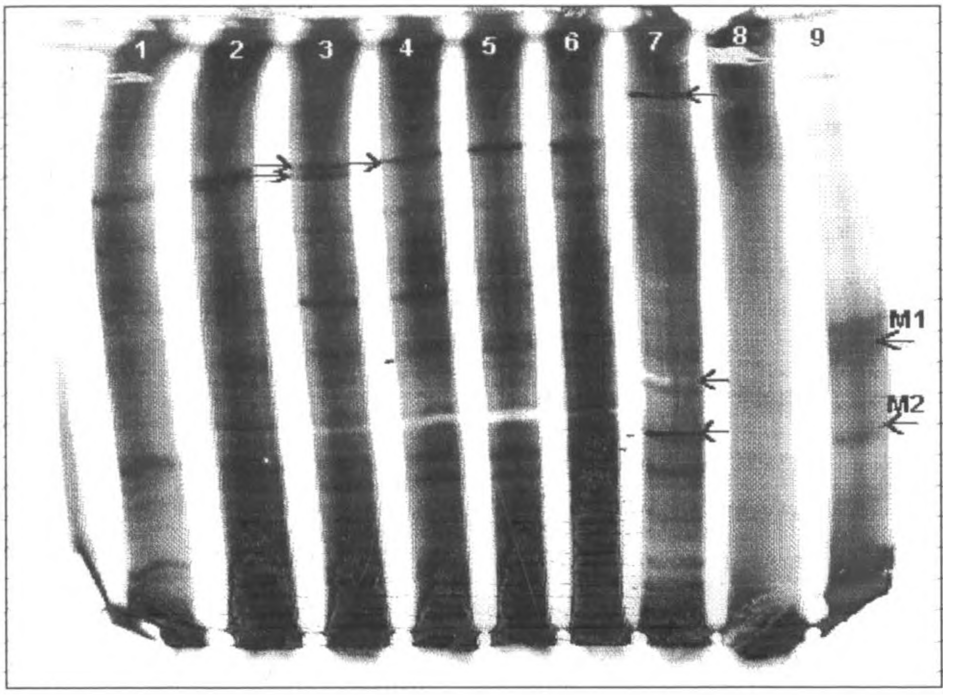


Fig. 7. Protein bands separated on a 10% SDS polyacrylamide gel and stained with silver nitrate. Lanes 1-6 are *C. kahawae* isolates S11.9, R2.3, S2.8, S12.8,R4.5, R6.8, respectively. Lane 7 = *C. acutatum*, Lane 8 = *C. gloeosporioides* and Lane 9 = marker proteins (M1 =67 kDa and M2 = 45 kDa)

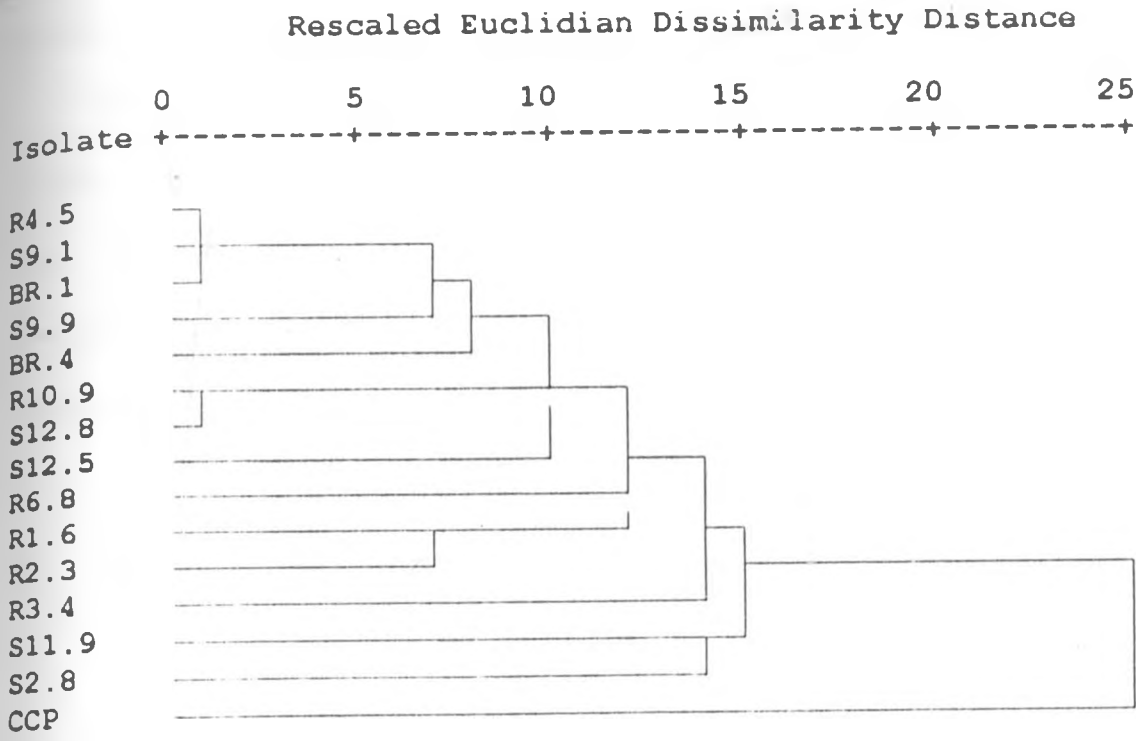


Fig. 8. Dendrogram derived from Euclidian Distance matrix based on analysis of total proteins.

C. acutatum isolate (CCP) was very divergent from *C. kahawae* isolates. Grouping of isolates was neither according to host/locality of origin nor according to Benomyl sensitivity/tolerance.

Table 15 indicates that protein profiles of *C. kahawae* isolates were highly identical with similarity coefficients between pairs of isolates ranging from 0.60 to 1.00. The similarity coefficients between *C. acutatum* and isolates of *C. kahawae* were generally low ranging from 0.20 to 0.40 indicating that the two species are genetically distinct.

4.3 Isozyme electrophoresis.

A total of 28 putative loci were resolved from nine out of 12 enzymes systems assayed. There was no activity observed for ME, AK and HEX. The loci *Est-B32, Est-B36, Est-B62, Est-B73, Est-B89, Est-B100, Mdh-B100, Gpdh-B100, Idh-B100, Pgi-B100, Mpi-B27, Mpi-B67, Mpi-B100, Fdp-B44 and Phi-100* were monomorphic and invariant for all isolates (Table 16). Polymorphism was exhibited by the *Est-B8, Est-B12, Est-B45, Est-B51, Est-B59, Ldh-B100, Ldh-B167, Pgi-B57, Mpi-B20, Mpi-B60, Mpi-B96, Fdp-B240* and *Fdp-B300* bands. Examples of polymorphic bands are presented in Figure 9.

A total of seven distinct multilocus ETs were distinguished (Table 16). ET 1 comprised of isolates S12.5, S12.8, S2.8, S9.1, S9.9 and S11.9. They were characterized by lack of activity of the *Est-B8* and *Est-B12* bands but carried the *Ldh-B167* and *Pgi-B57* bands. All the isolates were obtained from susceptible varieties. ETs 2 and 3 both comprised of isolates R1.6, R2.3, R3.4, R4.5, R6.8 and R10.9. They

Table 15. Simple matching coefficients generated from 14 isolates of *C. kahawae* and the non pathogenic species, *C. acutatum*

	R1.6	S12.8	S9.1	R3.4	R2.3	R10.9	R4.5	S2.8	R6.8	S9.9	S11.9	S12.5	BR.1	BR.4
R1.6														
S12.8	0.85													
S9.1	0.85	0.90												
R3.4	0.80	0.85	0.75											
R2 3	0.95	0.80	0.90	0.85										
R10.9	0.85	1.00	0.90	0.85	0.80									
R4.5	0.85	0.90	1.00	0.75	0.90	0.90								
S2.8	0.65	0.80	0.70	0.75	0.60	0.80	0.70							
R6.8	0.75	0.80	0.90	0.80	0.80	0.80	0.90	0.70						
S9.9	0.80	0.85	0.90	0.80	0.85	0.95	0.95	0 75	0.85					
S11.9	0.65	0.80	0.80	0.75	0.70	0.80	0 80	0.80	0.70	0 85				
S12.5	0.85	0 90	0.90	0.85	0 80	0.90	0.90	0.70	0.70	0.85	0.70			
BR 1	0.85	0.90	1.00	0.75	0 90	0.90	1.00	0.70	0.90	0.95	0.80	0.90		
BR. 4	0 80	0.85	0.95	0.70	0.85	0.85	0.95	0.75	0.75	0.90	0.85	0.85	0.95	
CCP	0.35	0.30	0.20	0.35	0.25	0.30	0.20	0.40	0.30	0.25	0.30	0.30	0.20	0.25

Key: Benomyl sensitive *C. kahawae* strains = R1.6, S12.8, S9.1, R3.4, R2.3, R10.9, R4.5, S2.8, R6.8, S9.9, S11.9 and S12.5, Benomyl tolerant *C. kahawae* strains = BR.1 and BR.4.CCP = *C. acutatum*

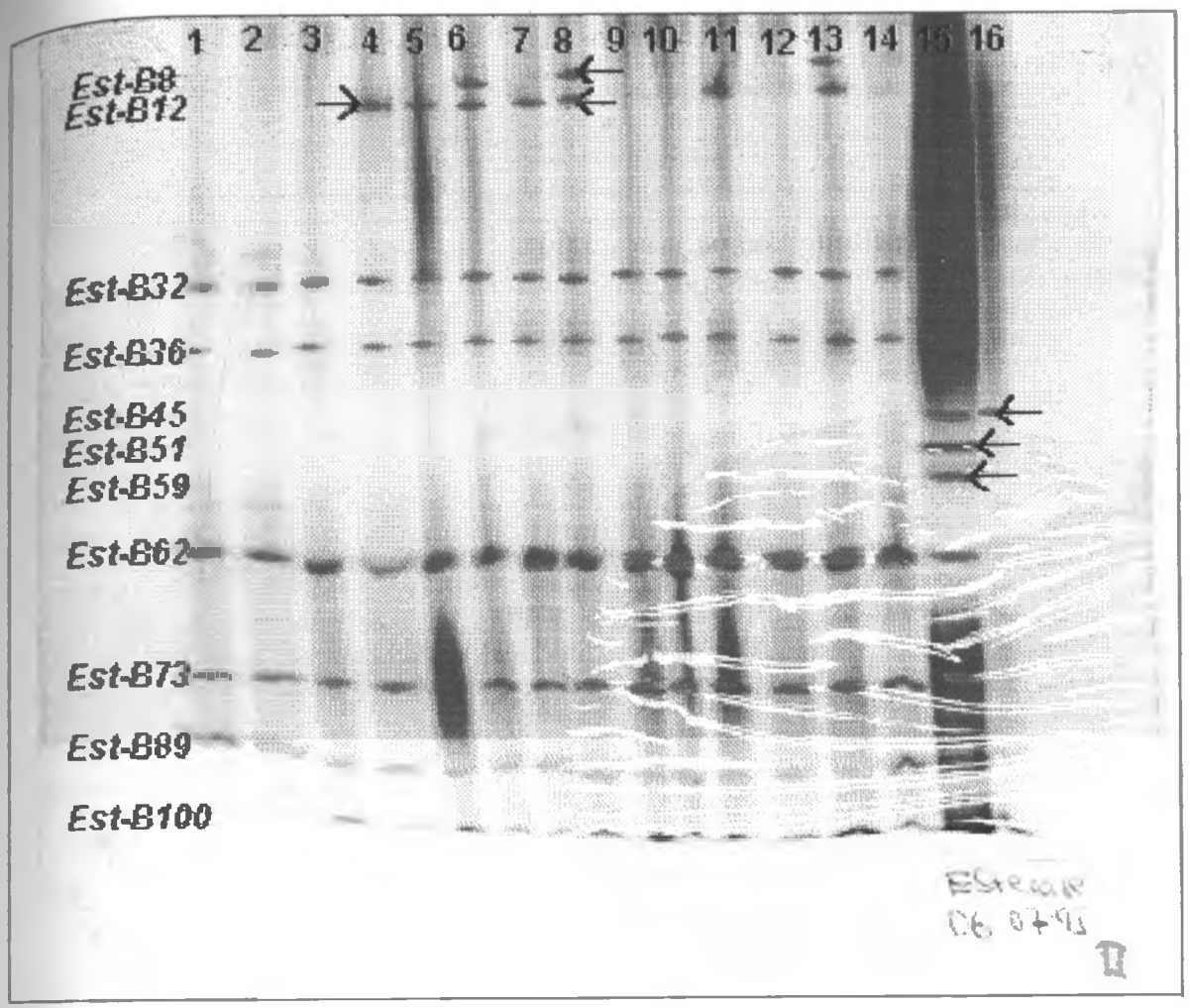


Fig. 9: Zymogram for esterase (EST), from *C. kahawae* isolates (Lane 1= S12.5, Lane 2 = S12.8, Lane 3 = S2.8, Lane 4 = R1.6, Lane 5 = R2.3, Lane 6 = R3.4, Lane 7 = R4.5, Lane 8 = R6.8, Lane 9, = S9.1, Lane 10 = S9.9, Lane 11 = R10.9, Lane 12 = S11.9, Lane 13 = BR.4*, Lane 14 = BR.1*) *C. gloeosporioides* (Lane 15) and *C. acutatum* (Lane 16).
*Benomyl tolerant strains.

Table 16. Electrophoretic types and their band combinations.

