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# *In vitro* Screening and Characterizing the Most Promising Antagonistic Microorganism as Biocontrol Agent(s) Against *Colletotrichum kahawae*

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## Abstract

Coffee berry disease (CBD) caused by *Colletotrichum kahawae* causes yield loss of more than 50% if not controlled with the appropriate fungicides or/and resistant cultivars. This study was aimed to screen indigenous coffee-associated microorganisms for their inherent antagonistic potential. From a total of 4323 microorganisms isolates tested, over 3% exhibited remarkable inhibition against *C. kahawae*. 13 bacterial isolate showed maximum inhibition of mycelial growth against the fungal pathogen tested. Among the yeast and fungal isolate 11 and 9 islates were most antagonistic to *C. kahawae* respectively. Twenty four isolates produced antibiosis, 5 isolates showed micoparacitic activity and 4 islates produced enzyme. The result indicated the species richness of indigenous coffee in Ethiopia which can be explored for their beneficial application as biological control. The in vitro results provide the first evidence of an antagonistic effect of coffee associated microorganisms against the CBD and potential of all bacterial, yeast and fungal groups for biological control of CBD.

**Keywords:** *Coffea arabica*; *Colletotrichum kahawae;* Antagonist; antibiotic; Siderophores; PGPR

## Introduction

Ethiopia is the origin for Arabica coffee (*Coffea arabica* L.) and coffee is the major agricultural export product. Coffee is grown by smallholders under various kinds of shade trees [1,2]. African coffee farmers are currently facing many difficulties, e.g. yield losses, due to serious fungal diseases [3-5].

Coffee berry disease (CBD) caused by *Colletotrichum kahawae* is one of the major constraints of *Coffea arabica* production in Africa [6]. The disease is confined in the African high lands, where there is high humidity and low temperature [7]. It causes yield loss of more than 50% if not controlled with the appropriate fungicides or/and resistant cultivars [8].

Currently, no appropriate control measures are in place against this coffee pathogen. The high cost of pesticides, the appearance of fungicide-resistant pathogens and other social and health-related impacts of conventional agriculture on the environment have increased interest in agricultural sustainability and biodiversity [9]. Thus, millions of coffee farmers in developing countries are facing problems not only with low coffee prices but also a growing interest in organically-grown coffee.

The combination of these matters necessitates eco-friendly control methods, e.g. biological controls, which have been studied as an alternative or complementary approach to physical and chemical disease control measures for over several decades [10]. Current developments in sustainability involve a rational exploitation of microbial activities affecting plant development. Among the plant growth-promoting rhizobacteria (PGPR), *Pseudomonas* and *Bacillus* spp. in particular have been utilized widely in biocontrol methods in low input agricultural production systems [11].

Antagonism towards plant pathogenic fungi involves a diverse array of mechanisms such as the production of antibiotics, siderophores, lytic (fungal cell wall degrading) enzymes and hydrogen cyanide (HCN), competition for nutrients and parasitism, as well as emission of potent antifungal volatile organic compounds [12-17] are produced by a diverse array of microorganisms and vary widely in their overall structure, but most of them contain hydroxamate or catecholate groups (Neilands).

Little is known about microorganisms associated with Coffea arabica regarding their antagonistic effect against coffee berry diseases in Ethiopia. However, reports have revealed close associations of useful microorganisms with Coffea arabica including *Bacillus, Burkholderia, Pseudomonas*, members of the Enterobacteriaceae and others against coffee wilt disease [18,19].

Therefore, the authors considered the advantageous to screen indigenous antagonist microorganisms against *C. kahawae* from Ethiopia. Therefore, our goals were to screen native microorganisms associated to *Coffea arabica* tissues which are antagonist to the pathogen mentioned and to evaluate the underlying mechanisms by studying their ability to produce antibiotics, mycoparasitic and lytic enzymes.

## Materials and Methods

### Collection of coffee tissues

Different tissues of coffee (leaf, twig and berry) were used. Eight districts of Oromia and SNNPR regional stats of Ethiopia, at latitude of 6˚16' -8˚98'N, longitude of 34˚35' -41˚0'E and altitude of 1420-2080 masl were randomly selected from coffee growing regions of the country in the South, Southwestern, Southeastern, Eastern and Western main coffee growing ecologies. These areas include some of the afromontane rainforests, coffee ecology with four plantation systems namely: forest, semi-forests and garden coffee plantations of the high, medium and low altitudes. Afromontane rain forest was represented by Harenna and Geba-Doge forests of Manana Harana (Dellomenna) and Yayu districts respectively while semi-forest and garden plantations were represented by Gera, Anfillo and Decha, and Yirgacheffe, Darolebu and Bedeno districts respectively.

Except Darolebu and Bedeno, most of these sites are found in the belt of Afro-montane rainforest between 60N and 90N latitude [20]. The belt is generally categorized in to four agro-ecologies which articulated as sub-humid hot to warm low to mid highland mountains/SH1-7; sub-humid tepid to cool mountains/SH2-7; humid hot to warm low to mid highland mountains/H1-7; and humid tepid to cool highland mountains/H2-7. The altitude, length of growing season, maximum and minimum average temperature and annual average rainfall ranges from 900-1800 masl,180-240 days, 25.5-18°C to 17.5-11°C and 2200-775 mm respectively [21].

Within a given agro-ecological zone, coffee tissue was collected from four different PAs. Collection was confined to fields of small-scale farmers or home gardeners to ensure that no fungicide applications had been made. Depending on the availability, several tissues were collected from the same field. Each tissue was collected from four randomly selected plants. Method of selection of individual coffee from a bunch varied depending on the experiment and is described at appropriate places.

A total of 384 (128 leaf, 128 twig and 128 berry) spacemen were collected from 128 plantation plots. The collected samples were aseptically processed under laboratory conditions and microorganisms were isolated from coffee plant parts surface using the washing technique [6,22].

### Isolation of antagonistic microorganisms from coffee phyllosphare

Four randomly selected tissues from each of the three coffee tissues (leaf, berry and twig) were used for isolation of microorganisms dwelling on the phyllosphare. These tissues were shaken separately in sterile containers for 10 min in sterile normal saline solution (i.e. 0.85% NaCl w/v) using a shaker at 1400 rpm to dislodge microorganisms from the phyllosphare. Serial dilutions so obtained were plated separately on nutrient agar (NA), malt extract agar (MEA) and Potato Dextrose Agar (PDA).

After incubation for 24 to 48 hr, visible colonies of bacteria and yeasts from each sample were grouped by their colony morphologies (such as color, consistency, and shape) and representative colonies were aseptically transferred to fresh plates of the respective medium. The initial MEA and PDA plates were incubated further at 25°C for 5-8 days and representative colonies of filamentous fungi were selected based on their gross morphology and transferred to fresh MEA plates. Pure cultures of fungi isolates were made by cutting a plug of 0.2 $cm^2$ of agar from the edge of the colony with sterilized laboratory knife and aseptically transferred to sterilized plates of MEA using sterilized forceps. Pure cultures of bacteria and yeasts were transferred to new NA (for bacteria) and MEA (for yeast) by striking a single colony using sterilized glass road. Further purification was done trough re-isolating to a new respective media. Purified organisms were stored as slant cultures at Haramaya University Plant Pathology laboratory at 4°C (**Figure 1**).

