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NATURE OF RESISTANCE OF LYAMUNGU HYBRIDS TO *Colletotrichum kahawae* STRAINS

KILAMBO D.L.[1]*, SILVA M.[2], MABAGALA R.B.[3], VARZEA V.M.P.[2] AND TERI J.M.[1]

[1]Tanzania Coffee Research Institute P.O.Box 3004 Moshi, Tanzania.
[2]Instituto de Investigação Científica Tropical, Quinta do Marquês 2784-505 Oeiras, Portugal.
[3]Sokoine University of Agriculture P.O. Box 3005 Morogoro, Tanzania.
*Corresponding Author: Email- dkilambo@gmail.com

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Abstract- Nature of resistance of Lyamungu coffee hybrids to coffee berry disease (CBD) caused by *Colletotrichum kahawae* were evaluated using conidia germination on cuticular wax extracted from the hybrids, stimulatory effects on the infection of the pathogen, and evidence of genetic resistance on studies using hypocotyls and green berries in the field. Significant differences (P £ 0.05) were found between the levels of conidia germination on the extracted wax of the coffee genotypes. Nine coffee genotypes showed no conidia germination and five showed conidia germination percentage of below 20%. Conidial germination percentage levels of up to 90% were found on CBD susceptible variety N39. Light micrographs showed that hyphal growth of *C. kahawae* strains were restricted in epidermal cells and cortex of CBD resistant genotypes 20498 and 20509. Callose and thick walls were observed in cortex cells of resistant genotypes 20498 and 20509. Field evaluation also revealed same genotypes which exhibit highest levels of CBD resistance. In order to understand resistance of Lyamungu coffee hybrids, pathogenicity of 26 *C. kahawae* strains from different eco-agricultural zone in Tanzania was studied under laboratory conditions. Pathogenicity tests both on detached green coffee berries and hypocotyls drew a distinction between highly and less pathogenic strains. The *C. kahawae* strain 2006/14 was found to be the most pathogenic because it showed high sporulation capacity and also induces CBD symptoms 3 days after infection both on green coffee berries and hypocotyls.

Keywords- Nature of resistance, CBD pathogenicity, Lyamungu coffee hybrids

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Introduction

Coffee berry disease (CBD) caused by *Colletotrichum kahawae* Waller and Bridge is the most serious disease of Arabica coffee (*Coffea arabica L.*) in Tanzania. The pathogen infect all stages of the crop from flowers to ripe fruits, but maximum crop losses occur following infection of green berries initially forming dark sunken lesions and eventually the rotting of the berry. However, according to the history of CBD, the genetic resistance appeared complete in *Coffea canephora* and partial in *C. arabica*. Hocking [11] demonstrated that there is a notable variation in CBD resistance within coffee varieties. Mulinge [16], on the other hand, found out that the susceptibility of berries to CBD varies with the age of the crop; berries are most susceptible when they are between 4th and 16th weeks after flowering, and at ripening stage but pin heads and hard mature berries (≥ 16th week) are resistant. Differences in the resistance of coffee trees to CBD are frequently observed under laboratory and field conditions. The high levels of resistance were found in Rume Sudan [32-34].

Studies carried out in Kenya by Van der Vossen and Walyaro [32] concluded that coffee resistance to CBD appears to be controlled by major genes on three different loci. The highly resistant variety Rume Sudan carries the dominant R- and recessive k-genes. Hibrido de Timor carries one gene for CBD resistance on the T-locus. In Arabica coffee resistance mechanism to *C. kahawae* are both preformed and induced, and operate at different stages of pathogenesis [10]. The coffee berry cuticle could act as a physical barrier to the penetrating pathogen. Masaba & van der Vossen [15] accounts for scab formation on the berry surface as resulting from cork berries formation. Similar studies on the occurrence and possible role of fungi toxic compounds either preformed or induced in coffee berries with *C. kahawae* [5,6,11]. They concluded that coffee berries posses inherent antifungal compounds that counteract infection from *C. kahawae* species. Accordingly, Steiner [30] reported that surface wax extracted with chloroform from green berries of varieties Rume Sudan and Blue Mountain contain substances that decrease significantly conidial germination of *C. kahawae*.

Breeding programme on CBD resistance was initiated in Tanzania in early 1960s; and by 1990s, there were a number of Lyamungu coffee hybrids which were reported to have acquired resistant genes to *C. kahawae* [17]. These hybrids when tested for reaction

to *C. kahawae* strains from Lyamungu (TZ 005) between 1992 and 1993 using an attached berry technique reveal a number of coffee lines with resistance to the pathogen [18]. However the nature of resistance of these coffee hybrids needs to be confirmed using different *C. kahawae* strains existing in coffee growing regions in Tanzania.

Materials and Methods

Nature of Resistance of Lyamungu Hybrids to *Colletotrichum kahawae*

The current study which focused on the influence of *Colletotrichum kahawae* strains on physical defense mechanisms of coffee, was done to establish whether Lyamungu hybrids possess cuticular wax on green berries that inhibit spore germination of *Colletotrichum kahawae*, whether they have stimulatory effect on the infection process and whether they have field resistance by applying artificial inoculation using four *C. kahawae* strains on the hybrids.

The Influence of Surface Wax from Green Berries on Spore Germination of *Colletotrichum kahawae*

Germination of spores of the *C. kahawae* strains (post-penetration stages) was evaluated on surface wax layer extracted from green coffee berries of hybrid coffee lines. From each of the hybrid lines [Table-1], 10 green berries approximately 17 weeks after flowering were collected for wax extraction. The 10 berries from each selection were shaken for 15 seconds in 100 ml chloroform which was then evaporated off in a water bath at 40°C. The residue was re-dissolved in 5 ml chloroform and filtered using Whatman no. 1 filter paper. Four 5 ml drops of wax were placed on the slides cleaned with acetone replicated three times. After drying, the wax deposits were covered with one 5 ml drops of an aqueous suspension containing 2 x 10^6 conidia per ml of *C. kahawae* of isolate 2006/16. Tween 80 (0.1 per cent) was added to improve wetting of the wax layer and enhancing conidial germination. The slides were incubated in damp chambers for 16 hrs at 22°C. In each drop the number of germinated conidia out of 50 was recorded. Rume Sudan VC298 and Hibrido de Timor1343 were included as resistant and N39 as susceptible controls.

Table 1- Coffee lines resistant to Colletotrichum kahawae strain TZ 005 from Tanzania

Code	Parentage
20497	Kaffa X (N 39 X OP 729) X HdT
20498	(N 39 X HdT 1343) X Rume Sudan
20499	(N 39 X Kaffa) X HdT
20500	Rume X (N 39 X HdT)
20501	(N 39 X OP 729) X HdT) X Illubabor
20502	(N 39 X HdT) X Illubabor
20503	(Bourbon x Kaffa) x (Rume x HdT) x HdT 1343
20504	(N 39 X OP 729) X HdT) X N 39
20505	(N 39 X OP 729) X HdT) X N 39
20506	(N 39 X OP 729) X HdT) X Kent
20507	KP 423 X HdT
20508	(N 39 X OP 729) X HdT 1343) X N 39 X HdT 1343
20509	Rume Sudan VC 298
20510	Hibrido de Timor 1343
20511	N 39
20512	KP 423

Stimulatory Effect on the Infection of *Colletotrichum kahawae* in Coffee Hypocotyls

Coffee genotypes 20498, 20509 and 20511 at the hypocotyl stage (5-6 weeks after germination) were spot inoculated with a 5 ml drop of *C. kahawae* strains Que 2 and T3 (2006/14). After inoculation, the coffee hypocotyls were maintained at 100% relative humidity in darkness for 24 hrs. Cross sections of the infected hypocotyl fragments at a thickness of 20-25 mm were made with a freezing microtome stained and mounted in a cotton blue lactophenol solution to evaluate fungal post-penetration stages. Hyphal length inside hypocotyl tissues were estimated by counting the number of cortex layers penetrated using a micrometric eyepiece. The data were recorded from at least 20 infection sites per genotype at 24, 48 and 72 hrs after inoculation. To detect callose deposition on coffee hypocotyls, cross sections of infected tissues were placed in 0.07 M, pH 8.0 phosphate solution (K_2HPO_4) for 10 min and then transferred into a 0.01% solution of aniline blue in the phosphate solution for 10 min before being mounted in the same solution. Callose deposition on coffee hypocotyls was identified by bright yellow fluorescence light, where absence of fluorescence was considered as negative.