Band	Electrophoretic types						
	ET1	ET2	ET3	ET4	ET5	ET6	ET7
Est-B8	00	11	11	00	11	00	11
Est-B12	00	00	11	00	11	00	11
Est-B32	11	11	11	11	11	11	11
Est-B36	11	11	11	11	11	11	11
Est-B45	00	00	00	00	00	00	11
Est-B51	00	00	00	00	00	11	11
Est-B59	00	00	00	00	00	11	00
Est-B62	11	11	11	11	11	11	11
Est-B73	11	11	11	11	11	11	11
Est-B89	11	11	11	11	11	11	11
Est-B100	11	11	11	11	11	11	11
Mdh-B100	11	11	11	11	11	11	11
Gpdh-B100	11	11	11	11	11	11	11
Idh-B100	11	11	11	11	11	11	11
Ldh-B100	11	11	11	00	11	11	11
Ldh-B167	11	11	11	00	00	11	11
Pgi-B57	11	11	11	11	11	00	11
Pgi-B100	11	11	11	11	11	11	11
Mpi-B20	00	00	00	00	00	00	11
Mpi-B27	11	11	11	11	11	11	11
Mpi-B60	00	00	00	00	00	11	11
Mpi-B67	11	11	11	11	11	11	11
Mpi-B96	00	00	00	00	00	11	00
Mpi-B100	11	11	11	11	11	11	11
Fdp-B44	11	11	11	11	11	11	11
Fdp-B100	11	11	11	11	11	11	11
Fdp-B240	00	11	11	11	11	11	00
Fdp-B300	00	00	00	00	00	11	11
Phi-B100	11	11	11	11	11	11	11
Frequency of isolates per ET	6	3	3	1	1	1	1

00 denotes absence of a band
11 denotes presence of a band

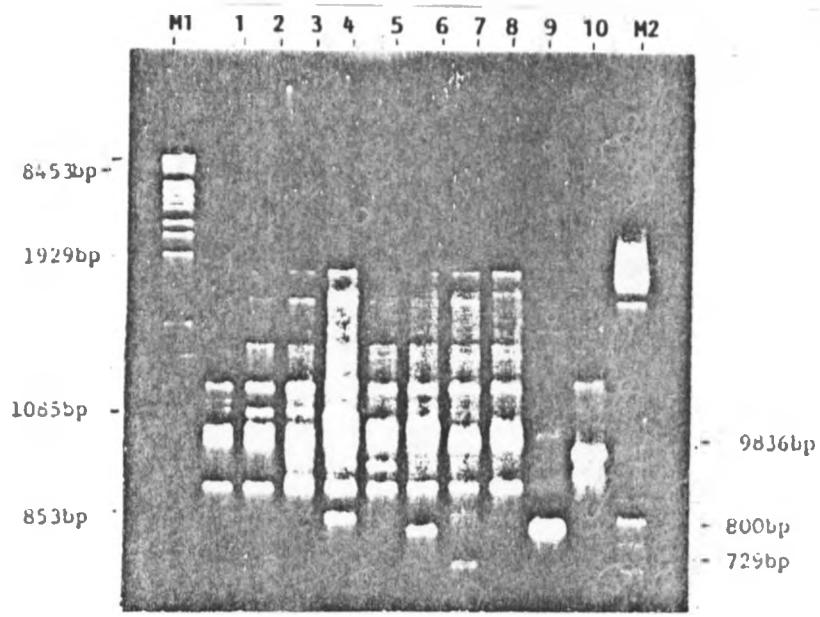
stained for *Est-B8* either singly or in combination with *Est-B12*. The isolates also carried the *Ldh-B167* and *Fdp-B240* bands but lacked the *Est-B45*, *Est-B51*, *Est-B59*, *Mpi-B20*, *Mpi-B60*, *Mpi-B96* and *Fdp-B300* bands. The Benomyl-tolerant strains, BR.1 and BR.4 were categorized in ET4 and ET5 respectively. ET4 not only lacked activity of the *Est-B8* and *Est-B12* bands but also did not stain for both *Ldh-B100* and *Ldh-B167* bands. ET5 carried the *Est-B8*, *Est-B12* and *Ldh-B100* bands but lacked activity of *Ldh-B167* band. *C. gloeosporioides* was the only species in ET6. The species carried the *Est-B51* and *Est-B59* bands but lacked activity of the *Pgi-B57* band which distinguished it from the other species. *C. acutatum* was categorized in ET7. The species was distinct for carrying the *Est-B45* and *Mpi-B20* bands.

4.4 RAPD analysis.

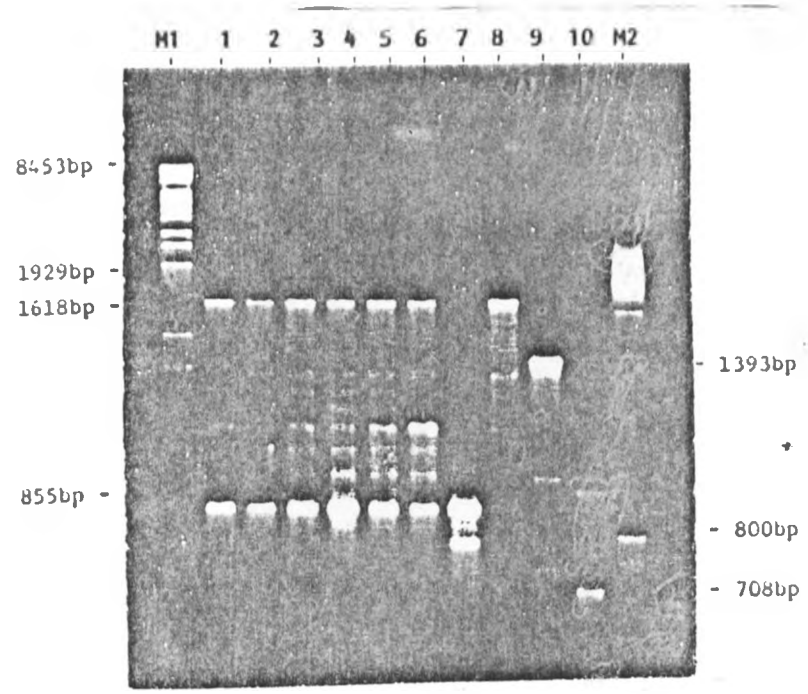
A total of 39 decamer oligonucleotide primers were assayed for amplification of DNA from eight isolates of *C. kahawae* and two non-pathogenic *Colletotrichum* species, *C. gloeosporioides*, and *C. acutatum*. Three primers, (5', 3'), CCG TCT CTT T, AAG GCA CCA G and GTG TTT CCG G failed to amplify DNA from both pathogenic and non-pathogenic species. The remaining 36 primers which generated a total of 487 amplification products are presented in Table 17. The avarage number of DNA bands per primer was 13.6. The fragment sizes ranged from 300 bp to 2500 bp [Figures 10 (a) to 10(l)].

Table 17: A list of primers used for Random Amplified Polymorphic DNA(RAPD) analysis of *Colletotrichum* species.

Primer	Sequence 5'-3'	RAPD	Primer	Sequence 5'-3'	RAPD
401	TAG GAC AGT C	12	427	GTA ATC GAC G	77
403	GCA AGG CTG T	13	428	GGC TGC GGT A	10
405	CTC TCG TGC G	6	429	AAA CCT GGA C	9
406	GCC ACC TCC T	12	430	AGT CGG CAC C	16
407	TGG TCC TGG C	12	431	CTG CGG GTC A	27
409	TAG GCG GCG G	13	432	AGC GTC GAC T	9
410	CGT CAC AGA G	9	433	TCA CGT GCC T	9
411	GAG GCC CGT T	16	434	TCG CTA GTC C	8
412	TGC GCC GGT G	10	435	CTA GTA GGG G	14
415	GTT CCA GCA G	6	437	AGT CCG CTG C	22
417	GAC AGG CCA A	14	439	GCC CCT TGA C	15
420	TAC GTG CCC G	9	440	CTG TCG AAC C	10
419	GCA GGG TTC G	21	443	TGA TTG CTC G	11
420	ACG GCC CAC C	14	444	GCA GCC CCA T	22
421	CAC CTG CGG G	18	445	TAG CAG CTT G	15
422	GGG TCT CGA A	14	446	GCC AGC GTT C	18
423	ACG GAG GTT C	16	447	CAG GCT CTA G	16
425	CGT CGG GCC T	16	448	GTT GTG CCT G	18



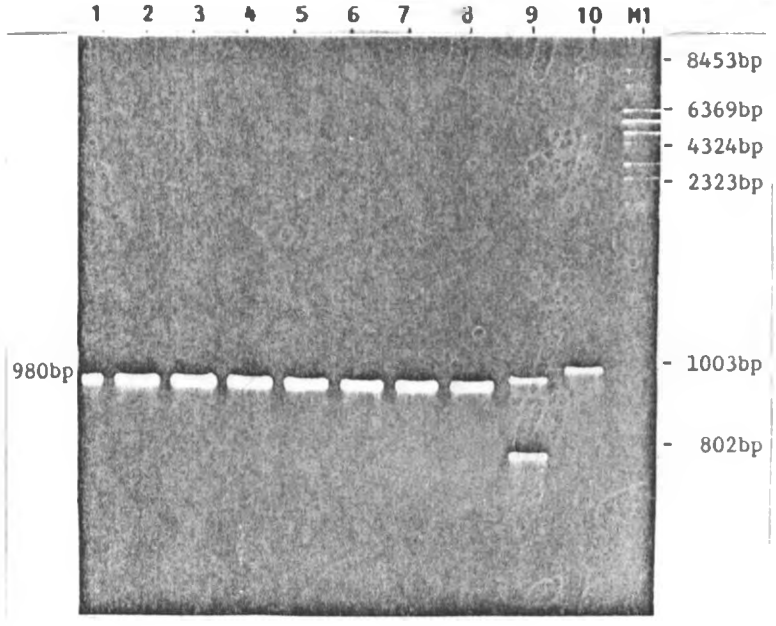
(a)



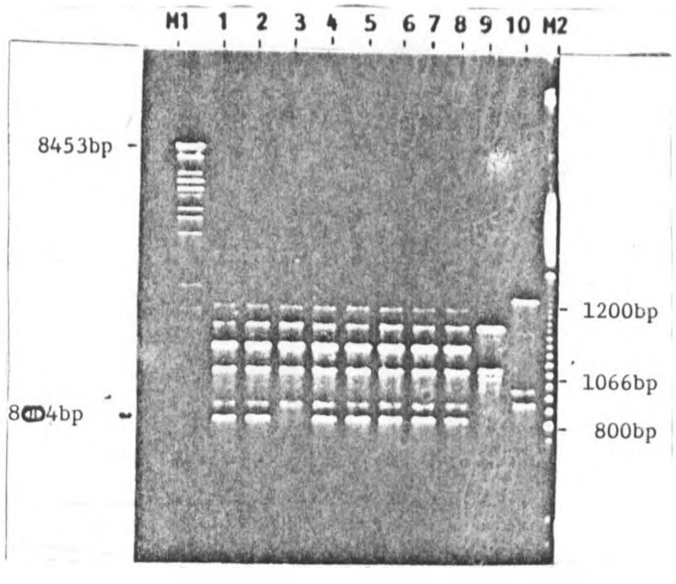
(b)

Fig. 10 (a). RAPD profiles generated by primer 406
Fig. 10 (b). RAPD profiles generated by primer 407.

Lanes M1 and M2 are λ-Bst E 11-digest and 100 bp DNA ladder markers respectively. Benomyl-sensitive *C. kahawae* isolates are represented by lane 1 = R1.6, Lane 2 =R2.3, Lane 3 = R2.8, Lane 4 = R3.4, Lane 5 = S9.1 and Lane 6 = S9.9. Benomyl tolerant strains of C. kahawae are represented by Lane 7 = BR.1 and Lane 8 = BR.4. Lanes 9 and 10 are *C. gloeosporioides* and *C. acutatum* respectively. The same markers and test isolates were used for all RAPD assays.