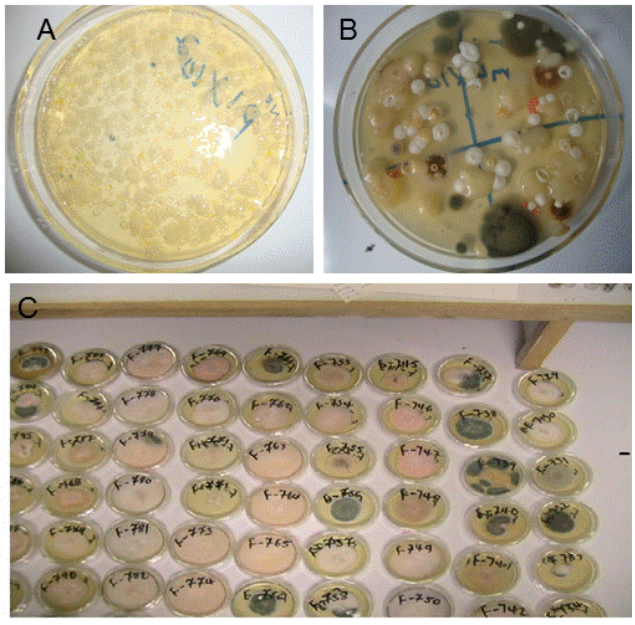


**Figure 1:** Isolating and purification of potential antagonist microorganisms: (A) NA plates and (B) MEA plates used for isolation and (C) Purification of isolates.

The samples yielded 4323 microorganisms (1472 bacteria, 1240 yeast and 1610 filamentous fungi). They were selected after categorizing all organisms by colony shape and color size.

Representative colonies of the isolates were selected for mass screening (**Table 1**).

**Table 1:** Microorganism isolated from *Coffea arabica* and their antagonism towards *Colletotrichum kahawae*.

| | Plant part[1] | | | | Biological activity[2] | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Microorganism[3] | L | T | B | Total | A | L | C | O` | N | Total |
| Bactria | 425 | 499 | 548 | 1472 | 259 | 47 | 128 | 0 | 1038 | 1472 |
| Yeast | 379 | 456 | 405 | 1240 | 48 | 33 | 334 | 0 | 825 | 1240 |
| Fungi | 507 | 540 | 564 | 1611 | 141 | 0 | 180 | 125 | 1165 | 1611 |
| Total | 1311 | 1495 | 1517 | 4323 | 448 | 80 | 642 | 125 | 3028 | 4323 |

[1]Plant parts: L, Leaf, B, Berry and T, Twig

[2]Biological activity of antagonists categorized as: A, Antibiosis L, Lytic, C, Checked the growth of the pathogen, O, Overlap/ mycoparacitic and N, No biological activity

[3]Microorganisms were tasted in their respective group: Bacteria, Yeast and fungi

## Identification of coffee berry diseases and isolation of causal organism

Nine coffee berries collected from three locations were used in this experiment. Mature but unripe, healthy looking berries were incubated in ventilated glass chambers till symptoms appeared from CBD that developed as natural infections. Diseases were identified based on symptomatology using standard disease compendia [23]. Causal organisms responsible for each disease were determined by isolation of pathogens and successful performance of Koch's postulates. Pathogen causing disease was isolated from the advancing edges of infections on to Potato Dextrose Agar (PDA). Cultures were incubated at 25°C for 3-4 weeks under continuous light till sporulation and pure cultures of pathogens were maintained in PDA. Pathogens were identified by the macro and micromorphological features of their cultures [23,24].

## Testing of antagonism *in vitro*

Morphologically different bacterial, yeast and fungal colonies appearing on NA/MEA/PDA were tested against *C. kahowae*, the causal organism of coffee berry disease by the dual culture method as described [25] to determine *in vitro* antagonism. As several different *C. kahowae* isolates were obtained from previous experiment, the most aggressive isolate was used for testing *in vitro* antagonism.

Microorganisms isolated from the berries, twigs and leaves were tested for their primary antibiotic activity against *C. kahowae* by using dual culture method (15 ml of MEA on 9 cm diameter plate) based on inhibition of the pathogen growth. For first mass screening, mycelial plug (0.5 cm diameter) of the test pathogen was seeded in the centre of a culture plate and three candidate organisms were seeded 4 cm apart from the pathogen at three corners of the plate 0.25 cm from the edge. Control plates were inoculated with the pathogen only. All cultures were incubated at room temperature for 12 days and radial growth of the pathogen was measured every 24 hours. The degree of inhibition of growth was calculated as a percentage of the colony radius in the control plates. Percent inhibition was calculated as; % inhibition = [(A-B)/A] × 100, where 'A'= average colony diameter in the control plate and 'B' = average colony diameter in the test plate. The circumference of the colony of pathogen in each plate was marked on the underside of the plate and it was further incubated for five days to confirm presence of fixed inhibition zone. Isolates showing greater than 50% inhibitory effects were selected for second inhibition test using one to one dual culture method.

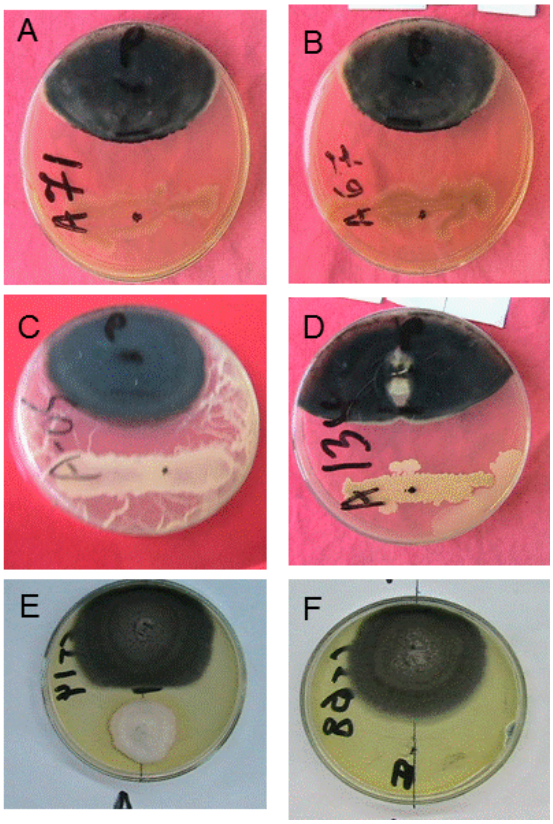


**Figure 2:** *In vitro* Inhibition of mycelium growth of the pathogen by the promising antagonists bacteria (A and B), yeast (C and D) and filamentous fungi (E) isolates test. (A and B) and control plates with pathogen only (F).

