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Cultural, Morphological and Pathogenic Variability of *Colletorichuma kahawae* Isolate of Gurage Zone

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ABSTRACT

Arabica Coffee is an important crop in the national economy of Ethiopia. Coffee berry disease caused by the fungus *Colletotrichum kahawae* Waller and Bridge is the most devastating threat to *Coffea arabica* L. production in Africa at high altitude. Hence, this study was carried out for Variation of a representative *Colletotrichum* isolates of Gurage zone of major coffee producing areas using cultural, morphological and pathological criteria. Out of 33 sample 13 representative *C. kahawae* isolates from the study area and one Gera isolate were isolated from infected green coffee berry which showed significant variations in their cultural, morphological characteristics and pathogenicity. Mean radial colony growth rate of isolate showed significant variation ($p<0.001$) with the range of 2.67 to 4.08 mm/24hrs on PDA in EZA and CA1 isolates, respectively. Conidial size also showed significant difference ($p<0.001$) in the range of 5 to 6.04 and 9.24 to 10.0 µm in width and length, respectively. Similarly, conidia production varied from 182.25 to 432.92 × 10^4conidia/ml of isolate EK1 and EZD, respectively. All isolates were found to be pathogenic to Arabica coffee with highly significant variation ($P < 0.01$) and infection percentage in the ranges of 45.83 to 68.06%. Aggressive isolate EZD should be used for screening of coffee variety for CBD resistance evaluations.

Keywords: Coffee berry disease; *Colletotrichum kahawae*; Pathogenecity

INTRODUCTION

Coffee is the most important cash crop worldwide; more than 125 million people in the coffee growing areas derive their income directly or indirectly from Coffee products [1]. Ethiopia is the center of origin and genetic diversity of Arabica coffee. In Ethiopia coffee farming provides a livelihood income for around 15-16 million peoples, based on four million small-holder farms,10 percent of agricultural production, and about 34 percent of total export earnings over the past decade [2-4]. The occurrence of major severe diseases is one of the main limiting factors of coffee production. Coffee berry disease (CBD) caused by the fungus *Colletotrichum kahawae* Waller and Bridge, is the most devastating threat to *Coffea arabica* L. production in Africa at high altitude.

C. kahawae attacks all stages of the crop from flower to ripe fruits and occasionally leaves, but the maximum crop loss occurs following the infection of green berries [5]. Yield loss due to CBD is estimated to range from 24% to 30%, but it may reach up to 100% in high rainfall, high humidity and high altitude areas [6,7]. Different research has been indicated that variation among coffee berry disease pathogen through morphological, cultural and pathogenic characteristics [6-9]. According to Tefestewold study indicated variation/similarities within *C. kahawae* isolates collected from Hararge, Illubabor, Kaffa and Sidamo areas and recorded a presence of variations in aggressiveness and absence of races within *C. kahawae* populations based on pathogenicity tests [9]. Eshetu and Waller also reported presence of physiologic races within *C. kahawae* isolates in Ethiopia, it would be useful to look at a profile of several isolates from widely differing coffee types existing in the country, in a locality over time [6]. Apart from the host, the variation in CBD severity could be associated with differences in virulence between *C. kahawae* isolates occurring in different coffee growing regions.

The variability within *C. kahawae* populations from different Gurage zone coffee producing areas of Ethiopia so far was not known. Therefore, the present study was carried out on variations within representative *C. kahawae* isolates from different Gurage zone coffee producing areas were studied using cultural, morphological and pathological criteria.

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MATERIALS AND METHODS

Description of the study area

The study was conducted in laboratory and growth room of Jimma Agricultural Research Center from July 2017 to June 2018.

Collection of samples

Green diseased coffee berries with active CBD lesions were collected from the Gurage zone three major coffee producing districts i.e., Cheha, Enemorina ener and Ezya districts. Gurage Zone is found in Southern Nations Nationalities and Peoples Regional State, located between 7.8° - 8.5° latitude and 37.5° -38.7° longitude [10]. From each districts three peasant associations were selected and a total of 33 coffee specimens were collected from randomly selected coffee farms. A total of fifteen to twenty green coffee berries from each farm with active CBD lesions were collected. Samples were picked using disinfected forceps, packed in perforated sterile plastic bags and transported to Plant Pathology Laboratory of Jimma Agricultural Research Center and maintained at 4°C for further studies.