(c)

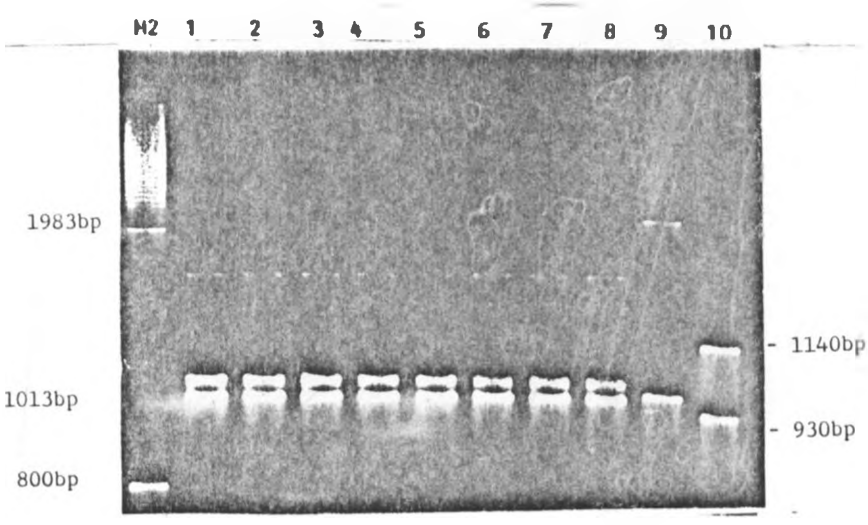


(d)

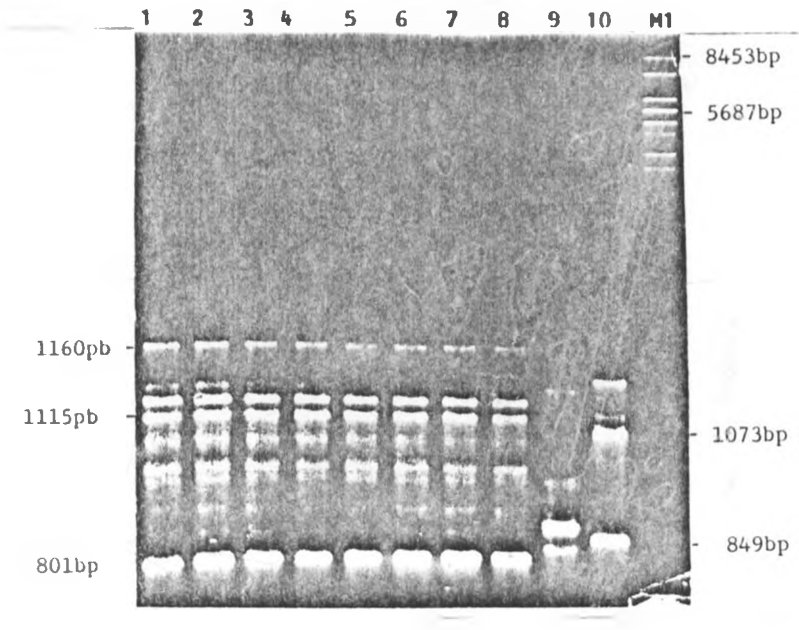
Fig. 10 (c). RAPD profiles generated by primer **419,**

Fig. 10 (d). RAPD profiles generated by primer **411.**

Description of isolates are presented on page 82



(e)

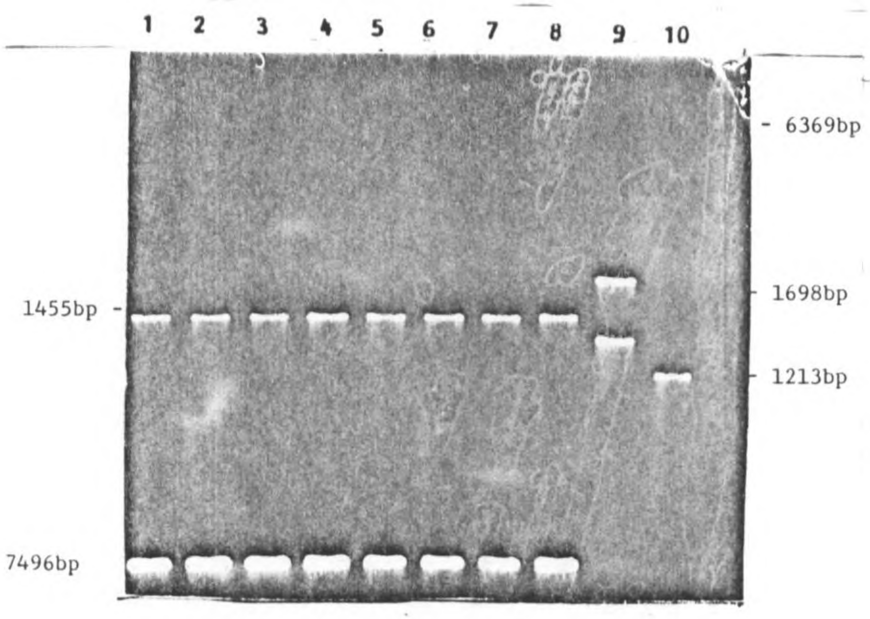


(f)

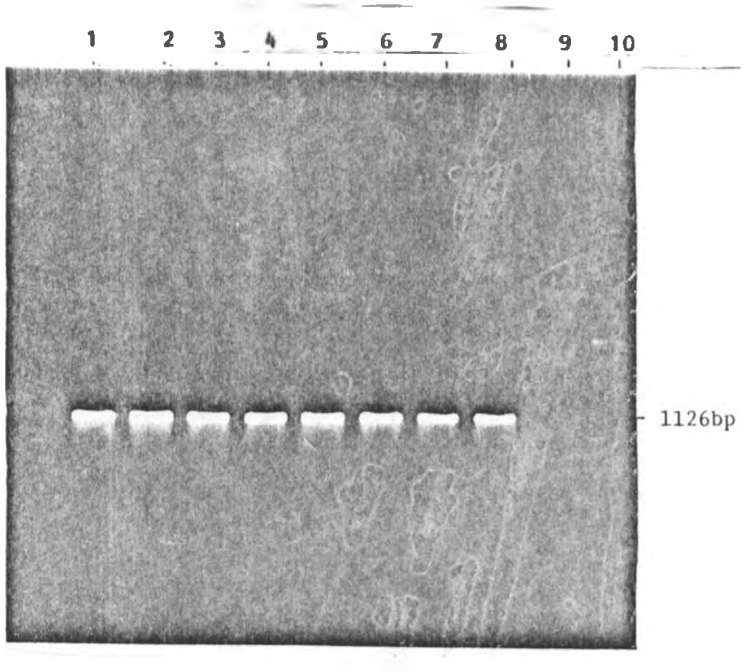
Fig. 10 (e). RAPD profiles generated by primer 421.

Fig. 10 (f). RAPD profiles generated by primer 425.

Description of isolates are presented on page 82



(g)

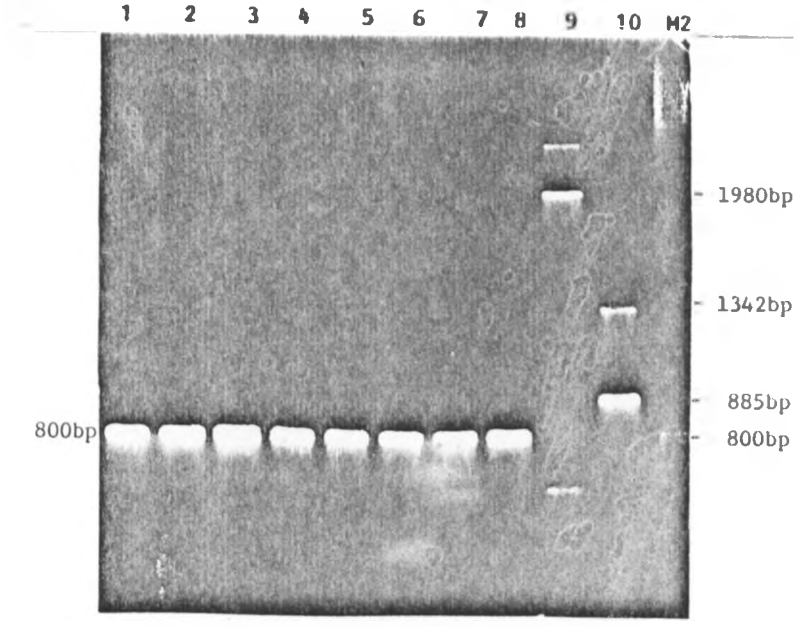


(h)

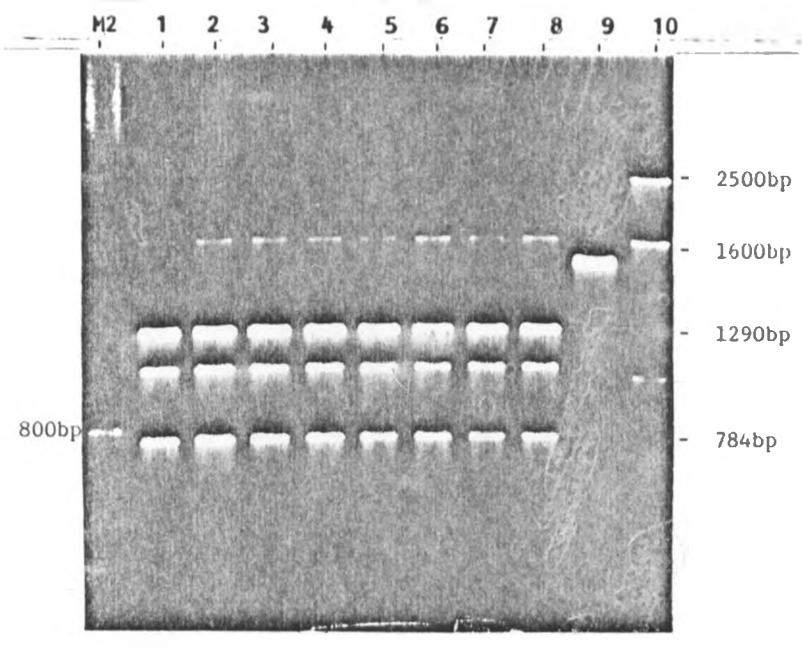
Fig. 10 (g). RAPD profiles generated by primer 428,

Fig. 10 (h). RAPD profiles generated by primer 429.

Description of isolates are presented on page 82



(I)

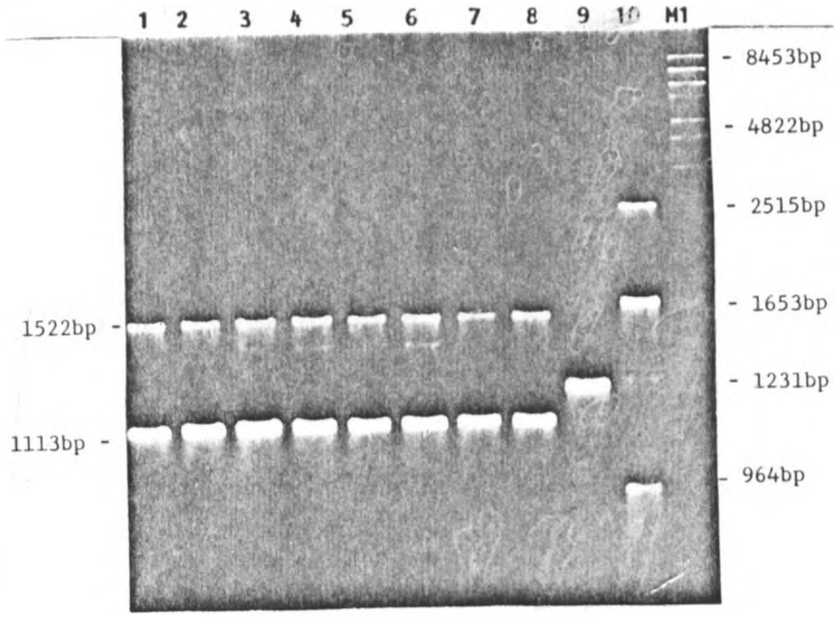


(j)

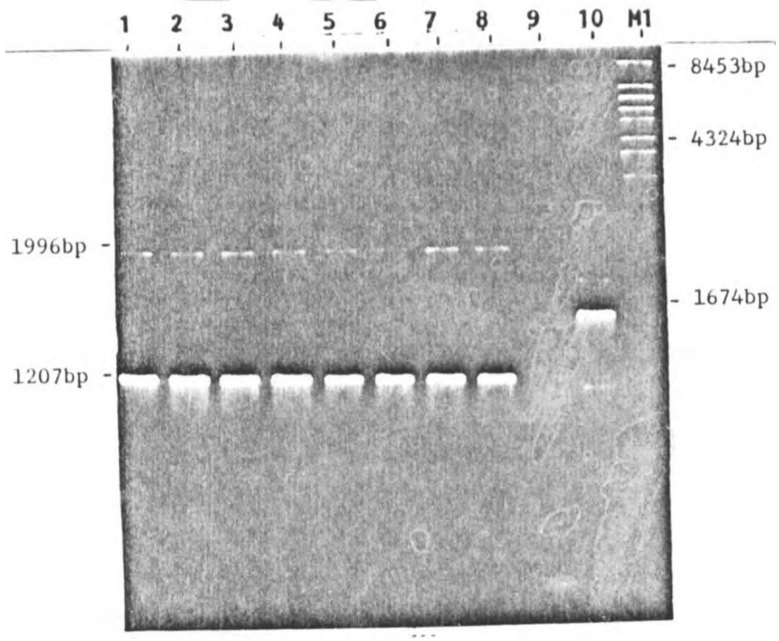
Fig. 10 (i). RAPD profiles generated by primer 432,

Fig. 10 (j). RAPD profiles generated by primer 433

Description of isolates are presented on page 82



(k)



(l)

Fig. 10 (k). RAPD profiles generated by primer 434.

Fig. 10 (l). RAPD profiles generated by primer 440.

Description of isolates are presented on page 82

Variation between pathogenic *C. kahawae* isolates and the non-pathogenic species was detected by all the 36 primers which generated reproducible bands. A total of 393 (80%) bands were polymorphic indicating that RAPDs detected variability with more precision than either protein or isozyme electrophoresis. Of the 80% polymorphic bands, 71% were species-specific suggesting that the species were highly divergent. Figures 10 (a) to 10 (l) indicate that isolates of *C. kahawae* were consistently different from the two non-pathogenic species. Within *C. kahawae* most amplification products were monomorphic. Strain-specific bands were only 9% of total polymorphic bands implying that there is limited variation within *C. kahawae* species. Some of the variation among isolates of *C. kahawae* are shown in Figures 10(a) - 10 (c).

For each primer, band levels were recorded and the presence or absence of an amplification product was respectively coded "1" or "0" for all the isolates. From this data, a matrix of simple matching coefficients (S) was calculated using the SPSS statistical programme. Within *C. kahawae* species, coefficients ranging between 0.88 and 0.93 were observed indicative of the close relationships among isolates within the species (Table 18). Although the similarity values were high, it can be observed that there was no S value of 1.00 (complete similarity) observed as in protein electrophoresis, confirming the sensitivity of this method in detecting variation compared to protein electrophoresis. The non-pathogenic species were not only different from each other (S=0.44), but were also distantly related to the *C. kahawae* isolates with simple matching coefficient values ranging from 0.35 to 0.42.