Evaluation of 16 Coffee Lines for Resistance to *Colletotrichum kahawae* Strains

A completely randomize design (CRD) arranged in a split-plot was used in the study. Four *C. kahawae* strains represented the main treatments and 16 genotypes were arranged as sub-plots with three replications. Forty hypocotyls (5-6 weeks old) of 16 coffee genotypes were spray inoculated twice with *C. kahawae* suspensions (2.0 x 10^6 spores/ml) at 48 hrs. intervals following [33] procedures. A temperature range of 22-24°C was used during the first four days of incubation to allow infection, while relative humidity in the boxes was maintained at 100%. Thereafter, inoculated coffee hypocotyls were incubated for three weeks at 19-20°C. Hypocotyls were individually scored for CBD symptom development using a scale of 0-4 (0 = absence of symptoms, 1 = one or two small brownish chlorotic lesions, 2 = coalescence brownish lesions, 3 = abundant black lesions, 4 = dead hypocotyls), developed by Van der Graaff [31]. The scoring days were at 7, 14 and 21 days at Lyamungu, Moshi, Tanzania and 3, 6, 9, 12, 15, 18 and 21 days at CIFC, Portugal. Different rating interval was attributed to a modification of the inoculation and assessment procedures done at CIFC [35]. Categories of reaction Disease Index Reaction (DIR) of the coffee hybrids were determined.

For each genotype DIR was determined using the formula shown below [25]:

$$DIR = 25\frac{\sum i \times ni}{in}$$

Where:

I = numerical value of disease description

ni = number of hypocotyls in disease description

n = number of hypocotyls in all description

Resistance categories were classified as; resistant (DIR 0-25), moderately resistant (DIR 26-50), moderately susceptible (DIR 51-75), and susceptible (DIR 76-100).

Evaluation of 16 Coffee Genotypes Under Field Conditions

Ten mature bearing coffee trees of 21 years old per genotype were artificially inoculated using four virulent strains of *C. kahawae*. Strains 2006/8, 2006/12, 2006/14 and 2006/22 of *C. kahawae* were inoculated to a set of two branches having five to six berry clusters per branch (about 40 berries per cluster) of green expanding coffee berries at 8 to 12 weeks old. The concentration of 2 x 10^6 spores/ml of spore suspension was used. Each coffee branch was sprayed twice at 48 hrs. intervals using the method described by Kilambo, et al [12]. The assessment of CBD development was done by counting

the number of CBD coffee infected berries against that of uninfected berries after 7, 14 and 21 days after the second inoculation. Coffee genotypes 20511 and 20512 which were N39 and KP423, respectively, and which were susceptible to *C. kahawae* were also used as controls. The mean CBD incidence for each variety was calculated and used in the correlation analysis together with the results obtained from hypocotyls reaction.

Determination of Pathogenicity Levels of *Colletotrichum kahawae*

CBD Pathogenicity Levels on Green Coffee Berries

The pathogenicity of *C. kahawae* was tested on 20 fully expanded soft green berries (£ 14 weeks old from the date of flowering) of the susceptible variety N39. Coffee berries were surface sterilized, placed on damp sterilized sand in plastic boxes and inoculated with a 0.02 ml drop of the standard *C. kahawae* spore suspension using a pipette. The inoculated green coffee berries were incubated at 25°C and observed for CBD symptom development for 14 days. Parameters on the percentage of infected berries; days for first symptoms appearance, enlargement of lesions, and appearance of sporulation and sporulation capacity after 10 days were recorded. Sporulation capacity was determined by counting spores per ml using haemocytometer.

CBD Pathogenicity Determination on Hypocotyls

Seeds of N 39 were planted with the parchment on, in plastic bags containing a sterilized soil mixture made up of compost and sand at a ratio of 2:1 respectively. The plastic bags were kept under moist conditions in the laboratory at a temperature of around 25°C. After two weeks of pre-germination, coffee seedlings (40 seedlings per tray) were planted in plastic trays.

Inoculum Preparation of *Colletotrichum kahawae* Strains and Inoculation

Spore suspensions of tentative *C. kahawae* were prepared from 17-day-old cultures on MEA. Five ml of sterile distilled water were pipetted on each culture to dislodge the spores. A sterile scalpel was used to scrape off colonies from the agar surface; the mixture was placed in sterile conical flasks and shaken well by hand to dislodge the spores further. The mixture was then filtered to obtain *C. kahawae* spore suspensions. Spore concentrations were estimated using the haemocytometer and calibrated to 2.0 x 10^6 spores/ml [33]. Forty hypocotyls (5-6 weeks old) from 16 coffee genotypes were spray inoculated twice at 48 hr intervals with suspensions of *C. kahawae* using the method described by Van der Vossen, et al [33]. Strains of *C. kahawae* 2006/14, 2006/22, 2006/12 and 2006/8 collected from Tanzania based on their pathogenic levels were used to inoculate 16 coffee genotypes. The *C. kahawae* strains Ca1 (Cameroon), Que2 (Kenya), and Z9 (Zimbabwe) were also used to assess nature of resistance of the same genotypes. In order to allow successful infection by *C. kahawae*, the seedlings were kept in propagation boxes at a temperature ranging from 22 to 24°C for the first four days after inoculation, and relative humidity in the boxes was maintained at 100% by spraying sterile distilled water three times a day (morning, mid-day and evening). After four days of incubation, the temperature was maintained at 19-20°C for the incubation period of three weeks. The numbers of infected seedlings per isolate were recorded at 7, 14 and 21 days and the pathogen was re-isolated from the infected seedlings to maintain isolate viability.

Sporulation Capacity on Green Coffee Infected Berries

Spore suspensions of *C. kahawae* isolates were obtained from clean sporulating 20 green berries by shaking them in a conical flask containing 5 ml of sterile distilled water. The suspensions were filtered through sterile paper towel. Spore concentration per ml was calibrated using a haemocytometer.

Results and Discussion

Determination of the Nature of Resistance to *Colletotrichum kahawae*

Influence of Surface Wax from Green Berries on Germination of *Colletotrichum kahawae*

The percentage germination of *C. kahawae* on the surface waxes of 12 coffee hybrids was compared with the field resistance of two known resistant coffee genotypes Rume Sudan (VC 298) (20509) and Hibrid de Timor 1343 (20510), N39 (20511) and KP 423 (20512) susceptible genotypes. The results obtained are presented in [Fig-1].

The results in [Fig-1] shows that there were highly significant differences (P£0.05) between the level of conidial germination on the wax of coffee genotype N 39, KP 423, the 12 hybrids, 20509 and 20510. Conidial germination percentage was highest on wax extract from coffee genotype 20511 than the rest. Germination percentage of conidia and disease resistance in the field were positively correlated ($r = 0.85^{***}$) ($P < 0.05$, d.f 14; $r_t = 0.497$, P £ 0.05; $r_t = 0.623$, $P< 0.01$).