Isolation and identification of the pathogen

The pathogens were isolated from infected coffee berries by following the methods described by Tefestewold [9]. The collected berries were cut into pieces with margin of diseased and healthy tissues using sterilized surgical blade. Then samples were surface-disinfected with 5% sodium hypo-chlorite solution for 2 minutes and then rinsed 5 times in sterilized water for 2 minutes. The sterilized samples were dried in laminar flow hood and then, five fragments (cut pieces) of each sample was taken and placed onto Petri dishes containing PDA supplemented with 0.04% streptomycin and incubated at 25°C for 3 to 5 days. The growing edges of any fungal hyphae (mycelial tip) developing from the tissues were sub-cultured aseptically to PDA and inoculated for 7–10 days at 25°C. After morphological and microscopic identification (conidial morphology, conidial lengths, colony growth rate, colony shape and colony colors) from 33 samples 13 representative pure cultures mono conidial *C. kahawae* isolates were preserved in 50% PDA slant method at 4°C for later use. *Phoma* spp., *Aspergillus* spp., *Penicilium* spp. and *Fusarium* spp. were grown on culture of some fragment samples and removed it and sterilize the petri dish to avoid contamination.

Cultural and morphological characterizations

Pure culture of 14 representatives *C. kahawae* isolates (for Cheha 4, for Ezya 5, Enemorina Ener 4 and 1 Gera isolate (hot spot area for CBD)) were isolated from infected green coffee berries. The plates were examined cultural, morphological and pathogenic characteristics of the pathogen isolates were studied following the methods and procedures used [9,11,12].

Cultural appearances: Ranges of cultural variation of 14 representative isolates were examined by culturing on PDA containing 0.04% streptomycin and incubated at 25°C in three replications in CRD design for all characters. An isolate was examined for a colony (mycelial) radial growth, colony color, colony shape and aerial mycelial growth characters. Mycelial (colony) radial growth (mm) of each isolate was measured from the reverse side of the Petri-dishes daily with ruler for 10 days starting from the 3rd day of incubation. The colony (mycelia) color on the upper side and types of pigments on the reverse side of the Petri-dish for each isolate was determined on PDA every 3 days using RGB color chart [13]. Hence, cultures were monitored for 12 days. Vigor of aerial mycelium growth types of each isolate was observed on upper side of a plate after 10 days of being cultured on PDA. Then, it was examined and recorded as dense (regular), irregular (scarce) or very scarce culture types. The colony form of each isolates was observed on reverse side of the plate on 8 days of being cultured on PDA. Then, it examined and recorded as round, irregular, filamentous, rhizoid or curled types of culture forms.

Morphological characteristics: Isolates was cultured on PDA medium in three replications for 10 days and then conidial size (length and width) were measured on 30 randomly selected conidia per replication per isolate. Length and width of conidia were measured with ocular micrometer (µm), at 400x magnification of compound microscope. Sporulation capacity of each isolates was determined from 10 days old culture of the isolates on PDA was washed by flooding with 10 ml sterilized distilled water, rubbed with sterilized scalpel and transferred to 50 ml sterilized beaker and thoroughly stirred for 15 minutes with magnetic stirrer to extract the spores from the interwoven mycelia. Finally, the mycelia were filtered into another sterilized beaker through double layer cheese clothes. The number of conidia per ml were counted using haemocytometer.

Pathogenicity test of *C. kahawae* isolates

The 14 representative isolates were evaluated for their pathogenic ability (virulence) on a detached green berry of susceptible variety (370) by following the methods of van der Vossen et al., and Bayetta [14,15]. Fifteen weeks old from date of flowering of the expanding coffee berries from bottom, middle and top of the coffee tree were randomly collected [16]. Berries were surfaced sterilized with 5% sodium hypochlorite solution for 2 minutes and rinsed three times with sterile distilled water for 2 minutes each and dried using sterile cotton cloth. The wounded stalk end of the berries was removed with a sterile scalpel to avoid contamination with saprophytic fungi. Eighteen berries per isolates were placed in 3 rows in plastic box on sterilized tissue paper for inoculation, in CRD design in three replications per isolates.