Table 18. Simple matching similarity coefficient matrix generated from eight isolates of *C. kahawae* and two non-pathogenic *Colletotrichum* species (*C. gloeosporioides* and *C. acutatum*).

	R1.6	R2.3	S2.8	R3.4	S9.1	S9.9	BR.1	BR.4	CCM
R1.6									
R2.3	0.91								
S2.8	0.91	0.92							
R3.4	0.88	0.89	0.91						
S9.1	0.91	0.92	0.91	0.92					
S9.9	0.88	0.89	0 89	0.90	0.93				
BR.1	0.88	0.88	0.89	0.91	0.92	0.92			
BR.4	0.89	0.88	0.88	0.91	0.91	0.91	0.92		
CMM	0.38	0.39	0.35	0.36	0.37	0.38	0.40	0.40	
CPP	0.39	0.41	0.38	0.38	0.38	0.37	0.37	0.42	0.44

Key: *Benomyl sensitive *C. kahawae* strains = R1.6, R2.3, S2.8, R3.4, S9.1 and S9.9,
Benomyl tolerant *C. kahawae* strains = BR.1 and BR.4.
CCM = *C. gloeosporioides*
CCP = *C. acutatum*

Using the SPSS statistical programme, an Avarage Linkage Hierarchical Cluster Analysis was performed on the data. Four cluster groups were distinguished (Figure 11). Benomyl sensitive isolates S9.1, S9.9, and R3.4, were clustered together with Benomyl tolerant strains BR.1 and BR.4 at a rescaled distance of 2 out of 25. However, according to virulence analysis (Figure 5), only two of the Benomyl sensitive isolates (R3.4 and S9.9) shared a cluster group number XI while the third, R3.4, belonged to a divergent cluster number XI. Another tight cluster at a rescaled distance of 3 out of 25 comprised of isolates R2.3, R2.8 and R1.6. Again, on the basis of virulence tests, these isolates belong to three different clusters, I, IX and VI, respectively. *C. gloeosporioides* (CCM) and *C acutatum* (CCP) formed single isolate cluster groups. RAPDs therefore confirmed the large genetic variation between species but variation within species was of a relatively lower magnitude. The observed intraspecific variation was not diagnostic for pathogenicity, Benomyl tolerance or host/locality of origin.

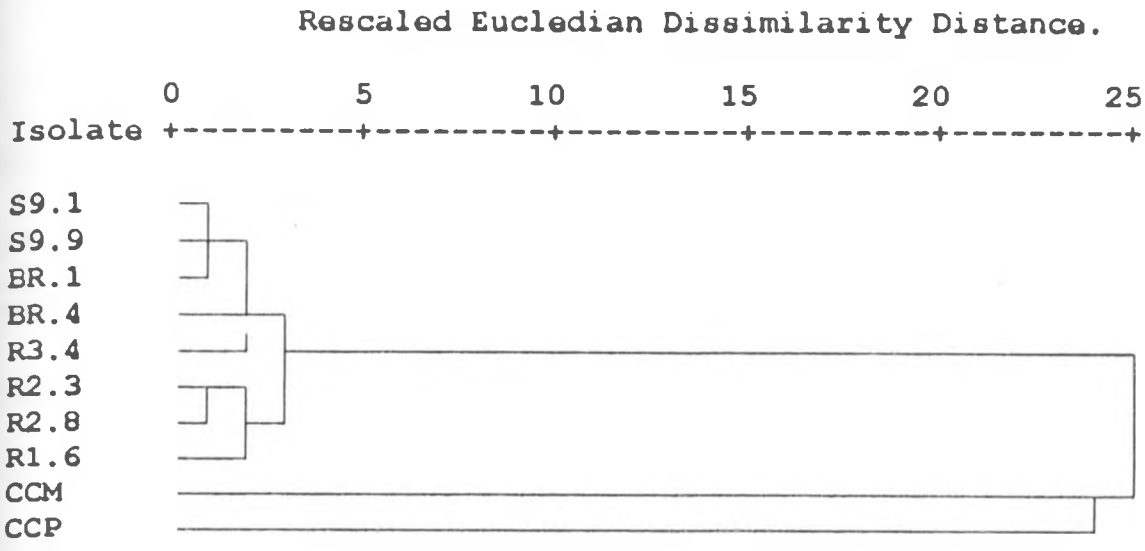


Fig. 11: Dendrogram derived from the Euclidian Distance matrix based on RAPD profiles.

4.5 Interactions between isolates of *C. kahawae* and Ruiru 11 genotypes.

Ruiru 11 genotypes differed significantly ($p \leq 0.05$) in their resistance to CBD when inoculated with isolates varying in virulence (Table 19). The mean grade of infection varied between 4.90 for progeny number 139 and 10.40 for progeny number 23 (Table 20). There were no progenies in the highly resistant classes of 1-3 on the scale of 1-12. On the medium resistant classes of 4-6, was the outstanding progeny number 139 and other statistically comparable progeny numbers 24, 25, 46, 67, 97, 144 and 148. The remaining progenies were only partially resistant (Classes 7-10). Results in Table 19 further indicate that the main effects of isolates were also significant ($p \leq 0.05$) reflecting differences in virulence. The differential effects of genotypes x isolates were, however, non-significant suggesting that the differences in virulence among isolates were genotype non-specific. Pathogen variation was therefore unlikely to be due to physiological specialization but could have been due to differences in aggressiveness or pathogen response to other environmental conditions that have nothing to do with the host. Estimates of variance components indicates that only a small proportion of variation for resistance was due to genetic effects. The broad sense heritability estimate was only 0.23.

Table 19. Analysis of variance for mean grade of infection on Ruiru 11 genotypes subjected to seven isolates of *C. kahawae*

Source of variation	df	Mean square	Estimated values of components of variance
Genotypes	65	22.97**	$\sigma^2 g = 1.32$
Isolates	6	180.15**	$\sigma^2 s = 1.33$
Genotypes x isolates	390	3.95	$\sigma^2 gs = 0$
Error	462	4.45	$\sigma^2 e = 4.45$

** significant at $p \leq 0.05$
$\sigma^2 g$ = Variance due to Ruiru 11 genotypes
$\sigma^2 s$ = Variance due to isolates
$\sigma^2 gs$ = Variance due to genotypes x isolates interaction
$\sigma^2 e$ = Error variance

Table 20: Mean grade of infection on Ruiru 11 genotypes inoculated with isolates of *C. kahawae*

Ruiru 11 progeny number	Mean grade		Ruiru 11 progeny number	Mean grade	
1	8.31	B-M	54	9.87	A-D
2	8.11	C-N	56	7.02	K-P
4	6.89	L-P	59	7.05	K-P
5	10.28	AB	60	7.47	G-O
7	9.48	A-G	61	9.00	A-K
8	8.45	A-L	62	10.28	AB
9	7.66	E-O	63	7.41	H-P
11	9.10	A-J	64	8.69	F-O
15	8.47	A-L	65	8.11	C-N
16	7.95	D-O	66	9.01	A-K
18	9.26	A-I	67	6.01	O-Q
21	9.18	A-I	69	8.98	A-K
22	9.97	A-D	71	7.36	I-P
23	10.40	A	72	9.28	A-I
24	6.05	O-Q	74	8.54	A-L
25	6.42	M-Q	75	9.01	A-K
29	9.78	A-D	76	7.31	I-P
31	7.31	I-P	77	7.02	K-P
34	6.97	L-P	79	8.69	A-L
35	7.63	E-O	80	6.96	L-P
36	8.17	C-M	81	9.43	A-G
38	8.28	B-M	82	8.39	A-M
39	8.69	A-L	84	9.80	A-D
40	9.41	A-H	85	9.49	A-G
41	9.07	A-J	86	9.50	A-F
42	8.61	A-L	89	8.74	A-L
43	8.64	A-L	91	9.23	A-I
44	10.04	A-C	97	6.45	M-Q
45	6.95	L-P	98	9.40	A-H
46	6.15	N-Q	100	7.17	J-P
48	9.65	A-E	139	4.90	Q
50	9.06	A-J	144	5.51	PQ
51	7.94	D-O	148	6.42	M-Q

Figures followed by the same letters are not significantly different at $p \leq 0.05$ according to DMRT.

4.6 Evaluation for yield, quality and field resistance to CBD in an advanced breeding population "B22A".

4.6.1 Variation analysis for yield.

Results in Table 21 indicates that there was significant variation ($P \leq 0.01$) for yield among families in population "B22A" when analysed separately and when compared to the standard check varieties, SL28, Catimor and Ruiru 11. The yield of families within the population ranged between 2.16 kg/tree in family number 17 and 4.51 kg/tree in family numbers 1 and 10 (Table 22). Ruiru 11 variety was the overall highest yielder with a mean of 4.73 kg/tree over the 5 years. The transgressive yield of Ruiru 11 which is higher than the test families in population "B22A" and the Catimor variety both of which are base populations from which Ruiru11 parents have been selected is an indication that the Ruiru 11 variety exhibits heterosis. In order to identify high yielding families in population "B22A", the yield of the popular Kenyan commercial variety SL28, was chosen as the truncation point. All the families except numbers 3, 5, and 17 were not significantly different ($p \leq 0.05$) from SL28. Yield exhibited a modest broad sense heritability estimate of 0.41 (Table 23).

Table 21. Analysis of variance for yield, quality and disease score on breeding population "B22A" separately and when compared with standard check varieties (in parenthesis).

Trait	Mean square and probability		
	Families	Years	Error
df	31 (34)	4 (4)	124 (136)
Yield	2.38** (2.44**)	33.78** (33.38**)	0.54 (0.69)
Bean grades			
"TT"	96.77** (102.57**)	1105.41** (1144.43**)	57.54 (56.88)
"PB"	9.29** (12.97**)	79.63** (91.11**)	4.20 (5.35)
"AA"	52.20** (74.96**)	24.74 (28.79)	19.30 (22.53)
"AB"	91.42** (97.27**)	978.65** (917.55**)	43.91(47.94)
"C"	13.80 (13.67)	140.50** (141.90**)	9.03 (9.24)
"T"	0.54 (0.51)	2.51* (2.50**)	0.83 (0.79)
Bean quality			
Raw beans	0.39* (0.36*)	1.34** (1.31**)	0.25 (0.23)
Roast beans	0.29 (0.27)	1.13** (0.92*)	0.30 (0.29)
Liquor quality			
Acidity	0.53 (0.54)	4.77** (4.71**)	0.48 (0.47)
Body	0.47 (0.48)	2.23** (1.85**)	0.35 (0.35)
Flavour	0.45 (0.52)	3.33** (3.03**)	0.35 (0.35)
Overall standard	0.31** (0.32**)	0.43 (0.37)	0.21 (0.21)
% active CBD	0.10 (1.04**)	0.15 (0.99)	0.10 (0.41)

* Significant at $p \leq 0.05$
** Significant at $p \leq 0.01$

Table 22. Mean yield of individual families of population "B22A"

Family No/variety	Yield (kg/tree)
1	4.51 AB
2	4.31 A-D
3	2.29 I-K
4	2.59 H-K
5	2.23 JK
6	3.20 C-K
7	4.02 A-E
8	3.71 A-H
9	3.41 B-K
10	4.51 AB
11	4.01 A-F
12	2.63 H-K
13	3.92 A-G
14	3.55 A-I
15	4.49 A-C
16	4.00 A-F
17	2.16 K
18	2.72 F-K
19	3.76 A-H
20	3.52 A-I
21	3.40 B-K
22	3.42 B-K
23	3.68 A-H
24	3.38 B-K
25	3.38 B-K
26	2.85 E-K
27	3.48 A-J
28	4.12 A-E
29	2.67 G-K
30	4.26 A-D
31	2.73 F-K
32	3.95 A-G
SL 28	3.75 A-H
Catimor	3.08 D-K
Ruiru 11	4.73 A

Means followed by the same letters are not significantly different at $p \leq 0.05$

Table 23. Estimates of genotypic variance, error variance and broad sense heritability.

Trait	σ^2_g	σ^2_e	H^2
Yield	0.37	0.54	0.41
Bean grade:			
TT	7.85	57.54	0.12
PB	1.02	4.20	0.20
AA	6.58	19.30	0.25
AB	9.50	43.51	0.18
C	0.95	9.03	0.10
T	0.00	0.83	0.00
Bean quality:			
Raw beans	0.03	0.25	0.11
Roast beans	0.00	0.30	0.00
Liquor quality:			
Acidity	0.01	0.48	0.02
Body	0.02	0.35	0.05
Flavour	0.02	0.35	0.05
Overall standard	0.02	0.21	0.09
% active CBD	0.00	0.10	0.00

Yearly variations were significant ($P \leq 0.01$) for yield (Table 21). Yields increased from year 1 to year 3, declining in year 4 before increasing again in year 5 (Figure 12).