Test isolates which did not yield inhibition zone, but which could attack hyphae of *C. kahowae* were further observed for their lytic or overlapping activity. The colony margin of the pathogen in each plate was marked upon contact with the antagonist colony. Plates were then incubated till the colony of the pathogen reached the edge of the plate and examined to check if hyphae of the pathogen were lysed back or overlapped by the antagonist colony. Antagonist organisms with lysis effects were all selected and tested as described below. Antagonist organisms which overlap the pathogen colony before it reached 50 percent growth were selected and tested as described below and the rest of isolates without any activity were damped.

Eight districts of Oromia and SNNPR regional stats of Ethiopia, at latitude of 6˚16' -8˚98'N, longitude of 34˚35' -41˚0'E and altitude of 1420-2080 masl were randomly selected from coffee growing regions of the country in the South, Southwestern, Southeastern, Eastern and Western main coffee growing ecologies (**Figure 2**). These areas include some of the afromontane rainforests, coffee ecology with four plantation systems namely: forest, semi-forests and garden coffee plantations of the high, medium and low altitudes. Afromontane rain forest was represented by Harenna and Geba-Doge forests of Manana Harana (Dellomenna) and Yayu districts respectively while semi-forest and garden plantations were represented by Gera, Anfillo and Decha, and Yirgacheffe, Darolebu and Bedeno districts respectively (**Table 2**).

**Table 2:** Category of microorganisms with antibiosis and mycoparasitic activity.

| Inhibition | Antibiosis activity[1] | | | | Pathogen | Mycoparasitic activity[2] | | | |
|---|---|---|---|---|---|---|---|---|---|
| Range | Bacteria | Yeast | Fungi | Total | Growth (%) | Bacteria | Yeast | Fungi | Total |
| 0 to 10 | 10 | 0 | 4 | 14 | 0 to 10 | 0 | 0 | 1 | 1 |
| 10 to 20 | 33 | 5 | 10 | 48 | 10 to 20 | 0 | 0 | 22 | 22 |
| 20 to 30 | 80 | 8 | 37 | 125 | 20 to 30 | 0 | 0 | 13 | 13 |
| 30 to 40 | 76 | 12 | 33 | 121 | 30 to 40 | 0 | 0 | 2 | 2 |
| 40 to 50 | 16 | 3 | 12 | 31 | 40 to 50 | 0 | 0 | 4 | 4 |
| 50 to 60 | 15 | 3 | 6 | 24 | 50 to 60 | 0 | 0 | 11 | 11 |
| 60 to 70 | 15 | 5 | 6 | 26 | 60 to 70 | 0 | 0 | 10 | 10 |
| 70 to 80 | 9 | 8 | 19 | 36 | 70 to 80 | 0 | 0 | 27 | 27 |
| 80 to 90 | 5 | 4 | 11 | 20 | 80 to 90 | 0 | 0 | 35 | 35 |
| 90 to 100 | 0 | 0 | 3 | 3 | 90 to 100 | 0 | 0 | 0 | 0 |
| Total | 259 | 48 | 141 | 448 | Total | 0 | 0 | 125 | 125 |
| Selected[3] | 44 | 20 | 45 | 109 | Selected 3 | 0 | 0 | 250 | 42 |

[1]Isolates with antibiosis activity were categorized based on their percent inhibition of the pathogen as compared to control (mean of 3 replications). Isolates with more than 50% inhibition were selected.

[2]Isolates with Mycoparasitic activity were categorized based on the growth of the pathogen as compared to control (mean of 3 replications). Isolates which did not allow the growth of the pathogen more than 50% were selected.

[3]Total numbers of isolates from each kind of microorganism selected for further experiment

Except Darolebu and Bedeno, most of these sites are found in the belt of Afro-montane rainforest between 60N and 90N latitude [20]. The belt is generally categorized in to four agro-ecologies which articulated as sub-humid hot to warm low to mid highland mountains/SH1-7; sub-humid tepid to cool mountains/SH2-7; humid hot to warm low to mid highland mountains/H1-7; and humid tepid to cool highland mountains/H2-7. The altitude, length of growing season, maximum and , minimum average temperature and annual average rainfall ranges from 900-1800 masl,180-240 days, 25.5-18°C to 17.5-11°C and 2200-775 mm respectively [21].

## Screening for antibiosis

Bacterial, yeast and fungal isolates which showed greater than 50 percent inhibition during primary antibiosis activity were further screened individually using one to one inoculation of the antagonist and the pathogen on dual culture. 5mm mycelia plug of the test pathogen was inoculated at the edge of the plate. Bacterial and yeast isolates were spotted opposite to the pathogen. For fungal isolates 5mm of mycelia plug were seeded opposite to the pathogen. Three replicate plates of each isolate were used in CRD at 22 to 27°C. Control plates were seeded with culture of the pathogen only. All cultures were incubated for 12 days and radial growth of the pathogen was measured every 24 hours.

Percent inhibition was calculated as; % inhibition = [(A-B) /A] × 100, where 'A'= average colony diameter in the control plate and 'B' = average colony diameter in the test plate. Percentage of inhibition was arcsine transformed before statistical analysis.

Isolates showing significant inhibitory effect as compared to the control were selected for further screening *in vivo* (**Figure 3**).

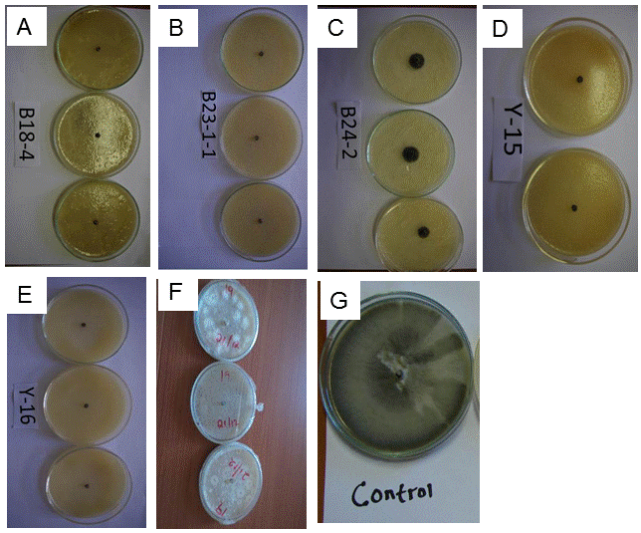


**Figure 3:** Inhibition of the mycelia growth of *C. kahawae* in *in vitro* by antagonists after under field condition test. Plates showing the effect of bacterial isolates BA18 (A), BA 23 (B), BA 24 (C) ; yeast isolates YA15 (D), YA16 (E) Fungal isolate (FA44) and control plates with pathogen only (G).