The wax surface extracted with chloroform from green berries of the 12 coffee hybrids suggest that they contain substances that decrease conidia germination. Correlation between conidia germination and field resistance was positive and significant ($r = 0.85^{***}$) ($P<0.05$), indicating that cuticular substances may contribute to the high levels of field CBD resistance. The study results revealed that CBD resistance of Lyamungu coffee hybrids is partly being contributed by wax surface on green coffee berries. Similar results were reported by Steiner [30] who found a significant decrease of conidia germination in the wax surface extracted from Rume Sudan. Other authors confirm that the cuticular wax layer has influence on the fungal growth, hyphal anastomosis and the production of penetration pegs from hyaline appressorium on green and red pepper and tomatoes [7,20].

Nature of the Resistance Coffea Arabica to *Colletotrichum kahawae* Strains at Hypocotyls Stage

Five to six week old hypocotyls of coffee genotypes 20498, 20509 and 20511 were inoculated with *C. kahawae* strains Que 2 and T3. The disease intensity reaction (DIR) and proportion of scabs and active lesions on the coffee hypocotyls are presented in [Table-2]. The DIR of coffee genotypes 20498 and 20509 indicated that the genotypes were moderately resistant to CBD, but coffee genotype 20511 was susceptible [Table-2]. The two genotypes (20498 and 20509) had higher proportions of scab lesions than genotype 20511, which had high proportions of active CBD lesions. The presence of scab CBD lesions formed resistant or moderately resistant coffee genotypes suggesting that the mechanism by which further invasion of the CBD pathogen is blocked [11,15]. Recently findings by Chen, et al [5] revealed that green coffee berries posses inherent antifungal compounds that counteract the infection of coffee by *C. kahawae* strains. Such antifungal compounds may exist in Ly-

amungu coffee genotypes that contributed to CBD resistance. However, more studies are needed to confirm the involvement of antifungal compounds in the resistance of Lyamungu coffee genotypes to CBD.

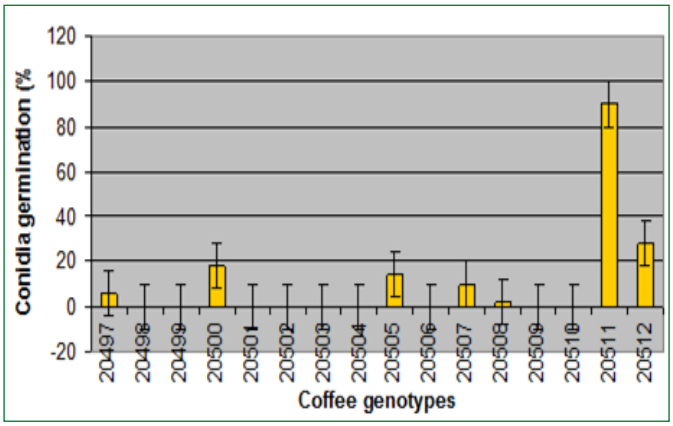


Fig. 1- Germination percentage of conidia of *Colletotrichum kahawae* (2006/16) strain on wax surface extracted from berries of different genotypes of *Coffea arabica*

Table 2- The reaction of three coffee genotypes to two Colletotrichum kahawae strains (Que 2 and T3)

Coffee Genotype	Disease Intensity Reaction* (DIR)		Proportion of scab and active lesions (%)*	
	Que. 2	T3	Que. 2	T3
20498	37	49	Scabs > 90	Scabs > 90
20509	36	48	Scabs > 95	Scabs > 95
20511	98	96	Actives 100	Actives 100

**Assessment was done 15 days after inoculation*

DIR = 0-25, Resistant; 26-50, Moderately Resistant; 51-75 moderately susceptible; 76-100 susceptible. Que2 = Kenyan C. kahawae strain, T3 = Tanzanian C. kahawae strain (2006/14)

Actives = active lesions, Scab = scabs

Growth of *Colletotrichum kahawae* in Epidermal and Cortex Cells of Hypocotyls

The results in [Table-3] show that the post-penetration of *C. kahawae* hyphae (24, 48 and 72 hrs.) between the three coffee genotypes was not significantly different from each other (P< 0.05). However, the penetration of *C. kahawae* strain Que 2 was deeper in genotype 20511 than it was in genotypes 20498 and 20509 [Fig-2].

Evidence of the Presence of Phenolic Compounds in Lyamungu Coffee Hybrids as a Source of Resistance to *Colletotrichum kahawae*

Infection sites per genotype were also located and fungal growth inside epidermal and cortex cells of the hypocotyls determined at 7 and 14 days after inoculation with T3 and Que 2 strains. The penetration of *C. kahawae* in each coffee genotype is shown in [Fig-3a] to [Fig-3e]. Cumulative data on the fungal penetration of *C. kahawae* inside hypocotyls were higher in the susceptible coffee genotype 20511 than they were in the resistant coffee genotype 20498 and 20509. [Table-3], [Fig-2]. Light micrographs also showed that hyphal growth of *C. kahawae* strains was restricted in epidermal cells and cortex for genotypes 20498 and 20509 [Fig-3a] to [Fig-3e]. On the other hand, setae, arcevuli and sporulation were observed on genotype 20511 14 days after inoculations [Fig-3e]. Similar results were reported by Silva, et al [28] who found that fungal hyphal length inside host tissues was significantly lower in the resistant coffee genotypes than in the susceptible ones. This might be because resistant plants develop defense mechanisms against attack by the pathogen [14,29]. In this respect, Masaba and van der Vossen [15] reported that scab formation on the green coffee berry surface is due to cork barrier formation which limits fungal hyphal growth inside the plant tissue. The formation of setae, arcevuli and sporulation after 14 days on genotype 20511 indicate susceptibility. The data confirm that Lyamungu hybrids can be good sources of CBD resistance.

Table 3- Post penetration growth of Colletotrichum kahawae strain Que 2 in hypocotyls of three coffee genotypes

Duration (hours)	Mean Fungal Hyphal Growth Inside Hypocotyls Tissues (mm)			DMRT (P<0.05)	SE	Variance	S.D	Mean
	20498	20509	20511					
24	6.95	11.25	12.2	18.8	1.6	7.8	2.3	10.1
48	10.15	12.25	19.7		2.9	25.2	5	14
72	10.55	13.2	21.7		3.4	35.2	5.9	15.2
Cumulative	27.65	36.7	53.6					

Key: 20498, 20509 and 20511 = Coffee genotypes, DMRT = Duncans' Multiple Range Test, SE = Standard error, S.D = Standard deviation, Mean followed by the same letter within the same column does not differ significantly following the mean separation by Duncans' Multiple Range Test