Inoculum preparation and Inoculation: Ten days old mycelia colonies culture of each isolate was washed by flooding with 10 ml sterilized distilled water, rubbed with sterilized scalpel and then transferred to 50 ml sterilized beaker to harvest conidia. The suspension of each isolate was stirred with magnetic stirrer for 15 minutes and filtered through double layers of cheese clothes. After repeating the procedure the spore concentration of each suspension was adjusted to 2×10^6 conidia/ml and 20 µl of conidia suspension was deposited on the berries using a pipette while shaking time to time when drawing the inoculums [17,18]. As a control (check) 20 µl distilled sterilized water was poured on the berries. Boxes were sealed to provide saturated humid conditions necessary for disease development. Regular opening after every three days was done for 10 minute to allow for aeration of the berries. The data on infection collected every three days starting from 3rd days post inoculation when CBD symptoms were visible. After 14 days, data on disease intensity (PSI),

expressed as pathogenicity level of each isolates were recorded using a scales of 0 to 6 [19]. After scoring each coffee berry individually, average infection percentage (AIP) on each isolates across the replicates was calculated as follows:

$$AIP = \sum [Ir1 + Ir2 + Ir3 + \cdots Irn]/N$$

Where, I is the sum of disease score; n is the number of replication; **Irn** is the sum of disease score in replication *n*; **N**is the total number of berries scored in the replications.

Data analysis

All the data were subjected to analyses of variance (ANOVA) using SAS program version 9.3 software [20]. Fishers least significant different (LSD) mean separation tests were performed for comparison of isolate characters means that showed significant difference. The relationships among pathogen characteristics were determined by Pearson correlation analysis using the SAS software (Proc procedure).

RESULTS AND DISCUSSION

Cultural and morphological characteristics of *C. kahawae* isolates

There was highly significant ($p < 0.001$) difference among isolates in their radial colony growth rate (Table 1). Mean radial colony (mycelial) growth rate was ranged from 2.67 ± 0.26 to 4.08 ± 0.26 mm/24 hrs in isolates of CA1 (Cheha districts) and EZA (Ezha districts), respectively (Table 1). Over all mean of radial growth rate of 3.11 ± 0.26 mm/24 hrs was recorded and this results indicated a faster mean growth rate as compared to Hindorf [11,21], i.e., 1.9 ± 0.5 and slower mm/24hrs for the average mycelia growth rate of CBD isolates at 22°C incubation on 2% Oxoid MEA. However, this study result was comparable with Waller et al., and Arega et al. the colony growth rate of 2-4 and 0.6 - 5.5 mm/24 hrs, respectively [22,23]. As *C. kahawae* species are slow growing nature in mycelial growth rate in culture medium, this may use as distinguishing criterion of CBD pathogen from other *Colletotrichum* species (like *C. gloesporioides, C. acutatum)* and could serves as indicator of variability within the species [24].

Based on visual observation of the upper side of culture plates of colony appearance (aerial mycelial growth), dense, irregular (scarce) and very scarce types of colony (texture) were identified. Half of the isolates showed dense types of aerial mycelia growth and the rest scare types on PDA media (Table 1). Seventeen *C. kahawae* isolates showed 47.1%, 11.8 and 5.8% dense, irregular and very scarce aerial mycelia growth on both PDA and MEA media, respectively, whereas the rest 35.3% isolates showed inconsistent aerial mycelia growth [23]. From 35 *Colletotrichum* spp. isolates 60.0%, 31.4% and 8.6% on PDA indicated dense, irregular (scarce) and very scarce types of aerial mycelial growth, respectively [9].

Different colony colors were observed on both sides of the culture plates. Five groups of mycelial color were observed in upper side of plate"s *viz.*; Gray white (35.71%), Dark gray white (28.57%), Ghost white (14.28%), Cottony white (14.28%) and floral white (7.14%) (Table 1). The reverse side of the culture plates also showed; light golden rod (28.57%), pale golden rod (21.42%), lemon chiffon (21.42%), Navajo white (7.14%), antique white (7.14%) and corn silk (7.14%) colony pigmentation (Table 1). Diverse colony colors have been previously reported on both sides of a culture plates. Light gray, dark gray, gray and white mycelia types of colony color were also observed from Hararghe *C. kahawae* isolates [24,25]. Abdi and Abu also indicated pale yellowish to pinkish with dense whitish-grey aerial mycelium and a few bright orange conidial masses on the tips of the active growing hyphae on MEA media [19]. Diverse colony colors of the pathogens were observed due to by using different growth media and the characteristics" the pathogens. In general, the mycelial colony color of the isolates are whitish at the 3-5 incubation days; light gray in 6-7 and then 8-10 incubation days changed to dark gray; a distinctive characteristic colony color of *C. kahawae* isolates.