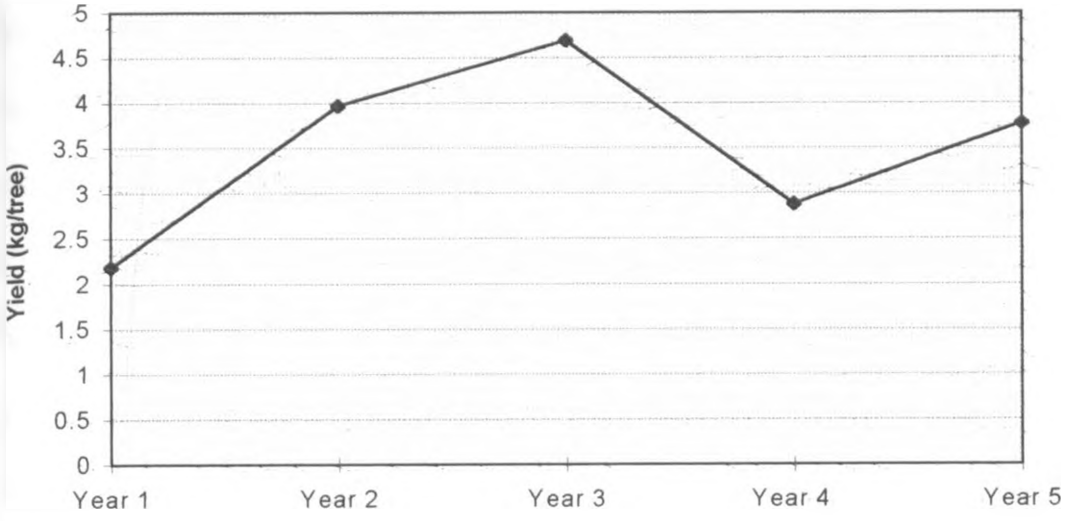


Fig. 12: Yearly variations for yield in population "B22A"

4.6.2 Variation analysis for bean grade characters.

Variation was significant ($P \leq 0.01$) for all bean grade characters among families within population "B22A" except "C" and "T" (Table 21). Families also differed significantly ($P \leq 0.01$) for the same characters when standard check varieties were included in the analysis. In coffee trade, the most important grades are "AA" and "AB". The highest "AA" producing family within population "B22A" was family number 5 (16.52%) while family number 14 (5.74%) produced the least (Table 24). The traditional commercial check variety, SL28 (23.50%) was significantly ($p = 0.05$) superior than the families in population "B22A". The proportion of "AB" grade was highest in family number 31 (50.48%) and lowest in family number 12 (33.05%). Although the proportion of grade "AA" was significantly ($p \leq 0.05$) lower in all test families than the best check variety, most test families recorded a higher proportion of the grade "AB". Phenotypic correlation between grades "AA" and "AB" was negative and significant ($r = -0.22$, $p \leq 0.05$) (Table 25). The results further indicate that bean grade "AA" had significant negative association with "TT" ($r = -0.36$, $p \leq 0.01$) and "C" ($r \leq -0.51$, $p = 0.01$) while association between "AA" and "PB" was positive and significant ($r = 0.53$, $p \leq 0.01$). Bean grade "AB" had weak association with "PB" ($r = -0.10$), "C" ($r = 0.06$) and "T" ($r = -0.10$) while its association with "TT" was negative and significant ($r = -0.71$, $p \leq 0.01$). The most important consideration in determining the criteria for selection for the commercial grades of interest, "AA" and "AB" could be the proportion of heritable variation. Characters with high heritability estimates are easier to improve.

Table 24. Means for bean grade characters "AA" and "AB" for population "B22A".

Family No./variety	AA	AB
1	14.34 B-H	41.39 A-F
2	16.18 B-D	39.71 A-F
3	11.32 B-J	40.10 A-F
4	14.74 B-G	43.95 A-D
5	16.52 BC	39.44 B-F
6	10.17 B-J	37.92 C-F
7	11.56 B-J	42.17 A-F
8	12.10 B-J	42.99 A-E
9	6.47 IJ	41.72 A-F
10	9.51 C-J	43.93 A-D
11	7.74 G-J	47.06 A-C
12	15.02 B-G	33.05 E-F
13	8.08 F-J	41.99 A-F
14	5.74 J	47.28 A-C
15	8.62 E-J	46.18 A-C
16	11.39 B-J	36.12 C-F
17	7.31 H-J	33.14 D-F
18	11.08 B-J	37.37 C-F
19	8.99 D-J	41.31 A-F
20	13.80 B-I	41.23 A-F
21	13.96 B-H	41.93 A-F
22	15.15 B-F	40.47 A-F
23	15.92 B-E	41.54 A-F
24	16.06 B-D	41.55 A-F
25	16.33 B-D	36.42 C-F
26	13.05 B-J	37.03 C-F
27	14.16 B-H	40.00 A-F
28	12.87 B-J	45.77 A-C
29	10.64 B-J	36.97 C-F
30	10.70 B-J	46.38 A-C
31	9.19 C-J	50.48 A
32	7.20 H-J	49.68 AB
SL28	23.50 A	31.58 F
Catimor	17.57 AB	40.93 A-F
Ruiru 11	17.15 B	40.88 A-F

Means on each column followed by similar letter(s) are not significantly different at $p \leq 0.05$

Table 25: Phenotypic correlation among bean grade characters

	TT	PB	AA	AB	C	T
TT		-0.26*	-0.36**	-0.71**	0.07	0.05
PB			0.53**	-0.10	-0.53**	-0.24*
AA				-0.22*	-0.51**	-0.19
AB					0.06	-0.10
C						0.37**
T						

* significant at $p \leq 0.05$

** significant at $p \leq 0.01$

Broad sense heritability estimates were 0.25 and 0.18 for bean grades "AA" and "AB" respectively. Apparently, these heritabilities are both low offering no significant advantage of basing selection on either character. The most outstanding family was number 31 with 50.48% of grade "AB" beans which was higher in absolute terms than the best check variety, Catimor, with 40.93% of grade "AB" beans. Family numbers 5, 6, 12, 16, 17, 18, 25, 26 and 29 produced significantly ($p \leq 0.05$) lower proportions of grade "AB" beans than the best overall family number 31. The yearly variations for all bean grade charaters is presented in Figure 13.

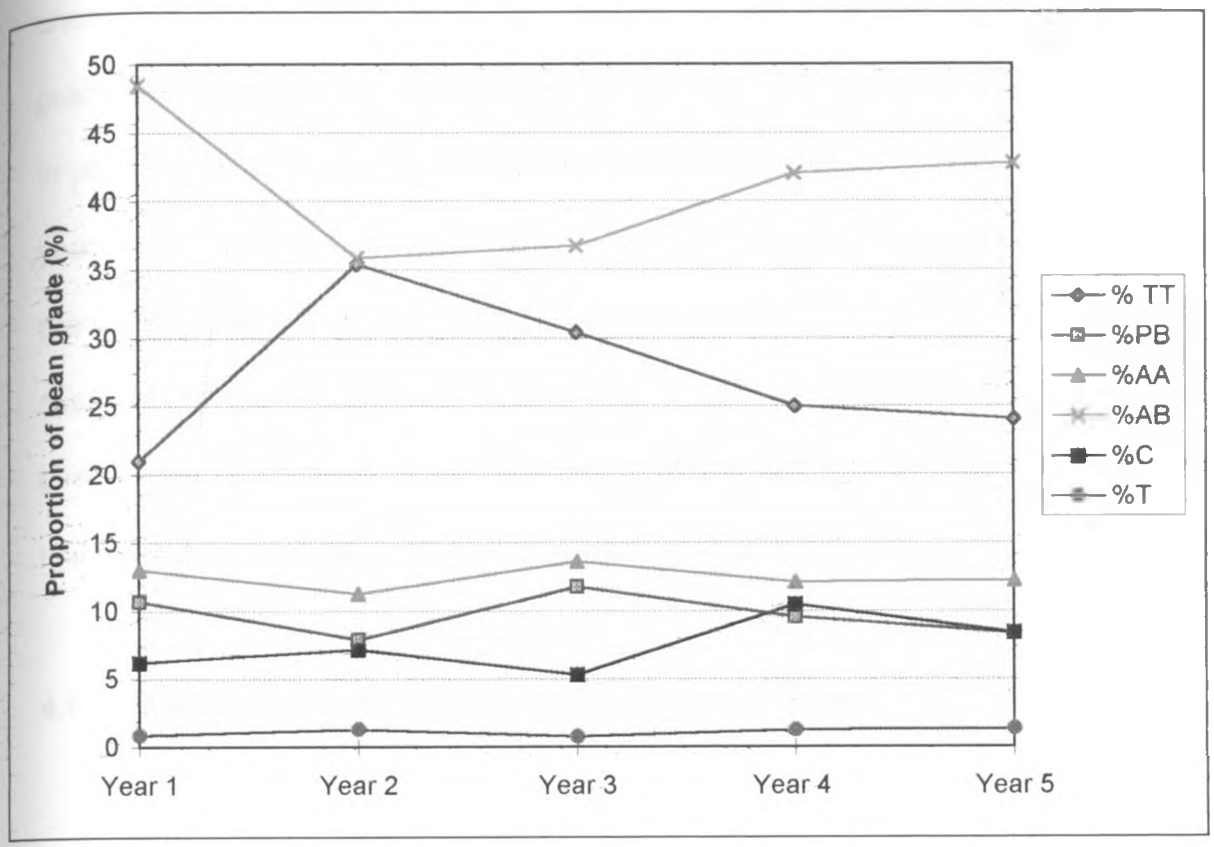


Fig. 13: Yearly variation for bean grade characters.

Significant ($p \leq 0.01$) yearly variations were observed for all bean grade characters except "AA" (Table 21). Results in Figure 13 indicate that the highest production of grade "AB" was in year 1, "AA" and "PB" in year 3, "TT" in year 2, "C" in year 4 and "T" in year 5. Lowest production of grades "AB", "AA" and "PB" was in year 2, "TT" in year 1, while "C" and "T" were in year 3. The minimum fluctuation for grade "AA" beans over the years favours the trait as a criteria for early selection of superior bean grade characters compared to "AB" but the proportion of grade "AA" beans were significantly ($p \leq 0.05$) lower in all the test families than the check variety making it difficult to apply truncation selection (Table 24).

4.6.3 Variation analysis for raw and roast bean quality.

Quality of raw beans differed significantly ($p \leq 0.05$) among families within population "B22A" and also when check varieties were included in the analysis (Table 21). Both forms of analyses revealed no significant difference ($p \leq 0.05$) among families for roast beans. Raw bean scores ranged between 4.60 for family number 4 and 3.26 for family number 15 compared to 3.89 for SL28, 3.86 for Catimor and 3.87 for Ruiru11 (Table 26). The best family with raw bean score (Family number 13) also combined high yield and high proportion of grade "AB" beans. The other outstanding families combining high yield with high proportion of grade AB beans and raw bean score statistically comparable ($p \leq 0.05$) to family number 13 were numbers 1, 2, 10, 11, 13, 19, 20, 21, 22, 31 and 32. The raw bean score of these families are also statistically comparable to the raw bean score of the standard check varieties. The proportion of genetic effects for the raw bean character was

Table 26. Means for bean quality score in population "B22A"

Family No./variety	Raw beanns	Roast beans
1	4.00 A-D	3.13 A
2	3.63 CD	2.60 A
3	3.93 A-D	2.73 A
4	4.60 A	3.33 A
5	3.80 B-D	2.73 A
6	3.86 A-D	2.93 A
7	4.26 A-C	2.46 A
8	4.06 A-C	2.73 A
9	4.40 AB	2.73 A
10	3.93 AD	2.86 A
11	3.73 B-D	3.20 A
12	4.13 A-C	3.00 A
13	3.26 D	2.73 A
14	4.33 A-C	2.93 A
15	4.25 A-C	2.73 A
16	3.86 A-D	3.13 A
17	4.33 A-C	3.22 A
18	4.00 A-D	3.00 A
19	4.00 A-D	3.26 A
20	3.86 A-D	2.73 A
21	3.66 B-D	3.00 A
22	3.67 B-D	2.83 A
23	4.12 A-C	2.70 A
24	4.33 A-C	3.17 A
25	3.80 B-D	3.22 A
26	3.66 B-D	2.93 A
27	4.19 A-C	3.23 A
28	4.13 A-C	2.86 A
29	3.69 B-D	3.03 A
30	4.13 A-C	3.40 A
31	3.69 B-D	2.59 A
32	3.86 A-D	2.86 A
SL28	3.89 A-D	2.86 A
Catimor	3.86 A-D	2.73 A
Ruiru11	3.87 A-D	2.82 A

Means on each column followed by the same letter are not significantly different at $p \leq 0.05$

however low, (Table 23), implying that only limited progress from selection is expected. Yearly variations were significant ($p \leq 0.05$) for both raw and roast bean qualities when scored on population "B22A" separately or when check varieties were included (Table 21). The quality of raw and roast beans was highest in the third and second year respectively but lowest in the 4th year for both characters (Figure 14).