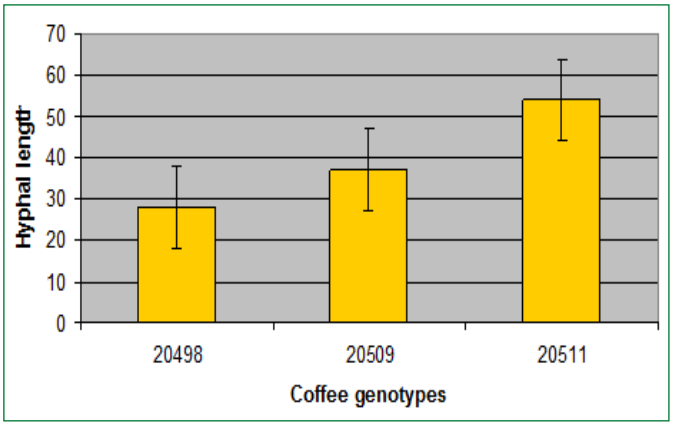
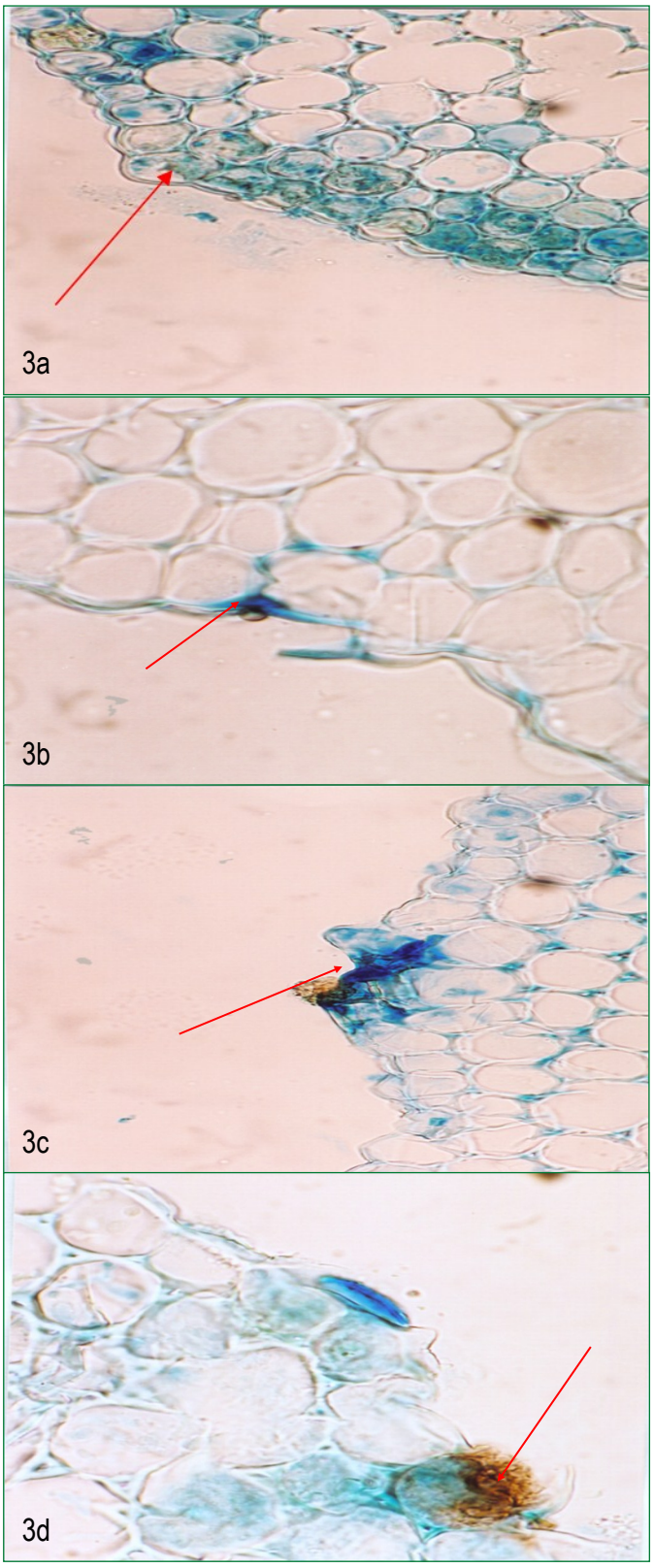


Fig. 2- Hyphal growth length (mm) of *Colletotrichum kahawae* strain Que in coffee hypocotyl tissues of resistant (20498 and 20509) and susceptible genotype (20511)

Light micrographs were used to detect callose deposition of coffee hypocotyls. The data presented in [Fig-4a] to [Fig-4d] revealed the presence of phenolic compounds enabling callose formation at infection sites in coffee genotypes 20498 [Fig-4a], coffee genotype 20498 showing callose formation and necrotic host cells [Fig-4b] and coffee genotype 20509 showing callose formation and thick walls [Fig-4c]. Coffee genotype 20511 showed callose formed around intracellular hyphae, but fungal hyphal growth of *C. kahawae* invading all over cortex cells [Fig-4d].

The use of histological characterization has been reported as being useful in detecting levels of resistance to species of root-knot nematodes related to accumulation of phenolics in *Capsicum annun* [24]. Silva, et al [26] reported that deposition of callose (a polysaccharide material) around intracellular hyphae is an early host response detected in the resistant genotypes. The authors also reported that thick walls are formed as a result of wall appositions (papillae) and callose (phenolic compounds) being deposited between the plasma membrane and outer wall of epidermal cell, at sites of attempted penetration by *C. kahawae*. More recent studies revealed that epi-

catechin and catechin present in green coffee berry pericarp of CBD resistant genotypes may prevent CBD by inhibiting appressorium penetration [3,4]. However, the amount of epicatechin and catechin present in Lyamungu hybrids requires investigation. These studies confirm further that Lyamungu coffee hybrids have good sources of resistance to *C. kahawae* strains and these can be used to improve the coffee breeding programme.



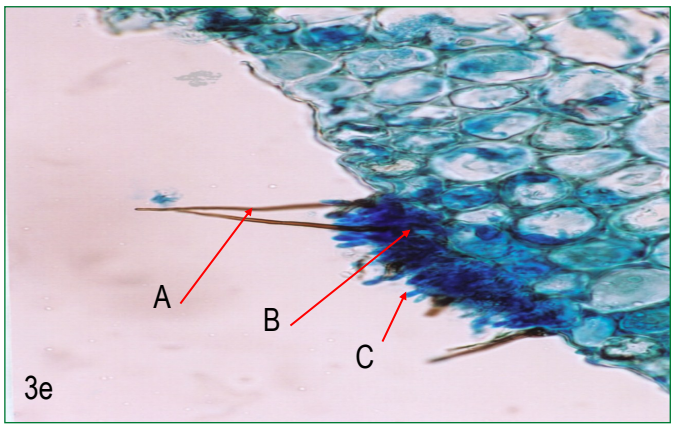


Fig. 3- Light micrographs showing post penetration growth of *Colletotrichum kahawae* in hypocotyls of coffee genotypes 20498, 20509 and 20511 all at Magnification X40. Hypocotyl sections stained with cotton blue lactophenol. Red arrows point to details below; hyphal growth of *Colletotrichum kahawae* strain T3 restricted in epidermal cells and 2nd layer of genotype 20498 seven days after inoculation [Fig-3a], Germinated conidia of Qu 2 without extend germtube when inoculated on genotype 20509 fourteen days after inoculation [Fig-3b], Necrotic reaction of genotype 20509 caused by reaction of *Colletotrichum kahawae* strain T3 seven days after inoculation [Fig-3c], Nectrotic reaction of genotype 20509 to *Colletotrichum kahawae* strain Qu2 fourteen days after inoculation [Fig-3d], Formation of setae (A), arcevuli (B) and sporulation (C) and high hyphal growth caused by *Colletotrichum kahawae* strain T3 fourteen days on genotype 20511 [Fig-3e].