The colony form of the Gurage *C. kahawae* isolates showed two types of colony form, which was most of the isolates was irregular and some isolates showed curled colony shape. These colony forms were the characteristics the filamentous fungi that the *Colletotrichum* spp. belongs.

Morphological characteristics *C. kahawae* isolate: There was highly significant ($p < 0.001$) difference among isolates in their conidia size (Table 2). All *C. kahawae* isolates had variable conidia length and width, which ranged between 9.03 to 10.49 ± 0.54 µm and 5.0 to 6.04 ± 0.22 µm, respectively. The average conidial length and width of isolates were 9.87 ± 0.54 µm and 5.38 ± 0.22 µm recorded, respectively (Table 2). Isolate EZA had the largest mean conidial length (10.49 ± 0.54 µm) and the smallest mean conidial length was recorded from isolate EZD (9.24 ± 0.54). While the widest conidial width was recorded on isolate EZS2 (6.04 ± 0.22), and the narrowest mean conidial width was from isolates EB (5.00 ± 0.22) (Table 2). In this study, all isolates showed variable conidial length and width similar previous observations by different authors. Tefestewold reported of variable mean conidia length (13.5 -19.3 µm) and width (2.9-5.2 µm) on PDA [8]. Kilambo et al. also recorded the conidia length ranged from 8 to 18 mm and width ranged from 2 to 6 mm and showed an overlap of conidia size between isolates thus, making it difficult to distinguish the strains of *C. kahawae* by conidia size [26]. Talhinhas et al. indicated variability in conidia size within and between strains when studying the diversity of *Colletotrichum* species in olive anthracnose and concluded that it is difficult to distinguish fungal strains using spore size [27].

Sporulation capacity of Gurage *C. kahawae* isolates has been evaluated on 10 days old cultures that revealed highly significant ($P<0.001$) differences among isolates (Table 2). Conidia production ranged between 182.25 to 432.92 × 10^4 conidia/ml of isolate EK1 and EZD, respectively. Hence, isolate EZD produced highly significant amount of conidia (432.92 ± 27.04 × 10^4) followed by isolates CW, GC and EZS3 but was not statistically different. While, isolate EK1 (182.25 ± 27.04 × 10^4) was produced the smallest amount of conidia which was highly significant difference among all isolates (Table 2). The high conidia production was the characteristics of the virulent pathogen of the *C. kahawae* isolates that produce enough inoculum sources for disease development. Tefestewold observed (1.2-5.2) × 10^5 conidia /ml and (6.84- 17.20) × 10^6 conidia /ml production from six isolates of *C. kahawae* on PDA medium and GCA (green coffee seed extract agar) [8]. Isolates of *Colletotrichum* species also produced an average number of conidia that ranged between 2.4 × $10^5$to 1.3 × 10^7 on PDA [9].

Table 1: Cultural characteristics of *C. kahawae* isolates of Gurage districts.