## Screening for lytic activity

Antagonists selected for their preliminary lytic effects during primary screening were tested specifically for their lytic activity as described by (Jeyarajan and Nakkeeran, 2000; Kefialew and Ayalew, 2008). Conidial suspension of *C. kahawae* was prepared by washing 10 days old MEA cultures with 5 ml sterile distilled water containing 0.01% Tween 80. Spores were suspended by gently scraping the colony surface with sterilized scalpel and transferred to 50 ml sterilized beaker and thoroughly stirred for 10-15 minutes with sterilized magnetic stirrer to extract the spores from the interwoven mycelia, and then filtered in to another sterilized beaker through sterilized double layer cheese cloths. Spore concentration was adjusted to $2 \times 10^6$ conidia/ml using a haemocytometer. The suspension was transferred to a sterilized test tube. One ml of suspension containing $2 \times 10^6$ conidia $ml^{-1}$ of the pathogen was mixed with 15 ml of sterilized MEA medium in sterilized test tube kept in a water bath at 40°C to maintain the optimum temperature and poured to sterilized culture plates. When sufficient growth of the pathogen occurs, the selected antagonistic organisms were spotted at three points (that is replication) over the test pathogen colony, and culture plates with no antagonistic isolates but inoculated with sterile distilled water were used as control. After 20 days, width of lysed mycelia around lytic organism colony was measured. Microscopic observation of lised mycelia was conducted after staining of the mycelia.

Lyses diameter was subjected to statistical analysis and bacteria and yeast isolates were treated in their respective group. Isolates showing significant lyses effect as compared to the control was selected for further screening *in vivo*.

## Screening for mycoparasitic activities

Fungal isolates which showed hyperparasitic activity before the pathogen reached 50 percent growth during primary mass screening activity were further screened individually using dual culture of the antagonist and the pathogen. Five mm mycelia plug of the test pathogen was inoculated at the edge of the plate and 5 mm plug of mycelia plug of fungal isolates were seeded opposite to the pathogen. Control plates were seeded with culture of the pathogen only and three replicate plates of each isolate were used in CRD. All cultures were incubated for 7 days at 25 to 27°C and over growth of antagonist and the pathogen were measured every 24 hours for 7 days. For confirmation of presence of hyperparasitic effect, coiling of hyphae of the pathogen was observed under microscope. Isolates showing significant hyperparsitic effect were selected for further screening *in vivo*.

## Data analysis

Analysis of variance (ANOVA) was carried out with the statistical software SPSS V. 20 (SPSS Inc., Chicago, USA). To assess the effect of the repeated experiments, a three-way ANOVA model was used by using repetitions as main plot and treatment combinations as sub effect. Percent data and scale units were subjected to Arc sign and square root transformation, respectively before analysis. The data from the repeated experimental runs were pooled and means were estimated through analysis of variance. A comparison among treatment mean was made with Duncan's Multiple Range Test (DMRT), ($P \leq 0.05$).

# Results

## Primarily screening of microorganisms for their potential biological activity

All the representative isolates were tested for antibiosis activity. Among these, a total of 448 isolates (259 bacteria, 48 yeasts and 141 filamentous fungi) were revealed antibiosis activity by forming distinct inhibition zone. The formation of inhibition zone was confirmed by incubation for additional 12 days. Among 448 microorganisms which showed inhibition of the growth of the pathogen by <50% were selected for the further experiment. Based on this a total of 109 microorganisms (44 bacteria, 20 yeasts and 45 filamentous fungi) were selected.

Observation was also made on the remaining isolates which did not yield antibiosis (inhibition zone), but which could result lytic or hyperparasitic activity. Thus 80 microorganisms (47 bacteria, 33 yeasts) showed lytic activity and 125 filamentous fungi showed hyperparasitic activity after contact with the pathogen. However, 642 microorganisms (128 bacteria, 334 yeasts and 180 fungi) made a contact with the pathogen by then the growth of both organisms was stopped. Therefore, these 642 microorganisms did not show any biological activity. The remaining 3028 isolates were covered by the pathogen which implies that they did not show any antagonistic activity rather than defeated by the pathogen. Therefore, 642 isolates with contact activity and 3028 isolates with no activities were

discarded. All 80 isolates (47 bacteria and 33 yeasts) with lytic activity were gone on for further lytic test. Among the 125 fungal isolates 42 isolates which showed hyperparasitic activity before the pathogen radial growth reached 50% from the control were also selected for further test. In general 109 isolates (44 bacteria, 20 yeasts and 45 fungi) with antibiosis, 80 isolates (47 bacteria and 33yeasts) with lytic and 42 filamentous fungi with hyperparasitic activities were selected for further antagonistic test **(Table 2)**.

## Antibiosis effect of antagonists on growth of pathogen

Analysis of variance revealed that there was a significant difference ($p<0.05$) among bacteria, yeast and fungi isolates tested for their potential antibiosis activities in their respective group. There was significant difference ($p<0.05$) among bacterial isolates and all of them significantly affect the growth of the pathogen as compared to the control. Percent inhibition of the pathogen growth by bacterial isolates ranged from 57 to 86.7 with isolate DM-016-L-2 -1 showed the highest percent inhibition (**Table 3**). All isolates showed more than 50 % inhibition of pathogen growth and among these 17 isolates resulted in >75% inhibition of pathogen growth. Among these 10 bacterial isolates (DM-016-L-2 -1, YC-111-L-4 -1, DL-025-T-3 -1, DM-010-L-2 -1, DA-121-L-4 -1, BO-048-L -1 -1, DA-118-B-5 -1, YU-070-T -1 -1, YU-069-L-2 -1 and AN-086-B-4 -1) were showed significantly higher ($p<0.05$) than all bacterial isolates. Therefore, these 10 bacterial organisms were selected for the further test *in vivo*.

**Table 3:** Bacterial isolates tested for antibiosis activity.

| isolates code | Inhibition (%)[1] | | | isolates code | Inhibition (%)[1] | | |
|---|---|---|---|---|---|---|---|
| DM-008-B-2 -1 | 71.7 | (57.80)[2] | Hi[3] | DL-025-B-5 -1 | 64.2 | (53.20)[2] | C[3] |
| BO-034-B-3 -1 | 72.5 | -58.4 | ij | BO-042-B -1 -1 | 68.3 | -55.8 | de |
| GA-063-B-2 -1 | 68.3 | -55.8 | de | AN-086-B-4 -1 | 83.3 | -65.9 | q |
| AN-083-B-3 -1 | 78.3 | -62.3 | o | DA-115-B -1 -1 | 75.4 | -60.3 | lm |
| YC-103-B-6 -1 | 57.9 | -49.6 | b | DM-010-L-2 -1 | 85.4 | -67.6 | rst |
| DA-118-B-5 -1 | 84.6 | -66.9 | qrs | BO-040-L-3 -1 | 76.7 | -61.1 | mn |
| DM-016-L-2 -1 | 86.7 | -68.6 | u | GA-059-L-3 -1 | 63.8 | -53 | c |
| DL-028-L-2 -1 | 70 | -56.8 | fg | DA-121-L-4 -1 | 85 | -67.2 | rs |
| BO-043-L-5 -1 | 57.1 | -49.1 | b | GA-056-T-5 -1 | 75 | -60 | kl |
| GA-053-L-4 -1 | 57.5 | -49.3 | b | YU-070-T -1 -1 | 84.2 | -66.6 | qr |
| GA-064-L-6 -1 | 74.6 | -59.7 | kl | YU-074-L-2 -1 | 63.3 | -52.7 | c |
| AN-083-L-6 -1 | 70.8 | -57.3 | gh | DL-030-L-2 -1 | 63.8 | -53 | c |
| YC-111-L-4 -1 | 86.3 | -68.3 | tu | GA-053-B-4 -1 | 71.7 | -57.8 | hi |
| BO-043-T-6 -1 | 72.9 | -58.6 | ij | DM-001-B-2 -1 | 78.3 | -62.3 | o |
| DA-119-T-2 -1 | 72.1 | -58.1 | hi | YU-071-B-6 -1 | 73.8 | -59.2 | jk |
| DM-006-T-5 -1 | 70 | -56.8 | fg | AN-094-B-6 -1 | 78.8 | -62.6 | op |
| AN-090-L-3 -1 | 69.6 | -56.5 | efg | DA-127-B-5 -1 | 80 | -63.4 | p |
| YU-069-L-2 -1 | 83.3 | -65.9 | q | DL-023-L-4 -1 | 77.9 | -62 | n,o |
| BO-036-L-3 -1 | 71.7 | -57.8 | hi | BO-048-L -1 -1 | 85 | -67.2 | rs |
| DM-005-L-2 -1 | 64.2 | -53.2 | c | YC-097-L-5 -1 | 67.9 | -55.5 | d |
| YU-077-B-4 -1 | 63.3 | -52.7 | c | DL-025-T-3 -1 | 85.8 | -67.9 | stu |
| BO-047-B-5 -1 | 63.3 | -52.7 | c | Control | 0 | 0 | a |
| DM-013-B-6 -1 | 69.2 | -56.3 | def | | | | |