Identification of Lyamungu Coffee Hybrids for Resistance to *Colletotrichum kahawae* Strains

Colletotrichum kahawae strains varying in pathogenicity were used to inoculate 16 *C. arabica* varieties. Both hypocotyls (5-6 week old) and berries in mature bearing plants (4th to 14th week) of the same coffee genotypes were inoculated with CBD spore suspension at 2 x 10^6 spore/ml. For the hypocotyls test, one experiment was done at Lyamungu using *C. kahawae* strains 2006/14, 2006/22, 2006/12 and 2006/8; the results are presented in [Table-4] and [Table-5]. A second experiment was done at CIFC, Portugal using *C. kahawae* strains Ca1, Que2 Z9 and T3 (2006/14); the results are presented in [Table-6]. A rating scale of 0-4 was used to score reaction on coffee hypocotyls per genotype. The mean percentage of seedlings that resisted *C. kahawae* infection per genotype was recorded 21 days after inoculation. Green coffee berries at 4 to 14 weeks old were inoculated at Lyamungu using strains 2006/14, 2006/22, 2006/12 and 2006/8, and the results are presented in [Table-7]. The mean percentage of the infected green berries per genotype was finally recorded 21 days after inoculation.

The results in [Table-4] (data scored using 0-4 rating scale, then arc sine transformed) present the reactions of *C. kahawae* strains 2006/14, 2006/22, 2006/12 and 2006/8 on coffee hypocotyls 21 days after inoculation. Coffee genotypes 20498 and 20509 significantly resisted CBD (P£ 0.05) as opposed to 20511. Likewise, the data on the percentage of coffee hypocotyls of the same genotypes that were resistant to *C. kahawae* strains are presented in [Table-5]. The percentage of coffee hypocotyls per genotype resistant to CBD varied significantly (P £ 0.05). Where as coffee genotypes 20498 and 20509 were resistant to all four *C. kahawae* strains (2006/14, 2006/22, 2006/12 and 2006/8), genotype 20511 was the most sus-

ceptible to almost all 4 *C. kahawae* strains. Furthermore, the DIR of 20498 and 20509 were observed to be resistant to CBD [Table-5].

The results from [Table-4] and [Table-5] show that coffee genotype 20498 and 20509 were resistant to CBD, suggesting a possibility of being used by coffee growers across coffee ecosystems in Tanzania.

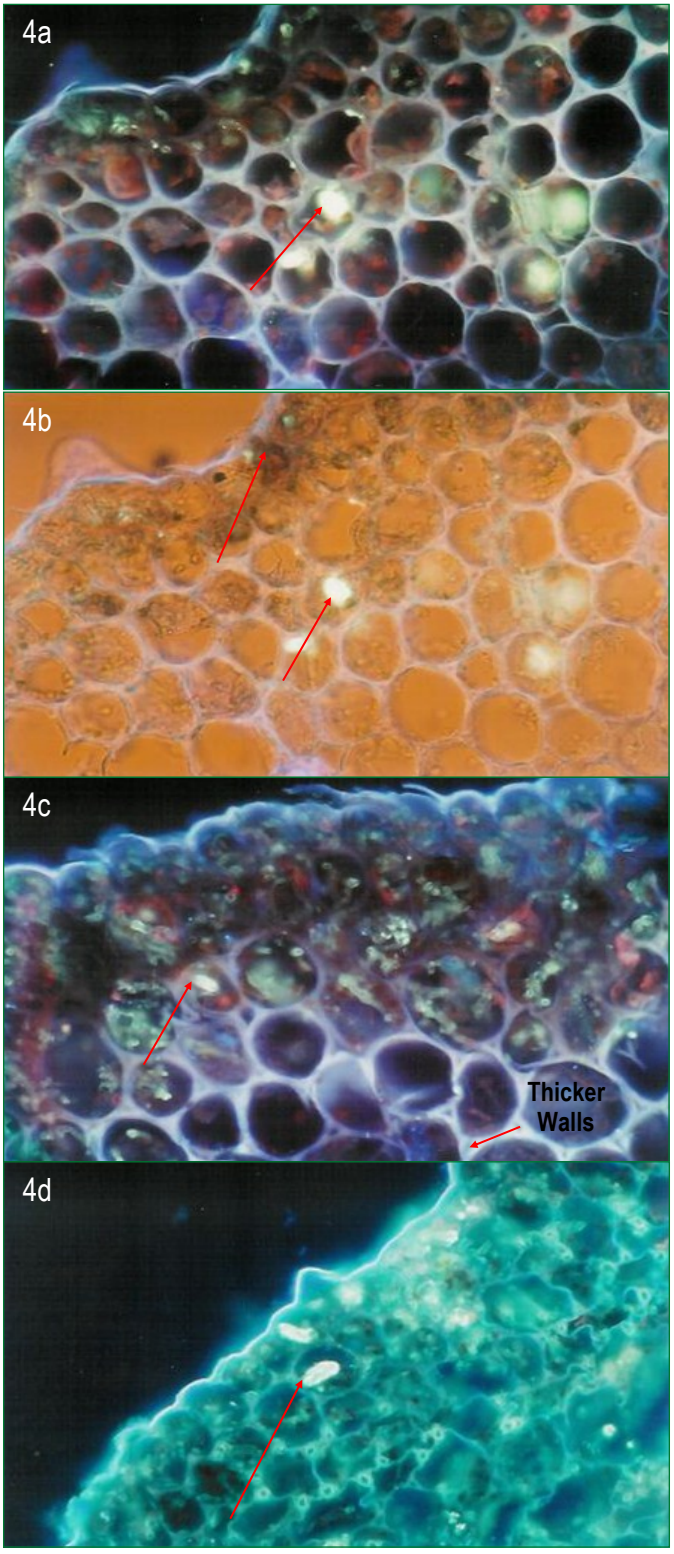


Fig. 4- Light micrographs of callose formation (arrow) in different coffee genotypes 20498 [Fig-4a] and [Fig-4b], 20509 [Fig-4c] and 20511 [Fig-4d] seven days after inoculation (Magnification x 25).

Table 4- Reaction of coffee genotypes to Colletotrichum kahawae strains 21 days after inoculation

Coffee Genotypes	*Colletotrichum kahawae* Strains			
	2006/14 (T3)	2006/22 (T12)	2006/12 (T26)	2006/8 (T4)
20497	7.3 A	3.8 ABC	7.1 ABC	6.5 AB
20498	0.0 B	0.0 C	1.9 CD	0.0 B
20499	8.1 A	5.7 ABC	8.7 ABC	7.9 A
20500	5.7 AB	0	2.7 BCD	4.6 AB
20501	7.3 A	0	4.6 BCD	6.5 AB
20502	9.9 A	6.5 ABC	9.4 AB	8.7 A
20503	6.5 AB	0.0 C	4.6 BCD	6.5 AB
20504	8.1 A	3.8 ABC	7.3 ABC	7.3 A
20505	9.9 A	7.3 AB	8.7 ABC	7.9 A
20506	8.1 A	1.9 BC	7.2 ABC	7.3 A
20507	6.5 AB	0.0 C	3.8 BCD	6.5 AB
20508	8.7 A	1.9 BC	6.7 ABCD	7.3 A
20509	0.0 B	0.0 C	0.0 D	0.0 B
20510	5.7 AB	1.9 BC	4.6 BCD	5.7 AB
20511	11.5 A	9.9 A	11.5 A	9.9 A
20512	8.1 A	0.0 C	8.1 ABC	5.4 AB
DMRT	6.015	6.015	6.015	6.015
Mean	6.963	2.669	6.056	6.125
S.E	0.782	0.795	0.764	0.677