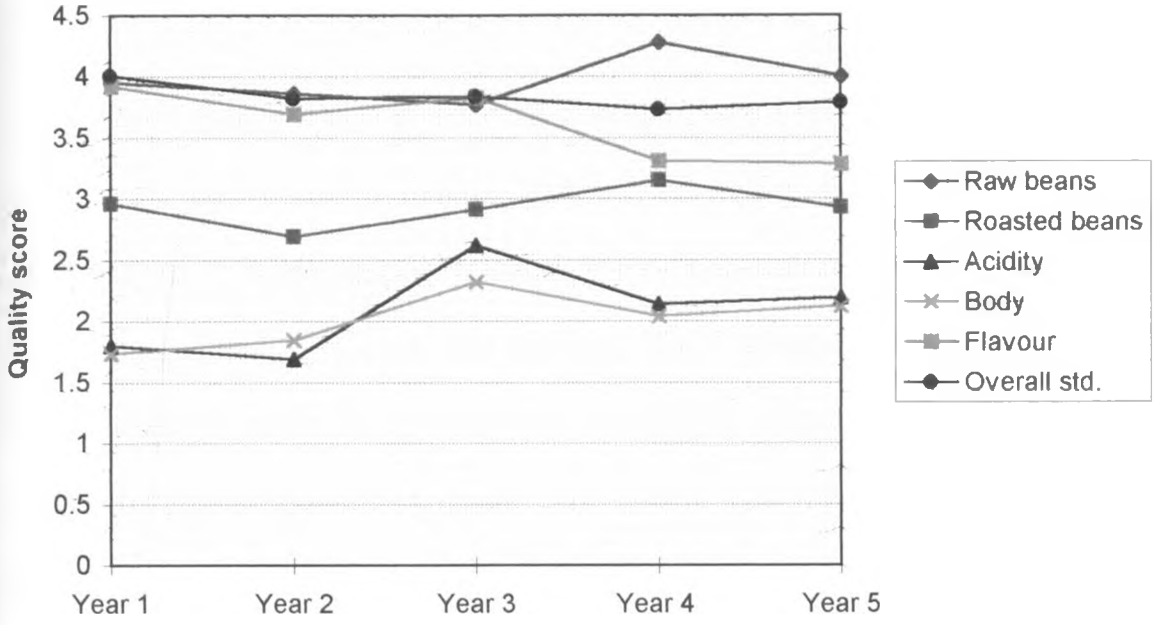


Fig. 14: Yearly variation for bean and liquor quality characters

4.6.4 Variation analysis for liquor quality characters.

Liquor quality is one of the most important characters determining the price of coffee at the Kenya coffee auction. Variation for acidity, body and flavour were not significant ($p \leq 0.05$) among families within population "B22A" when analysed separately or when compared with standard check varieties (Table 21). The means are presented in Table 27. The families identified in section 4.6.3 as combining high yield with high proportion of grade "AB" beans and raw bean quality also have acceptable acidity, body and flavour. Significant yearly variations ($p \leq 0.05$) were observed for all characters with both forms of analyses. Best scores for acidity, body and flavour were in years 2, 1 and 5 respectively (Figure 14).

Families within population "B22A" differed significantly ($p \leq 0.05$) for overall standard when analysed separately and when check varieties were included in the analysis (Table 21).

Table 27. Means of liquor quality characters in population "B22A"

Family No /Variety	Mean score			
	Acidity	Body	Flavour	overall std
1	2.00 A	1.80 A	3.80 A	4.00 A-E
2	2.00 A	2.10 A	3.37 A	3.63 B-E
3	1.80 A	2.00 A	3.53 A	3.66 B-E
4	1.80 A	1.60 A	3.80 A	4.20 A-C
5	2 .40 A	2.60 A	3.66 A	3.80 A-E
6	2.60 A	2.40 A	3.73 A	3.93 A-E
7	1.80 A	1.60 A	3.06 A	3.46 DE
8	1.90 A	1.80 A	3.40 A	3.73 A-E
9	2.40 A	2.40 A	3.80 A	4.06 A-D
10	2.20 A	2.40 A	3.66 A	3.86 A-E
11	2.40 A	2.40 A	4.13 A	4.40 A
12	1.60 A	1.60 A	2.73 A	3.33 E
13	1.40 A	1.60 A	3.66 A	3.80 A-E
14	2.60 A	2.40 A	3.93 A	3.86 A-E
15	2.20 A	2.20 A	3.20 A	3.66 B-E
16	2.60 A	2.00 A	4.13 A	4.13 A-D
17	1.99 A	1.95 A	3.68 A	3.95 A-E
18	1.80 A	2.20 A	3.40 A	3.60 C-E
19	2.20 A	2.00 A	3.86 A	3.86 A-E
20	2.20 A	1.60 A	3.74 A	3.86 A-E
21	1.80 A	2.20 A	3.33 A	3.86 A-E
22	1.70 A	2.00 A	3.17 A	3.70 A-E
23	1.80 A	1.90 A	3.20 A	3.30 E
24	2.60 A	2.00 A	3.73 A	3.97 A-E
25	2.36 A	2.15 A	3.68 A	4.17 A-D
26	2.40A	1.80 A	3.77 A	4.07 A-D
27	2.00 A	1.60 A	3.53 A	3. 80 A-E
28	1.86 A	1.75 A	3. 52 A	3.47 DE
29	2.13 A	1.80 A	3.76 A	4.01 A-E
30	1.80 A	1.60 A	3.53 A	3.93 A-E
31	1.86 A	1.95 A	3.45 A	3.70 A-E
32	2.40 A	2.50 A	3.80 A	3.73 A-E
SL28	1.73 A	1.90 A	3.26 A	3.61 B-E
Catimor	2.60 A	2.60 A	4.33 A	4.33 AB
Ruiru 11	2.11 A	2.03 A	3.48 A	3.82 A-E

Means on each column followed by the same letter are not significantly different at $p \leq 0.05$

Family number 23 had the best score of 3.30 compared to 3.61 for SL28, 4.33 for Catimor and 3.82 for Ruiru 11 (Table 27). Although this family was not outstanding for yield and bean quality characters, it provided an opportunity to relate its liquor quality character with outstanding family numbers 1, 2, 10, 11, 13, 19, 20, 21, 22, 31, and 32. Based on results in Table 27, all these families except number 11 were statistically comparable ($p \leq 0.05$) to the outstanding family number 23 and the commercial Kenyan cultivars, SL28 and Ruiru 11. The proportion of genetic effects for liquor quality characters were all less than 10%. Correlation analysis results indicate that the overall standard had significant ($p \leq 0.01$) association with raw beans ($r = 0.55$), roasted beans ($r = 0.51$), acidity ($r = 0.29$) and flavour ($r = 0.71$) (Table 28). Body had only weak positive correlation with overall standard ($r = 0.15$). Other significant positive corelations were between raw and roasted beans ($r = 0.51$, $p \leq 0.01$), acidity and body ($r = 0.66$. $p \leq 0.01$), flavour and roasted beans ($r = 0.25$, $p \leq 0.05$), flavour and acidity ($r = 0.47$, $p \leq 0.01$) and flavour and body ($r = 0.28$, $p \leq 0.01$). Yearly variations for overall standard were not significant (Table 21).

Table 28. Phenotypic correlation among bean and liquor quality characters

	Raw beans	Roast beans	Acidity	Body	Flavour	Overall std
Raw beans		0.51**	-0.04	-0.18	0.20	0.55**
Roast beans			0.04	0.00	0.25*	0.51**
Acidity				0.66**	0.47**	0.29**
Body					0.28**	0.15
Flavour						0.71**
Overall std						

* $p \leq 0.05$

** $p \leq 0.01$.

4.6.5 Variation analysis for field resistance to CBD.

Percent active CBD differed significantly ($p \leq 0.01$) among families only when check varieties were included in the analysis (Table 21). The susceptible SL28 variety had a significantly ($p \leq 0.05$) higher CBD score than all the other genotypes (Table 29). All the families already identified to combine high yield with superior bean and liquor quality also exhibited field resistance to CBD. Yearly variations for CBD score were not significant ($p \leq 0.05$, Table 21). The CBD scores were generally very low over the years except in the fourth year (Figure 15). The trait exhibited no genetic variation that could be exploited by selection ($H^2 = 0.00$). Therefore, in conclusion, out of the 32 families, 10 were outstanding. These included family numbers 1, 2, 10, 11, 13, 19, 20, 21, 22, 31 and 32. They combined field resistance to CBD with high yield and superior bean and liquor quality.

Table 29 Means for CBD score in population "B22A"

Family No /Variety	CBD score
1	0.05 B
2	0.07 B
3	0.00 B
4	0.00 B
5	0.00 B
6	0.00 B
7	0.09 B
8	0.00 B
9	0.00 B
10	0.00 B
11	0.06 B
12	0.00 B
13	0.00 B
14	0.02 B
15	0.80 B
16	0.00 B
17	0.00 B
18	0.00 B
19	0.08 B
20	0.00 B
21	0.00 B
22	0.00 B
23	0.00 B
24	0.00 B
25	0.03 B
26	0.00 B
27	0.00 B
28	0.03 B
29	0.02 B
30	0.00 B
31	0.00 B
32	0.00 B
SL28	2.61 A
Catimor	0.00 B
Ruiru 11	0.25 B

Means on each column followed by the same letter(s) are not significantly different at $p \leq 0.05$

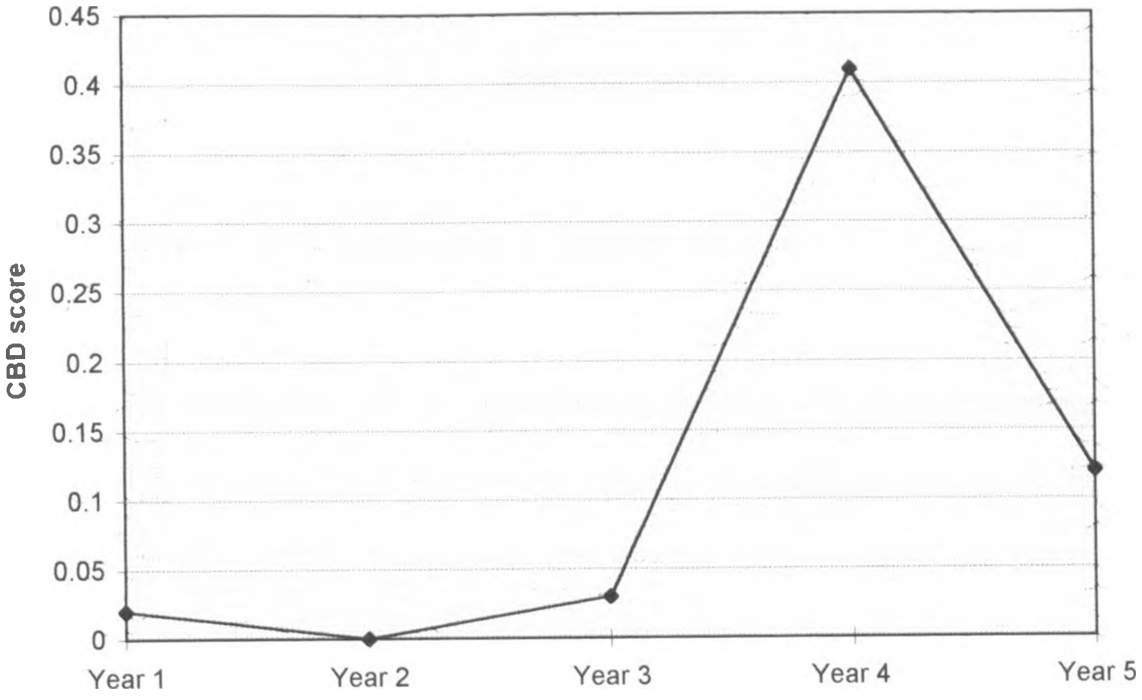


Fig. 15: Yearly variations for CBD score

CHAPTER FIVE.

DISCUSSION.

5.1 Virulence of *C. kahawae* on *C. arabica* varieties.

The observation that the main effects of varieties and isolates were significant ($P \leq 0.01$) is in agreement with the general outcome of studies on interactions between *C. arabica* varieties and *C. kahawae* isolates carried out in Ethiopia and Kenya (van der Graaff 1978, Masaba and van der Vossen, 1980). These authors observed that variation among isolates of *C. kahawae* was mainly due to differences in aggressiveness (variety non-specific). The unique feature of variation in aggressiveness is that the entire range of host differentials show an increase or a decrease of infection when inoculated with a specific pathogen strain. The apparent lack of variety specific variation associated with the existence of races was probably due to the fact that the pathogen co-evolved with a genetically narrow based host species. The greatest diversity of *C. arabica* species is found in the highlands of South West Ethiopia. CBD was however, never in Ethiopia until 1971 implying that there was hardly any substructuring of the pathogen population based on host selection pressure that would have resulted in physiological specialization. The genetic uniformity of the pathogen may also have been perpetuated by its clonal nature of reproduction. There is no conclusive evidence for the existence of a sexual stage in the pathogen. Sexual reproduction not only generates variation through gene recombination but also provides an opportunity to purge deleterious genes

accumulated over time in clonal lineages (Leung et al., 1993).

Analysis of variance further indicated that the single largest variation was due to varieties (49.83%). The large variation could be attributed to the fact that the varieties had not been previously subjected to selection for resistance and hence they reflected to a large extent the natural diversity. An important progenitor of CBD resistance, Rume Sudan expressed intermediate resistance to the test isolates implying that the isolates were highly virulent. Intermediate resistance was also expressed by varieties Pretoria, K7, and Padang. These varieties formed a distinct cluster group. van der Vossen and Walyaro (1980) carried out inheritance studies of CBD resistance on these varieties and concluded that the resistance was oligogenic. The variety Rume Sudan carries the dominant R- and the recessive k-genes. The R-locus has multiple alleles with R_1R_1 in Rume Sudan and R_2R_2 in Pretoria which also carries the recessive k-gene. Variety K7 carries the recessive k-gene. The inheritance of resistance in Padang is not clear. The variety Hibrido de Timor was only partially reisistant to these isolates. It carries the dominant T- gene for resistance. The partial resistance could be as a result of segregation in the variety. One of the most important underlying assumptions in virulence tests is that the host differentials are homozygous. This assumption was found to be valid with respect to yield and its components for the 11 varieties used in this study. It is however uncertain if this assumption is true for CDB resistance in Hibrido de Timor.

Virulence analysis revealed that the variety x isolate effect was significant. A similar outcome was reported by Rodrigues Jr. et al. (1992) in a study carried out with Angolan, Malawian and Kenyan isolates. Contrary to the earlier observation that virulence of the CBD isolates is entirely variety non-specific, the authors concluded that

physiological strains of the CBD pathogen might exist. However, in this study the proportion of variety x isolate interaction, usually associated with physiological specialization was significant but too small to suggest conclusively that races exists. It was further observed that grouping of varieties based hierarchical cluster analysis using ranks was similar to grouping obtained from mean grade of infection thus confirming that changes in rank among varieties were not significant and may have not been as a result of the presence of races. This conclusion is further confirmed by the fact that the partial contribution of individual isolates to the total variety x isolate interaction were all less than 3 % except in 4 isolates, S9.9 (3.09%), S11.6 (4.89%), S11.9 (3.08%) and S12.5 (3.41%). Using the same analysis, varieties Rume Sudan, Pretoria, K7 and Padang were identified as having high relative contributions to the total variety x isolate interactions of 16.18%, 14.28%, 16.02% and 17.23% respectively. It was further illustrated that the pattern of virulence of the 4 isolates on varieties with high contributions reflected large differences in aggressiveness with small differential interactions.