[1]Percent of inhibition recorded over the control (mean of three replications)
[2]Mean percent inhibition subjected to Arc sin transformation (mean of three replications)
[3]Means within a column followed by the same letters were not statistically significant ($P<0.05$), DMRT

All the yeast isolates showed significantly higher inhibition of pathogen growth as compared to the control. Growth of the pathogen was reduced by 52 to 86.7% due to the 28 yeast isolates and all isolates reduced pathogen growth by >50%. More than 75% growth inhibition of the pathogen was recorded from 14 isolates. Among these, 10 isolates (YU-070-L-2-2 ,

DA-116-L-5-2 , YU-078-B-5-2 , YU-067-B -1-2 , YC-097-B-6-2 , DM-004-B-3-2 , BO-033-B-3-2 , GA-049-B-6-2 , DM-004-L-2-2 and DM-015-B -1-2 ) were significantly higher ($p<0.05$) than other yeast isolates and selected for further in vivo test (**Table 4**).

**Table 4:** Yeast isolates tested for antibiosis activity.

| isolates | Inhibition (%)[1] | | |
|---|---|---|---|
| DL-023-T-6-2 | 52.5 | (46.40)[2] | b[3] |
| DL-024-B-3-2 | 73.8 | -59.2 | ef |
| DM-015-B -1-2 | 83.3 | -65.9 | k |
| YU-070-L-2-2 | 86.7 | -68.6 | k |
| BO-044-L-3-2 | 72.9 | -58.6 | e |
| BO-043-B-5-2 | 71.3 | -57.6 | d |
| AN-088-B-3-2 | 79.2 | -62.8 | h |
| GA-057-B-3-2 | 78.8 | -62.6 | h |
| YU-067-B -1-2 | 85.8 | -67.9 | k |
| DA-125-B-5-2 | 74.6 | -59.7 | i |
| AN-089-L-6-2 | 78.3 | -62.3 | gh |
| DA-116-L-5-2 | 86.7 | -68.6 | k |
| DA-119-B-5-2 | 77.1 | -61.4 | g |
| DM-004-L-2-2 | 83.8 | -66.2 | i |
| YU-078-B-5-2 | 86.3 | -68.2 | f |
| YC-105-B -1-2 | 58.3 | -49.8 | c |
| BO-033-B-3-2 | 84.2 | -66.6 | ij |
| YC-097-B-6-2 | 85.4 | -67.6 | jk |
| DM-004-B-3-2 | 85.4 | -67.6 | jk |
| GA-049-B-6-2 | 84.2 | -66.6 | ij |
| Control | 0 | 0 | a |

[1]Percent of inhibition recorded over the control (mean of three replications)
[2]Mean percent inhibition subjected to Arc sin transformation (mean of three replications)
[3]Means within a column followed by the same letters were not statistically significant ($P<0.05$), DMRT

All the filamentous fungi isolates showed significantly affected ($p<0.05$) pathogen growth as compared to the control. The maximum growth inhibition of the pathogen was recorded from isolate YC-101-B-5-3, resulting 85.6% inhibition of pathogen growth while the least inhibition (55.8%) was recorded from isolate DA-120-L -1-3. Only four isolates effectively reduced the pathogen growth by >75%. Among the filamentous fungi four isolates (AN-091-T-3-3, GA-064-T-2-3, DA-118-L-4-3, and YC-101-B-5-3) were showed significantly higher ($p<0.05$) inhibitory effect and they were selected for further in vivo test (**Table 5**). In general 10 bacteria, 10 yeasts and 4 filamentous fungi isolates were selected for further test *in vivo*.

**Table 5:** Fungal isolates tested for antibiosis activity.

| Isolates code | Inhibition (%)[1] | | | isolates code | Inhibition (%)[1] | | |
|---|---|---|---|---|---|---|---|
| YC-104-T-2-3 | 60 | (50.80) [2] | defg [3] | DM-006-B-3-3 | 69.2 | (56.30) [2] | q |
| DM-008-T-4-3 | 58.3 | -49.8 | cd | DA-126-T-6-3 | 66.3 | -54.5 | jk |
| GA-054-T-6-3 | 69.2 | -56.3 | mno | AN-084-B-4-3 | 67.9 | -55.5 | lm |
| DM-009-L-6-3 | 64.6 | -53.5 | hij | DA-121-T-4-3 | 65.4 | -54 | hijk |
| AN-094-T-5-3 | 66.7 | -54.7 | kl | DM-011-T-4-3 | 59.6 | -50.5 | cdef |
| BO-041-T-4-3 | 61.7 | -51.8 | g | DL-024-L-5-3 | 68.3 | -55.8 | lm |
| GA-059-T-5-3 | 60.8 | -51.3 | fg | DL-030-T-2-3 | 65.8 | -54.2 | ijk |
| BO-036-T-4-3 | 68.8 | -56 | mn | AN-083-T-2-3 | 58.8 | -50 | cde |
| GA-051-T -1-3 | 58.8 | -50 | cde | YC-098-L-2-3 | 65 | -53.7 | hijk |
| DA-113-B-4-3 | 69.2 | -56.3 | q | DM-016-T-2-3 | 60.8 | -51.3 | fg |
| AN-091-T-3-3 | 85 | -67.2 | st | GA-056-L-5-3 | 60 | -50.8 | defg |
| DM-004-T-2-3 | 65.4 | -54 | hijk | BO-037-B-6-3 | 65.4 | -54 | hijk |
| GA-064-T-2-3 | 84.6 | -66.9 | rs | YU-076-T-6-3 | 65 | -53.7 | hijk |
| DA-118-L-4-3 | 83.8 | -66.2 | r | AN-086-L-6-3 | 68.8 | -56 | q |
| YU-075-L-2-3 | 68.3 | -55.8 | p | BO-039-L-5-3 | 69.2 | -56.3 | no |
| GA-058-B -1-3 | 60 | -50.8 | defg | DA-116-T-2-3 | 68.3 | -55.8 | lm |
| DL-018-L-6-3 | 58.8 | -50 | cde | YU-070-B-4-3 | 68.8 | -56 | o |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| GA-062-B-6-3 | 64.6 | -53.5 | hij | DM-003-L -1-3 | 69.2 | -56.3 | q |
| DA-120-L -1-3 | 55.8 | -48.4 | a | YC-107-L-3-3 | 64.2 | -53.2 | hi |
| BO-033-L -1-3 | 63.8 | -53 | h | YU-071-T-4-3 | 60.8 | -51.3 | fg |
| GA-062-L-3-3 | 60.4 | -51 | efg | YC-101-B-5-3 | 85.8 | -67.9 | t |
| DL-022-T-3-3 | 57.9 | -49.6 | c | YC-099-T-4-3 | 65 | -53.7 | hijk |
| YC-110-T-3-3 | 60 | -50.8 | defg | Control | 0 | 0 | a |