Means followed by same letter within the column do not differ significantly following mean separation by Duncan's multiple range test. CIFC = Centro de Investigacao das Ferrugens do Cafeero, Portugal. SE = Standard error, S.D = Standard deviation Data scored 0-4, then transformed to arc sine

Table 5- Percentage of coffee hypocotyls per coffee genotype that indicated resistance to four Colletotrichum kahawae strains from coffee ecosystems in Tanzania

Genotype/ strains	2006/14 (T3)		2006/22 (T12)		2006/12 (T26)		2006/8 (T4)	
	%	DIR	%	DIR	%	DIR	%	DIR
20497	79.2 D	48.4	100.0 A	13	84.9 BC	36.6	87.4 C	35.2
20498	100.0 A	8.1	100.0 A	0	100.0 A	6.9	100.0 A	1.7
20499	63.0 F	55.5	96.7 A	21.9	71.1 E	51.6	77.5 EF	49.4
20500	93.0 B	30.9	99.1 A	4.1	96.6 A	20.7	94.1 B	20.1
20501	79.6 D	39.9	100.0 A	6.6	85.7 BC	25.4	86.8 C	29.9
20502	49.6 GH	74.9	90.0 B	30.3	62.5 F	65.9	67.5 G	51.9
20503	86.4 C	35.6	100.0 A	3.7	88.2 B	24.6	85.1 CD	27.9
20504	63.8 EF	55.2	98.3 A	17	88.0 B	37.9	75.6 F	42.9
20505	45.8 H	73.8	90.0 B	38.2	55.1 G	60.6	60.0 H	54.2
20506	63.4 EF	54.1	95.5 A	14.5	85.5 BC	32.1	81.3 DE	41.9
20507	87.4 C	28.8	99.1 A	6.4	87.3 B	20.8	86.7 C	27.2
20508	68.1 E	56.1	98.1 A	13	79.3 D	39.8	85.2 CD	38.7
20509	100.0 A	0.6	100.0 A	0	100.0 A	0.8	100.0 A	5.2
20510	97.4 AB	21.2	100.0 A	10.6	97.5 A	23.8	100.0 A	18.6
20511	3.3 I	97.9	68.7 C	48.2	10.8 H	93.9	40.0 I	73.8
20512	54.1 C	51.9	99.1 A	15.8	81.7 CD	38.6	85.0 CD	36.5
DMRT	1.5		1.5		1.5		1.5	
Mean	25.4		7.9		22.3		15.8	
S.E ±	6.3		1.9		5.6		3.9	

Means followed by same letter within the same column do not differ significantly following mean separation by DMRT test. DIR = Disease Intensity Reaction. DIR 0-25, Resistant; 26-50, moderately resistant; 51-75, moderately susceptible; 76-100 susceptible, % = Percentage

The data presented in [Table-6] indicate that the percentage of coffee genotypes resistant to CBD differed significantly (P £ 0.05). However, there was differential interaction between *C. kahawae* strains Ca1, Que 2, Z9 and T3 (2006/14) and *C. arabica* genotypes. Furthermore, the results on the DIR indicate interaction between *C. kahawae* strains and coffee genotypes. Coffee genotype 20509 was resistant to *C. kahawae* strains Ca1, Que2, and Z9; however, it was susceptible to strain T3. Similarly, coffee genotype 20498 indicated

resistance to strains Ca1, Que2 and Z9 but it was susceptible to strain T3. Coffee genotype 20510 shows susceptibility to strain Ca1, but it was resistant to the three strains of *C. kahawae*. These data imply that the *C. Kahawae* strains are likely to be adapted to coffee genotypes wherever they exist. However, further investigations are recommended to establish the possibility of the existence of physiologic races of *C. kahawae* strains. Earlier findings indicate that variation among strains of *C. kahawae* is predominantly due to differences in the degree of pathogenicity, but physiologic races do not exist [21,23].

Table 6- Percentage of coffee hypocotyls per coffee genotype that indicated resistance to four Colletotrichum kahawae strains from Cameroon, Kenya, Zimbabwe and Tanzania

Genotype/	Cal		Que 2		Z9		T3	
Strain	%	DIR	%	DIR	%	DIR	%	DIR
20497	10.4 E	37.5	81.3 C	32.9	55.8 C	36.1	59.5 C	31.5
20498	70.7 B	25	84.5 BC	32	55.6 C	48.9	26.0 F	64.3
20499	0.0 F	100	25.0 I	56.8	2.0 HI	93.8	4.4 I	90.9
20500	47.2 C	28	75.0 D	28.9	17.7 G	68	20.9 G	46.9
20501	0.0 F	100	73.9 D	28.8	37.5 D	24.9	34.3 E	37.5
20502	0.0 F	95.8	46.7 G	35.6	5.9 H	56.8	20.8 G	47
20503	4.6 F	76.7	89.1 AB	35.4	31.6 E	55.4	79.8 B	34
20504	16.7 D	51.9	60.9 E	41.7	25.0 F	63.3	22.5 FG	63
20505	4.0 F	92.9	36.3 H	49.9	0.0 I	88.5	10.5 H	58.4
20506	2.4 F	93.8	72.9 D	41.9	0.0 I	91	48.3 D	42.7
20507	4.0 F	75	60.1 E	40.8	23.9 F	72.6	35.5 E	36.5
20508	0.0 F	100	87.5 AB	29.2	36.7 D	63.5	59.3 C	37.5
20509	79.5 A	25	92.0 A	25	92.1 A	25	25.1 FG	63.1
20510	4.2 F	79.9	89.2 AB	27.7	66.1 B	27.3	97.9 A	26.1
20511	0.0 F	100	55.2 F	0.0 J	0.0 1	100	0.0 I	100
20512	2.0 F	92.9	64.1	100	2.3 HI	82.5	0.0 I	96.9
DMRT	4.6		4.6		4.6		4.6	
Mean	15.4		64.4		28.3		34	
S.E	6.5		6.6		6.9		7.1	

Means followed by small letter within the same column do not differ significantly following mean separation by Duncan's Multiple Range Test. DIR = Disease Intensity Reaction, S.D = Standard deviation, SE = Standard error DIR 0-25, Resistant; 26-50, moderately resistant; 51-75, moderately susceptible; 76-100 susceptible. Ca1, Que2, Z9 and T3 = C. kahawae strains % = Percentage of infected coffee hypocotyls.

The mean percentage of coffee berries per genotypes that were susceptible to *C. kahawae* strains are presented in [Table-7]. Susceptiblity of the coffee genotypes to *C. kahawae* differed significantly across genotypes (P £ 0.05). Coffee genotypes 20498, 20499, 20504, 20509 and 20510 were all slightly susceptible to CBD. Coffee genotypes 20511 and 20512 were significantly highly susceptible (P £ 0.05) to CBD.

Coffee genotype 20498 and 20509 were consistently resistant to CBD for both hypocotyls and green coffee berries. This implies that coffee genotypes 20498 and 20509 are reliable sources of CBD resistance, and can be used by farmers as well as in breeding programmes.