Isolate code	Form	Texture	Color		colony growth (mm/ day)
			Upper	Reverse	
EK1	Irregular	Dense	Gray white	Light gold rod	3.05 ± 0.26^{c}
CW	Irregular	Dense	Ghost white	Light rod	3.04 ± 0.26 ^{c}
EZS1	Curled	Dense	Cottony white	Lemon chiffon	2.99 ± 0.26 ^{c}
EKO	Irregular	Scarce	Gray white	Lemon chiffon	3.06 ± 0.26 ^{c}
EZS3	Irregular	Dense	Dark gray white	Light gold rod	3.12 ± 0.26^{bc}
EZA	Irregular	Dense	Gray white	Pale gold rod	**2.67 ± 0.26** ^{d}
EZD	Curled	Scarce	Gray white	Pale gold rod	3.36 ± 0.26 ^{b}
CA1	Irregular	Scarce	Dark gray white	Light gold rod	**4.08 ± 0.26** ^{a}
CS	Curled	Dense	Floral white	Light gold rod	3.01 ± 0.26 ^{c}
CA2	Irregular	Scarce	Gray white	Pale gold rod	3.05 ± 0.26 ^{c}
EK2	Irregular	Dense	Dark gray white	Antique white	2.88 ± 0.26^{cd}
GC	Irregular	Scarce	Dark gray white	Navajo white	3.33 ± 0.26 ^{b}
EB	Irregular	Scarce	Ghost white	Corn silk	2.91 ± 0.26^{cd}
EZS2	Irregular	Scarce	Cottony white	Lemon chiffon	2.97 ± 0.26 ^{c}
Mean					3.11
LSD					0.26
CV (%)					4.99

Means followed with the same letter are not significantly different LSD (0.05).
Notes: EK1; EK2; EKO and EB (Enemorina Ener district isolates), CW; CA1; CA2 and CS (Cheha district isolates), EZS1; EZS2; EZS3; EZA and EZD (Ezya District isolates) and GC (Gera isolate)

Table 2: Mean conidia size and conidia production of *C. kahawae* isolate of Gurage zone.

Isolate code	Conidia size (µm)		Conidia production
	Width	Length	(x10,000/ml)
EZS2	6.04 ± 0.22^{a}	9.77± 0.54^{bcd}	277.33 ± 27.04^{g}
CA2	5.64 ± 0.22^{b}	9.86 ± 0.54^{bc}	209.79 ± 27.04^{h}
GC	5.55 ± 0.22^{bc}	9.44 ± 0.54^{cde}	395.11 ± 27.04^{bc}
CA1	5.47 ± 0.22^{bcd}	10.22 ± 0.54^{ab}	380.45 ± 27.04^{cd}
EZS3	5.44 ± 0.22^{bcd}	9.03 ± 0.54^{e}	392.17 ± 27.04^{bc}
EZS1	5.36 ± 0.22^{cde}	10.14 ± 0.54^{ab}	256.00 ± 27.04^{g}
EK2	5.34 ± 0.22^{cde}	9.72 ± 0.54^{bcd}	355.75 ± 27.04^{de}
EK1	5.33 ± 0.22^{cde}	10.22 ± 0.54^{ab}	182.25 ± 27.04^{i}
EKO	5.31 ± 0.22^{de}	10.06 ± 0.54^{ab}	305.97 ± 27.04^{f}
EZD	5.29 ± 0.22^{de}	9.24 ± 0.54^{de}	432.92 ± 27.04^{a}
EZA	5.26 ± 0.22^{de}	10.49 ± 0.54^{a}	250.83 ± 27.04^{g}
CS	5.25 ± 0.22^{de}	10.08 ± 0.54^{ab}	344.06 ± 27.04^{e}
CW	5.14 ± 0.22^{ef}	9.83 ± 0.54^{bc}	416.33 ± 27.04^{ab}
EB	5.00 ± 0.22 ^{f}	10.06 ± 0.54^{ab}	340.25 ± 27.04^{e}
Mean	5.38	9.87	324.23
LSD	0.22	0.54	27.04
CV (%)	2.49	3.25	5.84

Means followed with the same letter are not significantly different at LSD (0.05)
Notes: EK1; EK2; EKO and EB (Enemorina Ener district isolates), CW; CA1; CA2 and CS (Cheha district isolates), EZS1; EZS2; EZS3; EZA and EZD (Ezha District isolates) and GC (Gera isolate).