The observation that differences exist among isolates makes their detection even more important. The classical method of characterizing pathogen populations using a set of host differentials is still widely used with its inherent limitations notwithstanding. This method of detecting genetic variability is however of special interest because it gives information on the physiological specialization of the pathogen. When a large differential set is available, the potential to detect variation in virulence can be enormous (Caten, 1987). Burdon (1993) indicated that when each variety-isolate combination gives two possible outcomes (compatible or incompatible), 2^n races can theoretically be differentiated, where n is the number of gene pairs. The three-gene model suggested by van der Vossen and Walyaro (1980) has the potential of detecting only 8 ($2^n = 2^3 = 8$)

different races. Very often however, the differentials used posses more than one resistance gene or unknown resistance factors. For instance, varieties Rume Sudan and Pretoria carry the dominant R - and the recessive k-genes in duplicate combination. This further limits the number of detectable races. Intensive efforts are required to develop genetically defined differentials, such as near-isogenic lines (NILs) with single resistance gene differences. In a perennial crop like coffee, the development of NILs is not only time consuming but also costly. Variation could therefore seriously be under-estimated if host differentials alone are used to characterize pathogen populations. Perhaps the most serious limitation of virulence analysis using host differentials is that variation do occur during disease assessment even under controlled conditions (Gowen et al. 1989). To overcome this problem, variety x isolate interaction effects require assessment over several experiments but usually at an added cost. More sensitive measures of variety x isolate interaction such as significant differences in isolate ranking and interaction index proposed by Royer et al. (1984) have also been suggested as alternatives to the F-statistic term in the analysis of variance as more reliable measures.

5.2 Protein electrophoresis

Protein analysis was based on presence or absence of bands and their relative mobility (Rf) according to molecular weights. Protein functions could not be inferred from this data. A large number of bands ranging between 20 and 30 were counted per isolate with profile variations between experiments that were mainly due to the sensitivity of the electrophoretic procedure. Biratu (1995), however, counted 68-76 migrating bands for each isolate. The large difference in the number of bands could partly be attributed to

the differences in the electrophoretic procedure and partly due to the differences in isolates used. In this study, the protein extraction buffer consisted of 1.0 M Tris, 10% (V/V) glycerol and 5% (V/V) 2-mercaptoethanol, while Biratu (1995) used 0.06 M Tris HCl, 2% SDS, 10% glycerol at pH 6.8. The presence of SDS in the extraction buffer could have been responsible for the high degree of protein dissociation. Biratu (1995) also used an 8-15% SDS gradient polyacrylamide gel instead of the 10% gel used in this study. Gradient gels achieve better separation of proteins than uniform gels (Cole et al., 1991, Laemli, 1970). Gels with low acrylamide concentration only separate proteins of high molecular weights, while those of low molecular weights are not sieved and therefore migrate to the bottom. Gels of high acrylamide concentrations separate proteins of low molecular weights, while the mobility of high molecular weight proteins are impeded. Gradient gels therefore separate high molecular weight proteins in the region of low acrylamide concentration and low molecular weight protein in the region of high acrylamide concentration.

Protein profiles clearly distinguished the non-pathogenic *C. acutatum* from the pathogenic *C. kahawae* isolates indicative of its potential applicability in delimiting species. These results corroborate the findings of Biratu (1995) that protein electrophoresis are reliable in detecting large interspecific differences. Shipton and MacDonald (1970) also reported that variation between species are easily detected by protein electrophoresis provided the intraspecies difference is smaller than the interspecies contrasts. From the simple matching coefficients, isolates of *C. kahawae* were closely related (S = 0.60 - 1.00) to each other but distantly related to *C. acutatum* (S = 0.20 - 0.40). Intraspecies differences were also observed among isolates of *C. kahawae*. The differences were however, not diagnostic for pathogenicity, Benomyl

tolerance or host variety of origin. Biratu (1995) also reported similarities and differences in protein profiles which were irregularly distributed in the *Colletotrichum* species. The major drawback of protein electrophoresis is that variation in protein components may not be detected by electrophoresis if they do not involve changes in molecular weights and hence different rates of mobility. Therefore genetically different isolates may have different co-migrating proteins which are scored as similar bands.

5.3 Isozyme electrophoresis

A total of 12 enzyme systems were assayed, out of which, 9 were clearly resolved and 28 putative loci detected. Isozyme diversity was observed not only between species but also within species. This is consistent with results obtained from analysis of total proteins. Isolates were classified into 7 electrophoretic types with *C. acutatum* and *C. gloeosporioides* forming single species phenotypes indicative of their separate taxonomic status. Important enzymes in species delimitation were EST, PGI, and MPI. Biratu (1995) also investigated isozyme variation between pathogenic (*C. coffeanum* = *C. kahawae*) and non-pathogenic (*C. acutatum* and *C. gloeosporioides* acervuli and mycelium forms) species using 7 enzyme systems, and found that diversity between species was revealed by EST and PGI in addition to IDH, LAP, and MDH. The author observed that *C. acutatum* was largely divergent from the closely related *C. gloeosporioides* and *C. coffeanum* and concluded that the pathogenic *C. coffeanum* should be renamed *C. gloeosporioides* f. sp. *coffeanum*. Classification at the forma specialis level was considered more conventional because of the specificity of the pathogen to *C. arabica* host species. Polymorphism for PGI has also been detected in *C.*

gloeosporioides from different hosts (Lenne and Burdon, 1990; Bonde et al. ,1991; Kaufman and Weidmann, 1993). PGI was also ranked with EST as the best enzymes in differentiating rust races in pines corresponding to symptomatology and shape of aeciospores (Power et al., 1989).

In this study, MDH and IDH were monomorphic while LAP was not assayed. A possible explanation for these contrasting observations is that the Kenyan isolates used were geographically of widely different origin from the Ethiopian isolates tested by Biratu (1995). The electrophoresis procedures such as buffer systems and gel types also differed. Isozyme phenotypes may differ over surveys carried out at different times or localities (Burdon and Roelfs, 1985). The importance of standard equipment in comparing results of isozyme electrophoresis has been emphasised by Cole et al., (1991). The author observed that alcohol dehydrogenase which is sensitive to oxidation did not stain when run on mini-gel apparatus because of localised heating which deactivated the enzyme. However, the enzyme stained when run on big gels totally imersed in circulating buffer during electrophoresis.

Isolates of *C. kahawae* were subdivided into 5 electrophoretic types. *Est* loci were the most variable, partitioning the isolates into 3 electrophoretic types. The remaining 2 electrophoretic types were as a result of variability at the *Ldh* locus. Isozyme variation for esterase has been reported in many pathogenic fungi (Bonde et al. ,1991; Power et al., 1989 and Biratu, 1995). Isolates carrying the *Est-B8* band singly or in combination with *Est-B12* were all obtained from resistant host varieties except the benomyl tolerant strain BR.4. Isolates lacking activity of the *Est-B8* and *Est-B12* bands were obtained from susceptible hosts. The apparent overlap of isolate BR.4 obtained from a susceptible variety with isolates obtained from resistant varieties is an indication that the

two bands, *Est-B8* and *Est-B12* are not diagnostic for host-related virulence. These results considered together with the small magnitude of host x isolate interactions supports the hypothesis that physiological races of the pathogen might not be present in the population.

The Benomyl tolerant strains could be distinguished from the Benomyl sensitive strains by variation at the *Ldh* locus. There was lack of activity for the *Ldh-B167* band in the Benomyl tolerant strains. The Benomyl sensitive strains of *C. kahawae* and the non-pathogenic species stained for the band. A large sample of the test isolates is necessary to confirm the apparent linkage between Benomyl tolerance/sensitivity with variation at the *Ldh* locus. On small sample size, Zervakis et al. ,(1994) reached the conclusion that isozyme electrophoresis in multi-allelic systems has the advantage that some degree of compensation for limited sample size may be obtained since estimates of genetic diversity are more affected by number of alleles rather than number of individuals. Isozyme analysis is also useful as a distinct but a complimentary method of assessing pathogen populations. Burdon and Roelfs (1985) using defined pathogen races of *Puccinia graminis* f. sp. *tritici* found weak correlations between mean virulence and isozyme differences between groups, yet the two systems of analyses divided the pathogen population into the same clonal groups. Due to the co-dominant nature of isozymes, more phenotypes are revealed as compared to morphological markers. Unlike protein markers, segregating populations can be used to study inheritance of characters. Isozyme variations are however, constrained by the need to retain enzymatic function and the fact that many allelic variations do not result in differences in the electrophoretic mobility of the proteins and hence are not detected (Murphy et al.,1990). Due to these limitations, isozymes have been gradually replaced by techniques that exploit

polymorphism available at the DNA level.

5.4 Random Amplified Polymorphic DNA (RAPD) analysis

A total of 487 amplification products were detected with 36 decanucleotide primers. The large number of bands detected support the conclusion that DNA-based analyses detect variation with more precision than analyses based on products of gene expression (Freeman et al., 1993). RAPD analysis also allows a large number of primers to be screened thus increasing the chances of detecting nucleotide polymorphism. In this study, 3 primers, (5', 3'), CCC TCT CTT T, AAG GCA CCA G and GTG TTT CCG G, failed to amplify DNA from both pathogenic and non-pathogenic species. Failure to amplify DNA may arise because of mismatch between the primer and the template DNA (Williams et al., 1990) or total lack of homology between the primer and the annealing sites (Kelly et al., 1994). If no amplification occurs because of mismatch of otherwise homologous primer/template combination, repeating the PCR process may produce amplification products. However, no amplification products were observed in the second PCR with the 3 primers. Therefore, it was concluded that there was no homology between the primers and the template DNA from all the 3 species.

An average of 13.6 bands were detected per primer and fragment sizes ranged between 300 and 2500 bp. These fragment sizes were within the range of most plant pathogenic fungi (Freeman et al., 1993; Leung et al. ,1993; Sreenivasaprasad et al. ,1993 and Huff et al. ,1994). Up to 80% of the total bands detected were polymorphic, the majority of which (71%) distinguished the non-pathogenic *C. gloeosporioides* and *C. acutatum* from the pathogenic *C. kahawae*. These results are consistent with the

outcome of protein and isozyme analysis indicating that there is greater variation between species than within species. Sreenivasaprasad et al. (1993) also observed large differences between *C. kahawae* isolates and *C. gloeosporioides* species with both RFLP and RAPD profiles. An underlying assumption of RAPD analysis is that marker bands of the same size originate from the same genomic locus (Huff et al., 1994). This assumption is reasonable for intraspecific comparisons (Huff and Bara, 1993; Welsh and McClelland ,1990), but it is most likely not true for interspecific comparisons. This is a serious disadvantage with most analyses based on electrophoresis with ultimate effect of underestimating the natural genetic diversity of species due to co-migration of different bands.

Only 9% of the total polymorphic bands were due to differences among isolates of *C. kahawae* indicative of the high level of uniformity within the species. Close relationship among isolates of *C. kahawae* was also revealed by protein and isozyme electrophoresis. Although measures of complete similarity were obtained with protein analysis (S=1.00), RAPD similarity coefficients ranged between 0.88 and 0.93 supporting the view that RAPD primers are more sensitive to single base changes in the primer-target sites making it a useful method for phylogenetic analysis among closely related individuals (Williams et al., 1990). Comparatively, more bands were detected among isolates of *C. kahawae* with RAPDs than proteins or isozymes. This may be attributed to the fact that RAPD markers detect both coding and non-coding sequences in the genome. Estimates based on isozyme variability may underestimate overall variability because they sample only coding regions that may be conserved to maintain the function of the enzyme. The non-coding regions of DNA are believed to be more variable and may in part be responsible for the comparatively high RAPD polymorphism

(Kongkiatngam et al. 1995).

Groups generated by cluster analysis using RAPDs and protein profiles comprised of similar isolates with few exceptions. The small dissimilarities could be as a result of the differences in sensitivity of the two methods in discriminating between individuals. Like proteins, RAPDs did not detect markers that could be traced back to specific virulence groups or Benomyl tolerance. It is believed that the virulence phenotype of plant pathogenic fungi is controlled by relatively few loci (Flor, 1971; Michelmore and Hulbert, 1987). Since arbitrary primers were used to analyse the fungal genome, it could be possible that the pathogenicity loci were outside the amplified regions. The same argument may be true for Benomyl tolerance. To detect variation in such loci, a large number of primers have to be screened. Borovkova et al. (1995) evaluated up to 500 10-base primers and only 3 produced reliable polymorphism linked to the stem rust resistance gene *rpg4* in barley. A possible source of bias when relying on RAPD markers for detecting variation is due to their dominant nature. The presence of a given RAPD band does not distinguish whether its respective locus is homozygous or heterozygous (Williams et al., 1990, Williams et al., 1993). Estimates of allele frequencies are therefore considerably reduced relative to analysis using co-dominant markers (Lynch and Milligan ,1994). Perhaps this may have contributed to the relative ease with which a marker possibly associated with Benomyl tolerance was detected with isozymes and not with proteins or RAPDs. To increase chances of detecting RAPD markers, a large number of primers have to be screened on a large sample of isolates.