[1]Percent of inhibition recorded over the control (mean of three replications)
[2]Mean percent inhibition subjected to Arc sin transformation (mean of three replications)
[3]Means within a column followed by the same letters were not statistically significant (P< 0.05), DMRT

## Lysis effect of antagonists on growth of pathogen

The mycelium of *C. kahawae* was significantly lysed due to the application of bacteria and yeast isolates (**Tables 6 and 7**). Lysis diameter due to bacterial and yeast isolates ranged from 1.7 to 15.3 and from 1.6 to 12.3 mm respectively. All bacterial isolates were significantly lysed ($p<0.05$) the pathogen mycelia as compared to the control (**Table 6**). Among these bacterial isolates 3 isolates (DA-125-L-2 -1, YU-065-T-3 -1, and DL-031-T-4 -1 were statistically became superior to all bacterial isolates. Therefore, these bacterial isolates were gone on to the further in vivo test. All the yeast isolates significantly lysine ($p<0.05$) the pathogen mycelia as compared to the control. Among these yeast organisms one isolate (DM-012-L-6-2) showed statistically higher ($p<0.05$) than all other yeast isolates and selected for further in vivo test (**Tables 8 and 9** ).

**Table 6:** Bacterial isolates tested for lytic activity.

| Isolate code | Lysis diam. [1] (mm) | | | Isolate code | Lysis diam. [1] (mm) | | |
|---|---|---|---|---|---|---|---|
| AN-084-T -1 -1 | 7 | (2.70) [2] | jk [3] | YC-105-L -1 -1 | 4 | (2.10) [2] | de [3] |
| DA-125-L-2 -1 | 15 | -3.9 | l | GA-061-L-3 -1 | 8 | -2.9 | k |
| YU-065-T-3 -1 | 15.3 | -4 | l | BO-046-L-4 -1 | 4 | -2.1 | de |
| DL-031-T-4 -1 | 14 | -3.8 | l | DL-018-T -1 -1 | 4 | -2.1 | de |
| YU-072-L-2 -1 | 3.7 | -2 | d | GA-050-T-5 -1 | 4.7 | -2.3 | defg |
| DL-021-B-4 -1 | 4 | -2.1 | de | GA-054-L -1 -1 | 4 | -2.1 | de |
| DL-020-L -1 -1 | 5.3 | -2.4 | fghi | DL-030-B-5 -1 | 5.3 | -2.4 | fghi |
| YU-066-L-4 -1 | 5 | -2.4 | efgh | DA-114-L-2 -1 | 2.7 | -1.8 | c |
| DM-002-L -1 -1 | 4 | -2.1 | de | GA-050-B-3 -1 | 4 | -2.1 | de |
| DM-012-T-4 -1 | 6.3 | -2.6 | lj | YU-074-B-6 -1 | 4 | -2.1 | de |
| DA-117-L-4 -1 | 7.7 | -2.9 | k | AN-095-L-3 -1 | 4 | -2.1 | de |
| DA-123-B-2 -1 | 8 | -2.9 | k | GA-050-L -1 -1 | 4 | -2.1 | de |
| BO-038-T -1 -1 | 5.7 | -2.5 | ghi | BO-038-B-4 -1 | 5 | -2.4 | efgh |
| YC-100-T-5 -1 | 4.7 | -2.3 | defg | YU-079-T-2 -1 | 4 | -2.1 | de |
| AN-087-L-4 -1 | 4 | -2.1 | de | YU-068-B -1 -1 | 5.3 | -2.4 | fghi |
| YC-100-L-6 -1 | 5.3 | -2.4 | fghi | YC-110-B-5 -1 | 6 | -2.6 | hi |
| DM-002-T-2 -1 | 4 | -2.1 | de | BO-038-L-6 -1 | 2.7 | -1.8 | c |
| BO-034-L -1 -1 | 4.3 | -2.2 | def | DL-026-L -1 -1 | 5 | -2.4 | efgh |
| DM-016-B-6 -1 | 5.7 | -2.5 | ghi | YU-076-L -1 -1 | 4 | -2.1 | de |
| DM-007-L -1 -1 | 4 | -2.1 | de | YC-107-B-6 -1 | 5 | -2.4 | efgh |
| DM-003-B-6 -1 | 5 | -2.4 | efgh | YC-108-L-4 -1 | 5 | -2.4 | efgh |
| GA-057-L-5 -1 | 5.7 | -2.5 | ghi | GA-059-B-6 -1 | 5.3 | -2.4 | fghi |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| YU-078-L-2 -1 | 1.7 | -1.5 | b | YU-080-B-2 -1 | 4 | -2.1 | de |
| DM-013-L-5 -1 | 4.3 | -2.2 | def | control | 0 | -0.7 | a |

[1]Diameter of lysed mycelium (mean of three replications)

[2]Mean lysed mycelia subjected to square root (X+0.5) transformation (mean of three replications)

[3]Means within a column followed by the same letters were not statistically significant (P< 0.05), DMRT