Virulence determination of *C. kahawae* isolate of Gurage zone

The result revealed that all isolates were pathogenic to variety 370 and showed distinct and highly significant ($p < 0.001$) variations in the level of aggressiveness (Figure 1). The highest level of berry infection was recorded in isolate EZD with 68.06% infection from Ezha districts but statistically not significantly different from GC, CW, EB and CS isolates. The lowest berry infection level were recorded on isolate EZS2 (45.83%) but statistically not significantly different from EK2, CA2, EKO, EK1, CA1, EZA and EZS1 isolate (Figure 1). The isolate EZD produce highest amount conidia production and more virulent isolates as compare to the other isolates. *C. kahawae* strain can exhibited high virulence because of high sporulation capacity and germination of conidia in the host tissues [28,29]. The aggressiveness of the pathogen can be considered as quantitative

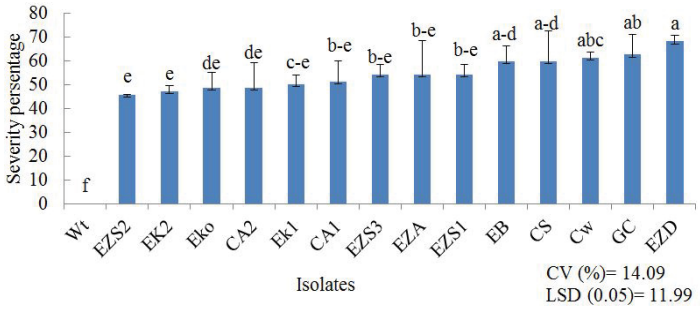

Figure 1: Virulence of *C. kahawae* isolates from Gurage zone on green expanding coffee berries. Means followed with the same letter are not significantly different at LSD (0.05). Distil sterilized water was applied us control (Wt).

Table 3: Pearson correlation coefficients of morpho-cultural characteristics and virulence of the 14 *C. kahawae* isolate of Gurage zone.

	Growth rate	Conidia	Width	Length	Virulence
Growth rate	1.00	0.42ns	0.17ns	-0.15ns	0.14ns
Conidia		1.00	-0.24ns	-0.59*	0.63**
Width			1.00	-0.23ns	-0.49*
Length				1.00	-0.34ns
Virulence					1.00

*and **=Significant at 0.05 and highly significant at 0.01 level respectively, ns = non-significant

measure of the level of the disease reached over time. This indicates that the most aggressive pathogen reached at a specific disease level faster than the less aggressive one. The situation can be measured via latent period, spore production, infection, lesion size and disease severity [30].

Correlation between virulence and morpho-cultural characteristics: Pearson correlation analysis revealed that highly significant ($P<0.001$) and strong positive correlation of virulence with the conidia production of isolates (r=0.63) (Table 3). The isolate EZD produce high amount of conidia which infected coffee berry more severely than the remaining isolates. A related finding was also reported earlier by Varzea et al. [28]. Kilimbo et al. also indicated that the conidia productions were weak positively correlated to virulence of isolates (r=0.15) [17]. Pearson correlation result revealed that virulence of the isolates was positive but non-significant correlation with conidia growth rate of the isolates (r=0.14) (Table 3). Positive correlation was found between enlargement of lesion size and sporulation capacity, lesion size and percent berry infection, as well as sporulation capacity and percent berry infection [17]. But this result was not similar to Kilimbo et al. results, in which the lesion size was positively correlated to percent berry infection [17].

In this study the conidia size of the isolates were highly variable within and among the *C. kahawae* isolates. In fact, since there was high variation between the same isolate of the pathogen on its conidial size, there was insignificant relationship between pathogenicity (virulence) and conidial size [23]. The variability in fungal pathogenicity and the close relationship between sporulation and virulence could provide a useful information base for screening coffee germplasm collections for resistance to the pathogen and subsequent breeding programs for durable resistance through the selection of highly sporulated and virulent fungal isolates [17]. Hence, the virulent isolate EZD found in this study should be used as screening isolate in coffee CBD resistance evaluation.

CONCLUSION AND RECOMMENDATIONS

Thirteen representative *C. kahawae* isolates of Gurage zone of major coffee producing areas of Ethiopia and one isolates from the CBD hot spot area of Gera were studied based on their cultural, morphological and pathological criteria. The study revealed the existence of variations in cultural, morphological and pathogenicity characteristics among *C. kahawae* isolates. All *C. kahawae* isolates collected from Gurage zone were pathogenic to susceptible Arabica coffee variety 370. However, isolates showed highly significant variation among them on their level of aggressiveness. The difference in virulence and aggressiveness implies that care should be taken and for further development of resistant varieties aggressive isolates should be used for a successful screening of coffee germplasms before the release of new developed cultivars.

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