5.5 Interactions between isolates of *C. kahawae* and Ruiru 11 genotypes

This study follows a previous investigation of resistance to CBD on Ruiru 11 using bulked inoculum obtained from a field with natural infection (Omondi,1994). Monoconidial isolates already shown to be genetically distinct by morphological, biochemical and molecular characterization were used in this study to investigate the influence of genetically diverse isolates on the cultivar Ruiru 11. Analysis of variance revealed significant ($p \leq 0.05$) main effects (genotypes and isolates) while their interaction was not significant. The combined effects of genotypes and isolates accounted for 41.71% of the variation, while interaction effect accounted for 24.95%. These results further confirm that variation for virulence is not genotype specific and the differences in resistance among Ruiru 11 genotypes are probably due to differences in aggressiveness of the isolates or heterogeneity of the variety.

Partitioning variance components revealed that only a small proportion of the phenotypic variance (for resistance) was due to genetic effects. Broad sense heritability was 0.23. This is consistent with the low narrow sense heritability of 0.04 previously estimated in the same Ruiru 11 population using offspring-midparent regression analysis (Omondi, 1994). Low heritability implies limited variability and therefore selection for resistance within the Ruiru 11 population will only be rewarding if directed in favour of genotypes with multiple resistance genes. It can be concluded that the low heritability for resistance in Ruiru 11 is because the variety combines favourable attributes of two parent populations previously screened and selected for CBD resistance. An analysis of general (GCA) and specific combining abilities (SCA) within the Ruiru 11 population revealed that SCA which is usually due to non-additive genetic effects was more

important in determining resistance than GCA (Omondi, 1994). On the contrast , van der Vossen and Walyaro (1980) observed a higher proportion of GCA than SCA when analysis was based on *C. arabica* varieties that had not been previously screened for resistance. These results further support the view that the proportion of GCA, which is a function of additive genetic variance, is higher in previously non-selected populations than in populations which have undergone screening and selection. In Ruiru 11 where SCA is predominant, superior parental combinations that exploit the desired epistatic and dominance effects, the principle components of non-additive genetic variation, should be identified and propagated vegetatively to avoid loss of the favourable non-additive effects by recombination. Due to the high variability for aggressiveness among isolates even within localities, Ruiru 11 should be distributed to growers as a composite of all the possible genotypes.

Further partitioning of the components of variance revealed that the variance due to genotype x isolate interaction was negative. Theoretically, variance estimates cannot be negative. However, the occurrence of negative estimates of genetic variance has been reported (El Rouby and Penny, 1967; Leone et al., 1968, Lindsey et al.,1962; Omondi and Ayiecho, 1995). The negative estimates are usually attributed to some combination of an inadequate genetic model (no epistatic effects in the model), sampling error; inadequate experimental design (competition effects among the individuals) and assortative mating (Lindsey et al. ,1962). It was estimated by Bridges and Knapp (1987) that the probability of obtaining negative estimates of genetic variance components due to sampling error alone was often greater than 0.20. They also indicated that the probability was lower for estimates of additive genetic variance (σ^2_A) than dominance variance (σ^2_D). Estimates from factorial mating designs (Design II) had lower

probabilities of negative variance estimates due to sampling error than estimates from nested mating designs (Design I). In this study, the negative values of variance components was interpreted as lack of variance and no estimation as to the relative importance of each attribute contributing to the occurrence of negative variance was performed.

5.6 Evaluation for yield, quality and field resistance to CBD in an advanced breeding population "B22A".

Evaluation of breeding populations should not only identify genotypes which can withstand pathogen variability but should also ensure that such genotypes combine high productivity with superior quality that satisfy the demands of both growers and consumers. Yield as a measure of productivity was evaluated in the breeding population "B22A". Of the total variation for yield, a moderate proportion was due to genetic effects as indicated by significant variation among families and a broad sense heritability estimate of 0.41. Walyaro and van der Vossen (1979) also observed highly significant genotypic effects for most growth and yield characters in a number of Arabica coffee varieties with high to moderate heritabilities. This is despite the fact that the character exhibits continuous variation and is therefore expected to be under quantitative or polygenic control with high environmental influence (Walyaro ,1983). Population "B22A" is derived from a hybridization programme involving genetically diverse parents which might have created new gene combinations resulting in moderately high genetic effects. In the genetically heterogeneous germplasm consisting of Turrialba collections and populations of the 1964 FAO mission to Ethiopia, Carvalho and Monaco (1972) found

that a large proportion of the total variation for yield was due to genetic effects. On the contrary, several authors observed low levels of heritability for yield in genetically narrow based population of coffee (Stoffels, 1941; Gardner,1950; Elguetta, 1950; Mendes et al., 1941; Castillo, 1957).

A unique feature of assessing yield in a perennial crop like coffee is that several measurements are required due to the seasonal/yearly variations. Results indicate that mean yields increased from year 1 reaching a maximum in year 3. This may be attributed in part to the growth of the tree increasing in the number of bearing surfaces. In year 4, a drop was observed followed by an increase in year 5. This could be explained by the biannual bearing pattern of coffee. Biannual bearing is described as a production pattern where a year of high yield is followed by a year of low production in a cyclic manner. This is a physiological phenomenon which is often observed in coffee. Due to the large seasonal/yearly variations, Walyaro and van der Vossen (1979) used estimates of phenotypic and genotypic variances and covariances of a number of growth characters and components of yield to construct selection indices. It was concluded that selection based on the first 2-4 years yield record combined with measurements such as girth and percentage bearing primaries were sufficient for efficient selection for higher productivity in Arabica coffee. Through this method, the length of the breeding cycle could be reduced to 5 years. Based on the 5 years yield record, Ruiru 11 was the overall highest yielder with a mean of 4.73 kg/tree compared to the range of 2.16 - 4.51 kg/tree in the test families of population "B22A". The yields for SL28 and Catimor were 3.75 kg/tree and 3.08 kg/tree respectively. Ruiru 11 variety is derived from outstanding selections of population "B22A" and Catimor. The transgressive yield of the variety could therefore probably be due to heterosis. Despite

the variation, most families except numbers 3, 5 and 17 had yields that were statistically comparable to the traditional SL28 variety and Ruiru 11.

Although productivity is an important trait in coffee breeding, quality is even more important because it determines the price and ultimate income to the farmer. The Kenyan coffee quality character renown worldwide has helped put Kenya coffee on a competitive edge fetching attractive premium prices. The breeding programmes at the Kenya's Coffee Research Station, Ruiru, have been sensitive to quality demands. The important quality parameters are bean grade and liquor or cup quality. Bean grades "AA" and "AB" are the premium grades fetching higher prices. Families in population "B22A" differed significantly for bean grades "AA" and "AB" but the proportions of the variations due to genetic effects were generally low implying that only limited progress is expected from selection. A significant negative correlation was observed between bean grades "AA" and "AB" indicating that increased proportion of one grade will result in a negative correlated response of the other grade. Walyaro (1983) observed similar relationship between bean grades "AA" and "AB" and concluded that selection should be directed on "AA" which is considered more superior than "AB" and ignore the negative correlated response of "AB". Adams (1967) and Crafius (1978) reported that the observed correlation between traits are due to genetic linkages, pleitropic effect of genes or physiological and developmental relations. Negative relationships when present indicate some form of competitive inhibition. This often arises from the fact that the traits compete for the same total amount of metabolic substrates produced by the plant. Studies on trait relationships have useful applications in selection (Omondi and Ayiecho, 1992). Since the broad sense heritability estimates for both grades were low there was no advantage of using one grade as a criteria over the other. However grade "AB" was

used instead of "AA" because all test families had lower proportion of grade "AA" beans than the check varieties. An optimum ratio of bean grades "AA" and "AB" will therefore be determined by the genotype and the growing conditions. Due to significant yearly variations for bean grade" AB", selection for this trait requires several measurements. This is a disadvantage because superior genotypes cannot be determined early in the selection programme. Several families combined high yield with high proportion of grade "AB" beans.

Families were also evaluated for quality of raw and roast beans. Family effects were significant ($p \leq 0.05$) for raw bean quality but non-significant for roast bean quality. Broad sense heritability estimate was low implying that genetic effects were of limited contribution. The non-significant effect of roast bean character could be attributed to the physical effect of roasting rather than genetic effects. Outstanding genotypes were therefore identified on the basis of raw bean score. Yearly variations were significant for both traits indicating that selection must be based on repeated measurements.

Variation for liquor quality characters were not significant among genotypes of population "B22A" and were similar to the check varieties. The limited variation is probably due to the fact that the traditional fine quality Kenyan selections like SL28, SL34, K7 and SL4 were included in the pedigree to impart the good quality. Therefore the families identified as combining high yield, with a high proportion of grade "AB" beans and good raw bean quality also had good acidity, body and flavour. Due to the significant yearly variations, these characters require repeated measurements. Significant family effects were observed for overall standard. However, yearly variations for the same trait was non-significant. Overall standard which is regarded as the ultimate determinant of quality was positively and significantly correlated with raw and roast bean

characters as well as acidity and flavour. Body had only a weak positive correlation with overall standard. Ten outstanding families including 1, 2, 10, 13, 19, 20, 21, 22, 31 and 32 were identified in population "B22A" as combining high yield, with good bean and cup quality.

Field resistance to CBD exhibited non-significant variation among families of population "B22A" . It is important to note that these genotypes were subjected to the pre-selection test in the laboratory before field planting. This confirms the conclusion that there is high correlation between pre-selection test based on hypocotyl seedlings and field resistance to CBD. Therefore, the already identified outstanding families were also highly resistant for CBD. Variation was significant when standard check varieties were included in the analysis mainly because of the susceptible SL28 which had a high percentage CBD score than all the other genotypes. CBD resistance was uniformly expressed across all the five years. Preselection tests based on hypocotyl seedlings is therefore a reliable procedure for CBD screening.

CHAPTER 6.

CONCLUSIONS AND SUGGESTIONS FOR FURTHER RESEARCH.

6.1 Scope of the study.

This study addressed four main areas:

6.1.1 the nature of interaction between *C. Kahawae* isolates and varieties of *C. arabica* varying in resistance.

6.1.2 the magnitude of genetic diversity in the pathogen population.

6.1.3 the nature of interaction between the CBD resistant cultivar Ruiru 11 and isolates of *C. Kahawae* varying in virulence.

6.1.4 Identification of genotypes combining field resistance to CBD with high yield and good quality in advanced breeding populations of *C. arabica* .

6.2 Conclusions

6.2.1 Variation for virulence within *C. kahawae* is unlikely to be due to the existence of races but could be predominantly due to differences in aggressiveness in combination with other environmental factors.

6.2.2 The magnitude of genetic variation is higher among coffee varieties which have not undergone selection than in a natural population of isolates.

6.2.3 Interspecific variation among *Colletotrichum* species colonizing coffee is larger than intraspecific variation within *C. kahawae*.

6.2.4 There is no evidence of adaptation of *C. kahawae* to the resistant *C. arabica* cultivar Ruiru 11. However, variation for resistance could be observed within the variety perhaps due to its composite nature and environmental factors which favour severe disease epidemics.

6.2.5 Elite families of *C. arabica* that can withstand the high pathogen variability in the field across seasons and produce high yields of fine quality coffee are present in the advanced breeding population, B22A. The tall-statured families could be recommended for release as a commercial variety to supplement the limited supply of the hybrid Ruiru 11 cultivar due to their unique trueness-to-type especially for CBD resistance.

6.3 Suggestions for further research

6.3.1 The dynamism of pathogen variability over space and time requires regular virulence surveys especially if the resistant varieties have been widely grown.

6.3.2 Due to the limited range of host differentials, some of which have duplicate combinations of resistance genes, more biochemical and molecular markers for pathogenicity should be screened.

6.3.3 Due to the large pathogen variability in the field especially for aggressiveness, the breeding programme should identify families with broad based resistance from the elite populations. By use of testcrosses and/or marker aided selection (MAS), genotypes carrying multiple genes of resistance can be identified.

6.3.4 The elite breeding populations should be evaluated to identify genotypes with all the desired characters fixed to be released as a true-to-type seed variety and to hasten the pace of replacing traditional varieties.

6.6.5 Since all the Kenyan commercial varieties are susceptible to bacterial blight of coffee and the disease appears to be spreading to other coffee growing areas from the Solai and Mt Elgon areas where it was originally confined, it is becoming a priority to introgress BBC resistance into commercial varieties with high yield, fine quality and resistance to CBD and leaf rust.

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Appendix 1. Coffee quality attributes

RAW			ROAST			LIQUOR		
Size and shape	**Colour**	**Quality**	**Type**	**Centre-Cut**	**Quality**	**Acidity**	**Body**	**Flavour**
O Bold 1 Medium Bold 2 Medium 3 Mixed 4 Small	O Greyish Blue 1 Greyish Green with blue tinge 2 Greyish Green 3 Greyish Green with brown tinge 4 Brownish Grey Green 5 Greenish 6 Brownish	O Fine 1 Good to Fine 2 Good 3 Fair to Good FAQ fully 4 FAQ 5 Poor to Fair 6 Poor 7 Very Poor	O Bright 1 Ordinary 2 Dull	O White 1 Normal 2 Brownish	O Fine 1 Good to Fine 2 Fair to Good FAQ fully 3 FAQ 4 Poor to Fair 5 Poor	O Pointed 1 Medium 2 Light Medium 3 Light 4 Lacking	O Full 1 Medium 2 Light Medium 3 Light 4 Lacking	O Fine 1 Good to Fine 2 Good 3 Fair to Good FAQ fully FAQ 5 Poor to Fair 6 Poor

Overall Quality

O Fine
1 Good to Fine
2 Good
3 Fair to Good FAQ fully
4 FAQ
5 Poor to Fair
6 Poor
7 Very Poor