**Table 7:** Yeast isolates tested for lytic activity.

| Isolate code | Lysis diam. [1] (mm) | | | Isolate code | Lysis diam. [1] (mm) | | |
|---|---|---|---|---|---|---|---|
| DL-022-B -1-2 | 2.3 | (1.70) [2] | Bc[3] | DL-025-L -1-2 | 4 | (2.10) [2] | d |
| YU-073-B-2-2 | 6 | -2.6 | ef | BO-045-T-3-2 | 5 | -2.4 | de |
| GA-052-B-4-2 | 4 | -2.1 | d | YC-102-L-2-2 | 7.3 | -2.8 | g |
| DA-116-B-6-2 | 5.3 | -2.4 | de | AN-090-B-2-2 | 4 | -2.1 | d |
| DM-012-L-6-2 | 12.3 | -3.6 | i | DM-007-B-5-2 | 4 | -2.1 | d |
| DL-018-B-4-2 | 5 | -2.4 | de | BO-045-B-6-2 | 7 | -2.7 | gh |
| DA-128-B-6-2 | 5 | -2.4 | de | GA-063-L-3-2 | 7.3 | -2.8 | h |
| YC-111-B-3-2 | 4.7 | -2.3 | de | GA-052-L-4-2 | 4.7 | -2.3 | de |
| YU-079-L -1-2 | 4 | -2.1 | d | BO-040-B-5-2 | 1.7 | -1.5 | b |
| YC-106-L-4-2 | 5 | -2.4 | de | AN-085-B-3-2 | 7.3 | -2.8 | g |
| DL-028-B-4-2 | 5 | -2.4 | de | AN-093-B-4-2 | 2.7 | -1.8 | c |
| DA-128-L-5-2 | 4 | -2.1 | d | DM-011-B-4-2 | 4 | -2.1 | d |
| YC-100-B-3-2 | 5 | -2.4 | de | AN-081-B-6-2 | 7 | -2.7 | g |
| GA-064-B-6-2 | 7 | -2.7 | fg | DA-121-B-5-2 | 4.3 | -2.2 | d |
| BO-036-B-5-2 | 4.7 | -2.3 | de | DL-031-B-3-2 | 4.7 | -2.3 | de |
| YC-102-B -1-2 | 5 | -2.4 | de | GA-062-T -1-2 | 5 | -2.4 | de |
| DA-124-L-4-2 | 2.7 | -1.8 | c | Control | 0 | -0.7 | a |

[1]Diameter of lysed mycelium (mean of three replications)
[2]Mean lysed mycelia subjected to square root (X+0.5) transformation (mean of three replications)
[3]Means within a column followed by the same letters were not statistically significant (P< 0.05), DMRT

**Table 8:** Isolates tested for mycoparasitic activity.

| Isolate code | Colonization[1] (%) | | | Isolate code | Colonization[1] (%) | | |
|---|---|---|---|---|---|---|---|
| AN-089-T-5-3 | 74 | (8.60) [2] | Qp[3] | AN-093-L-4-3 | 79.7 | (9.00) [2] | R[3] |
| YC-103-L-3-3 | 73.7 | -8.6 | op | BO-048-T-6-3 | 80 | -9 | r |
| DL-019-T-4-3 | 72.3 | -8.5 | lmno | DL-027-T-4-3 | 80.3 | -9 | r |
| YC-106-T-5-3 | 65.3 | -8.1 | ef | DA-113-T-5-3 | 73 | -8.6 | mnop |
| YU-078-T -1-3 | 68.3 | -8.3 | gh | AN-096-T-2-3 | 72 | -8.5 | klmn |
| GA-061-T-6-3 | 71.7 | -8.5 | jklm | AN-082-L-2-3 | 61 | -7.8 | c |
| YU-071-L-6-3 | 63.7 | -8 | d | DA-120-B-2-3 | 72.3 | -8.5 | lmno |
| DL-031-L-6-3 | 64.3 | -8.1 | def | DL-021-L -1-3 | 72.3 | -8.5 | lmno |
| DM-009-T -1-3 | 72 | -8.5 | klmn | DM-007-T-2-3 | 65.7 | -8.1 | f |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| AN-092-T-3-3 | 49 | -7 | b | AN-087-T-3-3 | 68.3 | -8.3 | gh |
| AN-081-T-2-3 | 80 | -9 | r | GA-057-T-3-3 | 75.3 | -8.7 | qr |
| YU-074-T-3-3 | 72 | -8.5 | klmn | BO-044-T-4-3 | 48.3 | -7 | b |
| YC-102-T-3-3 | 71 | -8.5 | jkl | BO-039-T -1-3 | 67.7 | -8.3 | g |
| YC-108-B-5-3 | 80 | -9 | r | DA-128-T-3-3 | 71.3 | -8.5 | jklm |
| DM-014-T -1-3 | 63 | -8 | d | BO-048-B-3-3 | 71.3 | -8.5 | jklm |
| YU-079-B-6-3 | 72 | -8.5 | klmn | DL-023-B -1-3 | 69.3 | -8.4 | Hi |
| GA-049-L -1-3 | 70 | -8.4 | lj | DM-014-L-5-3 | 71 | -8.5 | Jkl |
| DA-118-T-4-3 | 71 | -8.5 | jkl | BO-033-T-5-3 | 64 | -8 | De |
| YU-066-B-3-3 | 70.3 | -8.4 | ljk | DM-001-T-6-3 | 71 | -8.5 | Jkl |
| YC-112-L-5-3 ) | 71 | -8.5 | jkl | AN-095-B-5-3 | 70 | -8.4 | ij |
| DA-123-T-6-3 | 63 | -8 | d | control | 0 | -0.7 | a |
| YU-067-T -1-3 | 73.3 | -8.6 | nop | | | | |

[1]Colonization percent of pathogen mycelium (mean of three replications)
[2]Mean colonized mycelium subjected to arcsine transformation (mean of three replications)
[3]Means within a column followed by the same letters were not statistically significant (P< 0.05), DMRT

**Table 9:** Number of microorganisms selected for further test *in vivo*.

| **Biological** | **Group of organism**[1] | | | |
|---|---|---|---|---|
| Activity | Bacteria | Yeast | Fungi | Total |
| Antibiotic | 10 | 10 | 4 | 24 |
| Lytic | 3 | 1 | 0 | 4 |
| Mycoparasitic | 0 | 0 | 5 | 5 |
| Total selected [2] | 13 | 11 | 9 | 33 |

[1]Biological activity were compare for each group as antibiotic, lytic or mycoparasitic activity
[2]Isolates selected for further experiment *in vivo*

## Hyperparacitic and mycelia colonization effect of antagonists on growth of pathogen

There was significantly difference ($p<0.05$) among filamentous fungi of hyperparasitic activity. All hyperparasitic filamentous fungi were significantly higher in their hyperparasitic activity compared to the control. The maximum and minimum percent colonization of antagonists on mycelia of *C. kahawae* was recorded from isolate BO-044-T-4-3and DL-027-T-4-3resulting 80.3 and 48.3 percent colonization, respectively. Among hyperparasitic filamentous fungi five isolates (AN-081-T-2-3, YC-108-B-5-3, AN-093-L-4-3, BO-048-T-6-3, and DL-027-T-4-3) showed highly significant ($p<0.05$) parasitic activities therefore these hyperparasitic filamentous isolates were selected for further *in vivo* test.

In general a total of 33 isolates (10 antibiosis bacteria, 10 antibiosis yeasts, 4 antibiosis filamentous fungi, 3 lytic bacteria, 1 lytic yeast (isolate LY05) and 5 hyparasitic filamentous fungi selected for the test *in vivo*.