Resistance to CBD shown by the coffee genotype 20498 may possibly be genetical as it carries the R-gene derived from Rume Sudan and T-gene from Hibrido de Timor [32]. These genes have been found to carry resistance to CBD elsewhere. Mechanical barriers during penetration may also be involved because the germination of conidia of *C. kahawae* strains on the wax surface extracted from berries of this genotype was low [Fig-1]. Data from this study support the finding of Akinsanmi, et al [1], who reported that the distribution pattern of resistance in coffee is possibly caused by more than one mechanism. Lin, et al [13] reported that genes for CBD resistance in coffee were pathogen specific. When re-evaluating the resistance of *Coffea arabica* cv. Ruiru II with different strains of *C. kahawae*, Omondi, et al [22] reported that broad based resistance combining several genes in one plant may cause durable resistance. Other authors have also reported the importance of resistance genes in coffee to confer durability under different environment. Omondi, et al [22] and Noir, et al [19] reported that hypersensitive-like reactions caused by resistant genes have been shown in some *C. arabica* varieties against *Meloidogyne exigna*. Also studies shown the presence of resistance genes in some coffee plants as being due to hypersensitive reaction during infection by *Hemileia vastatrix* [8,9,27]. This study confirms the existence of potential CBD resistant coffee genotypes within Lyamungu germplasm collection, which can be used to improve the coffee breeding programme in Tanzania.

Table 7- Mean percent berries of Coffea arabica genotypes indicating percentage susceptibility to Colletotrichum kahawae strains from coffee ecosystems in Tanzania

Coffee Genotype	2006/14 (T3)	2006/22 (T12)	2006/12 (T26)	2006/8 (T4)
20497	11.6 BCDE	5.7 BC	5.5 D	11.1 CD
20498	3.1 FG	3.6 BC	0.0 E	0.0 G
20499	0.0 G	0.0 D	0.0 E	4.2 EFG
20500	7.9 DEF	3.6 CD	0.0 E	0.0 G
20501	14.3 BC	5.3 BC	3.0 DE	0.0 G
20502	7.1 EF	0.0 D	0.0 E	8.9 DE
20503	9.5 CDE	0.0 D	21.0 C	14.3 C
20504	0.0 G	6.7 BC	1.6 DE	5.7 EF
20505	0.8 G	10.0 B	3.3 DE	4.0 EFG
20506	13.6 BC	0.0 D	6.7 D	5.0 EFG
20507	12.5 BCD	4.0 CD	3.9 DE	1.6 FG
20508	15.4 B	2.9 CD	4.3 DE	5.0 EFG
20509	0.0 G	0.0 D	0.0 E	0.0 G
20510	3.2 FG	2.1 CD	0.0 E	0.0 G
20511	92.5 A	65.0 A	86.7 A	82.3 A
20512	88.4 A	61.1 A	77.8 A	73.1 B
DMRT (P£0.05)	4.6	4.6	4.6	4.6
Mean	17.5	10.6	13.4	3.5
S.E	7.3	5.2	6.9	6.4

Means followed by small letter within the same column do not differ significantly following mean separation by Duncan's Multiple Range Test. 2006/14, 2006/22, 2006/12 and 2006/8 = C. kahawae strains

Determination of Pathogenicity of *Colletotrichum kahawae* Strains

Determination of Pathogenicity of *Colletotrichum kahawae* Strains on Detached Green Coffee Berries

Pathogenicity of *C. kahawae* strains were tested on detached green expanding coffee berries at Lyamungu, Tanzania based on days of the first observable symptom appearance, enlargement of lesions, sporulation capacity and percentage of the infected berries. The data reveal that all 25 *C. kahawae* strains infected green coffee berries with some variations in the level of pathogenicity. Strains 2006/26, 2006/5 and 2006/7 had induced CBD symptoms on green coffee berries significantly earlier (at 2 days) than was the case with the rest of the strains (P < 0.05) [Table-8]. Strains 2006/27 and 2006/6 took five days to induce CBD symptoms on green coffee berries. On the same token, strains 2006/20 and 2006/1 induced significantly larger lesions than did the rest of the strains. Strain 2006/27 induced the smallest lesions (P < 0.05). Sporulation of *C. kahawae* appeared 5 days early on green berries for strains 2006/1 and 2006/7, while strains 2006/25 and 2006/27 sporulated 9 days late. The number of days to sporulation for the rest of the strains

was not significantly different (P £ 0.05) [Table-8]. Sporulation capacity for *C. kahawae* strain 2006/14 was significantly higher (P < 0.05) than was the case in the rest of the strains. Strains 2006/15 and 2006/13 sporulated the least [Table-8].

Data in [Table-8] indicate that the 25 *C. kahawae* strains differed significantly (P < 0.05) in the number of days to first symptoms appearance, lesion size (mm), days to sporulation, sporulation capacity (spores/ml) and percent infected berries, reflecting differences in pathogenicity (ability to infect) and virulence (degree of infection).

Strain 2006/14 (T3) was consistently observed to be highly pathogenic to green coffee berries of *C. arabica* genotypes used in this study. The high pathogenicity ability was attributed to high sporulation capacities [Table-8] which usually lead to a high percentage of germinated conidia and appressorium formation in the host tissues, resulting high infection levels. Accordingly, Silva, et al [26] report that *C. kahawae* strain Ca1 exhibit high virulence because of high sporulation capacity and germination of conidia in the host tissues. Omondi, et al [23] when studying the degree of pathogenicity using *C. kahawae* strains from Ethiopia and Kenya found virulence variability among the strains. This study has provided useful preliminary information on strengthening the coffee breeding programme in Tanzania. Strain 2006/14 of *C. kahawae* should be used in future studies for screening resistance of *C. arabica*.

Table 8- Virulence of Colletotrichum kahawae strains from different ecosystems on green expanding coffee berries

Strain	Location Collection of isolate	First symptoms appearance (days)	Lesion size* (mm)	Sporulation (days)	Sporulation capacity* (Spores/ml)	Infected Berries (%)*
2006/25	Lyamungu	4 B	2-3 E	9:00 AM	20 C	20 P
2006/26	Lyamungu	2 D	3-8 C	7 B	410 H	80 E
2006/27	Lyamungu	5:00 AM	1-2 F	9:00 AM	40 S	90 C
2006/28	Lyamungu	3 C	2-10 B	7 B	750 C	65 H
2006/29	Lyamungu	3 C	2-7 C	7 B	260 J	95 B
2006/14	Kibosho-Kombo	3 C	2-7 C	7 B	850 A	100A
2006/15	Marere-Mwika	3 C	2-5 E	7 B	50 R	90 C
2006/16	Machame Foo	3 C	2-3 E	7 B	120 P	25 O
2006/19	Rombo Mokala Juu	3 C	2-8 C	7 B	220 K	70 G
2006/20	Kerio	3 C	6-10 A	7 B	50 R	40 L
2006/21	Babati Haraa	3 C	3-8 C	7B	445 G	35 M
2006/22	Karatu Ngorongoro	3 C	3-10 B	7 B	790 B	75 F
2006/4	Mbozi Mahenje	3 C	3-6 C	7 B	30 T	50 K
2006/5	Mbozi Malenje	2 D	3-8 C	7 B	140 N	80 E
2006/6	Mbozi General	5:00 AM	2-3 C	7 B	450 F	20 P
2006/7	Mbinga Mbuji	2 D	2-4 E	5 C	290 I	65 H
2006/8	Mbinga Mkumbi	3 C	3-10 B	7 B	480 E	35 M
2006/9	Mbinga Ngima	4 B	2-4 E	7 B	150 M	55 J
2006/10	Mbinga Lugari	3 C	2-7 C	7 B	100 Q	30 N
2006/11	Mbinga Myanje	3 C	2-6 D	7 B	200 L	50 K
2006/1	Mbeya Lunji	3 C	3-12 A	5 C	20 V	85 D
2006/23	Mbeya Lunji 4	4 B	2-3 E	7 B	130 O	60 I
2006/24	Tarime Kimusi	3 C	3-7 C	7 B	150 M	70 G
2006/12	Kigoma Kalinzi	3 C	3-8 C	7 B	520 D	85 D
2006/13	Kigoma	4 B	2-3 E	7 B	13 V	90 C
DMRT (P <0.05)						0.82
SE						0.28

**Evaluated 7 days after inoculation*

Means followed by a common letter within a column do not differ significantly according to DMRT (P £ 0.05).

Determination of Pathogenicity of *Colletotrichum kahawae* Strains on Coffee Hypocotyls

Strains of *C. kahawae* were selected from three coffee growing zones based on their pathogenicity on detached green coffee berries. The strains included; 2006/14 and 2006/22 from Northern Tanzania, 2006/12 from Kigoma and 2006/8 from Southern Tanzania. [Fig-5] presents results of the reaction of these strains to coffee hypocotyls of the susceptible variety N39. Strain 2006/14 was more pathogenic than strains 2006/12, 2006/8 and 2006/22 (P <0.05) [Table-9]. This strain rapidly caused seedlings to shrivel and die within 7 days after inoculation and it took 14 days for other strains to kill the same coffee variety. Strain 2006/22 produced brown and black lesions only on coffee hypocotyls.

Results in [Fig-5] and [Table-9] confirm that *C. kahawae* strain 2006/14 was the most pathogenic strain than the rest of the strains. The strain rapidly caused coffee hypocotyls to shrivel and die within 7 days after inoculation, while other strains took 14 days for the same results. This signifies that the strain can be used very successfully to determine initial CBD resistance information of the coffee hybrids that is pre-selection for CBD resistance using hypocotyls [33].

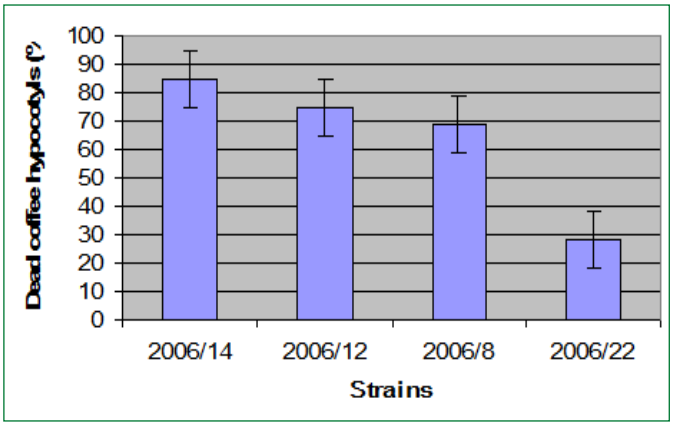


Fig. 5- Pathogenicity of four *Colletotrichum kahawae* strains on coffee hypocotyls of genotype N39

Table 9- Reaction of coffee hypocotyls of N39 susceptible varieties to four most pathogenic Colletotrichum kahawae strains from four coffee ecosystems in Tanzania

Strain Code		Location	Percent Dead Hypocotyls	Days to Shrivel
TaCRI	CIFC			
2006/14	T3	Kibosho-Kombo	85 A	7
2006/22	T2	Karatu	28 D	14
2006/12	T26	Kigoma-Kalinzi	75 B	14
2006/8	T4	Mbinga-Mkumbi	69 C	14
Mean			64.25	
S_X			1.28	
DMRT (P < 0.05)			5.8	

TaCRI = Tanzania Coffee Research Institute, Moshi, Tanzania; CIFC = Centro de Investigacao das Ferrugens do Cafeeiro, Portugal

Means followed by a common letter within a column do not differ significantly according to DMRT (P £ 0.05).

At CIFC Portugal, the virulence of strain 2006/14 (T3) from Tanzania was compared to *C. kahawae* strain Ca1, Qu2 and Z9. All strains were pathogenic on hypocotyls of N 39 [Table-9]. Accordingly, Varzea, et al [36] reported that *C. kahawae* strain Ca1 is the most pathogenic strain compared to other strains of the pathogen because of high sporulation capacity and rapid conidial germination on the host tissues. Strain 2006/14 (T3) demonstrated equal pathogenic to Ca1 [Table-10], [Fig-6]. Therefore, *C. kahawae* strain 2006/14 can be used in screening resistant to CBD in Tanzania.

Table 10- Reaction of four Colletotrichum kahawae strains on coffee from Cameroon Ca1, Kenya Qu2, Zimbabwe Z9 and Tanzania 2006/14 (T3) on coffee hypocotyls of cultivar N 39

Strain	Hypocotyl reaction (5 days)	(8 days)	Percent proportion of scabs & active lesions
Ca1	Brown (scab) lesions	Dead hypocotyls	100 % actives
Qu2	Brown (scab) lesions	Mixtures of scabs and actives	12 % scabs, 88 % dead
Z9	Brown (scab) lesions	Mixtures of scabs and actives	44 % scab, 56 % dead
T3	Scabs and actives	Dead hypocotyls	100 % actives

The number of coffee hypocotyls killed by the four *C. kahawae* strains was also assessed 9 days after artificial inoculation. [Fig-5] shows the percentage dead coffee hypocotyls of the cultivar N39 by Ca1, Qu2, Z9 and T3 (2006/14). The higher percentage of dead coffee hypocotyls were recorded on *C. kahawae* strains Ca1 and 2006/14 than in the Qu2 and Z9 strains. This confirms further the pathogenicity of *C. kahawae* strain 2006/14.

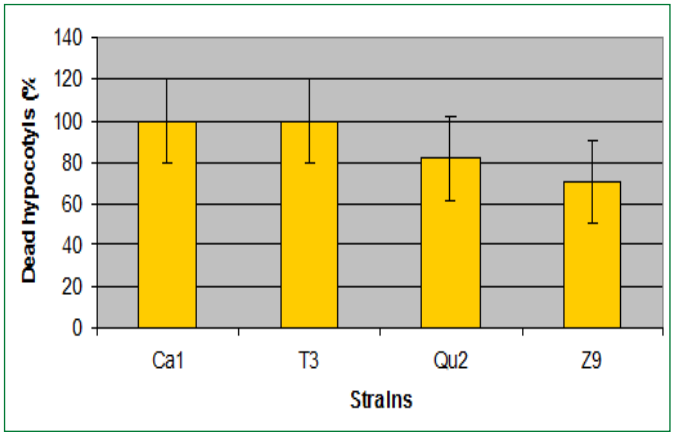


Fig 6- Percentage of dead coffee hypocotyls of cultivar N 39 caused by *Colletotrichum kahawae* strains nine days after artificial inoculation

Conclusions

The nature of resistance of Lyamungu coffee hybrids to *C. kahawae* strains was confirmed to have been influenced by surface wax from green berries of *C. arabica* genotypes and deposition of polysaccharide materials at the sites of attempted penetration by the pathogen. More studies are needed to confirm the involvement of antifungal compounds in the nature resistance of Lyamungu coffee genotypes to CBD. This study also indicates that *C. kahawae* strains are likely to have adapted to the coffee genotypes. However, further investigations are needed to establish the possibility of the existence of physiologic races of the *C. kahawae* strains.

The study also found out that some of the *C. kahawae* strains are highly pathogenic while others were less virulent when compared to strains from Cameroon, Zimbabwe and Kenya. The most pathogenic strain of *C. kahawae* found in Tanzania was 2006/14 (T3). The strain showed significant pathogenicity because of the high sporulation capacity and earliness in causing CBD symptoms appearance both on detached coffee green berries and hypocotyls in laboratory tests. From these findings it can therefore be concluded that high sporulation capacity and earliness to CBD symptom appearance are important factors to consider in selecting virulent *C. kahawae* strains for screening resistance of *C. arabica* varieties to the pathogen.

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