## Discussion

The pathogen (*C. kahawae*) was isolated for this test from three representatives areas were resulted high pathogenecity and conidial production. These show that the collected pathogen isolates were among the aggressive *C. kahawae* isolates of the country. Previous investigators were also successful in isolation of this pathogen from the same locations [20,26]. However the reference isolate obtained from JARC was the most aggressive strain and it was used throughout this experiment. According to Arega et al. [20] reported that this isolate showed that the highest pathogenecity and conidial production potential. This shows that the target pathogen isolate selected for this experiment was the most appropriate and applicability of the result to the other strains also. In our tests, *C. kahawae* caused deeply sunken lesions on the berry surface, then invaded and destroyed the berries as reported in previous studies [27,28]. Some of the isolates seldom infected the coffee beans, although they produced sunken lesions on the pericarp.

Selecting representative microorganisms from hundreds of thousands of colonies from dilution plate is economical, time saving and effective method which has been adapted by the several authors [29-31]. In the preliminary mass screening only 15% of the microorganisms were showed biological activity interims of antibiotic, lytic and hyperparasitic. Among these 10.4%, 1.9% and 2.9% were antibiotic, lytic and hyperparasitic activity respectively. This showed that only a fraction of microorganisms tested have a potential of antagonistic activity. After screening of hundreds of thousands of microorganisms several investigators also come up with only few active isolates [18,29].

According to Kohl [32,33] observation variability in the efficacy among the isolates of *Trichoderma* spp. against *Botrytis*

*cinerea* [34] tested 44 isolates of *Trichoderma spp*. and found that only 14 could infect and kill all the sclerotia inoculated, 13 could kill only some of the sclerotia, and 17 did not affect the sclerotial viability.

Previous studies also reveal that antagonistic activity has been expressed interims of several mod of actions including antibiotic, lytic enzymes production, and hyperparasites etc. [35,25]. Activities of antibiosis were determined by the formation of clear inhibition zone between the pathogen and the antagonist on the dual culture plate. On the other hand lytic and hyperparasite activity determined by the presence of lysed and growing over the pathogen respectively after contact has been made between the pathogen and the antagonist. Additionally both the pathogen and the antagonist stop growing after they contact each other and the pathogen starts growing over the antagonist was indicate that there is no biological activity observed by the candidate antagonistic microorganisms against the pathogen. The presence of clear inhibition zone as indicator of antibiotic production has been reported by several author [36-38].

More over the presence of lysed mycelia and the continuation of the antagonist growth over the pathogen has been used as an indicator of lytic enzyme production and hyperparasitic activity respectively by several authors [39,40]. However these indicators should be farther confirmed in additional test.

Antagonistic interactions between *Trichoderma* and *Gliocladium spp*. and other fungi have been classified as antibiosis, mycoparasitism, and competition for nutrients [41,42]. These mechanisms are not mutually exclusive, and a given antagonistic mechanism can fall into several of these categories. For example, the control of Botrytis on grapes by *Trichoderma* involves both nutrient competition and mycoparasitism of sclerotia, both of which result in suppression of the pathogen's ability to cause and perpetuate disease [43]. Both antibiosis and mycoparasitism may be involved in competition for nutrients [44]. Other evidence has shown that antibiotics and hydrolytic enzymes are not only produced together but act synergistically in mycoparasitic antagonism [45]. Some antagonistic interactions do not fall readily into any of the classical categories. For example, it has recently been suggested that the bio-control agent *T. harzianum* T39 reduces the pathogenicity of *B. cinerea* by reducing the amount of pectin-degrading enzymes produced by the pathogen [40]. The importance of a given antagonistic mechanism has been shown in many studies to be dependent on the antagonist strain, the target organism, and the environmental conditions [45,37]. Selection of biological control agents should take into consideration the intended application as well as the target pathogen [46-48].

Secondary screening was conducted to confirm and quantify the final biological control *in vitro* in this experiment. This secondary test confirmed the presence of biological activity by showing either antibiotic, lytic or hyperparasitc activity. However there was variation among antagonistic isolates interims of degree of biological activity [49]. This phenomenon was also confirmed by the work of several authors in the previous tests [50-52].

From the result of secondary screening a total of 33 antagonistic microorganisms were selected for their farther superiority of biological activity screened *in vivo*. Selecting few numbers of antagonists with highest activity is economical, time saving and efficient means of screening to select the most suitable antagonist for farther test and application [53].

Screening potentially useful crop protection biological agents has been practiced for many years [54] and used in this experiment. Unfortunately, *in vitro* screens for antagonism do not consistently reflect antagonism under field or greenhouse conditions [12]. Both successful and unsuccessful screenings of biological agents have been reported. For example, a positive correlation between the lytic activity of several strains of *T. harzianum* on cell walls of *Sclerotium rolfsii, Rhizoctonia solani*, and *Pythium aphanidermatum*, and the degree of biological control of those pathogens *in vivo* has been found [55]; however, no correlation was found between the production of lytic enzymes in liquid culture by *Trichoderma spp*. isolates and their control of fusarium wilt on muskmelon and cotton [56], or of *R. solani* on lettuce [57]. The proposed an explanation [58] of the lack of correlation between *in vitro* chitinase production by *Trichoderma spp*. and bio-control of *F. solani*. They postulated that *F. solani* is protected from chitinases and 1,3-glucosidases by a protein layer masking the carbohydrate polymers, making additional enzymes necessary for successful antagonism against this pathogen; however, Isolates of some authors [42,59] showed that purified endochitinase and chitobiosidase from *T. harzianum* P1 were highly active against *F. solani*.

## Conclusions

The relations among the isolated organisms with environmental factors, ecology and geographical parameters determined in this experiment will not only give information on where to find the abundance of microorganisms but also give the highlight for the final application of the antagonistic organisms as well as the manipulation of this parameters for the advantage of antagonists. By reversing the process leading to the iatrogenic increase of CBD on coffee there is the possibility of enhancing biological control of the disease by augmentation of the relevant components of the coffee microbiota at least as part of an integrated management strategy for this troublesome disease.

In this study, 4323 microorganisms (1472 bacteria, 1240 yeast and 1610 filamentous fungi) have been isolated from the tissues of healthy leaf, twig and berry from naturally affected area by CBD. Thirty three (13 bacterial, 11 yeasts and 9 filamentous fungal) isolates with high bio-control activity against *C. kahawae* were selected to use in vivo testes. The antagonistic isolates exhibited dual antifungal mechanism through direct antagonism.

The evidence from this studies points to a direct biological control mechanism operating through the microflora of coffee trees that limits the development of coffee berry disease. This seems to act through direct competitive antagonism in limiting the infection, colonisation and sporulation of the pathogen. These processes occur throughout the epidemic cycle of the disease, reducing both the primary inoculum produced from the

maturing bark of coffee stems and the secondary inoculum produced from disease berries. The *in vitro* findings indicate the potential application of these indigenous microorganisms as biocontrol agents due to their efficient inhibitory traits against *C. kahawae*. Further greenhouse and field trials could ascertain their future applicability for inoculum development.

By reversing the process leading to the iatrogenic increase of CBD on coffee there is the possibility of enhancing biological control of the disease by augmentation of the relevant components of the coffee microflora at least as part of an integrated management strategy for this troublesome disease. Bacteria, yeast and fungal isolates would seem to be potential candidates in a test for such a strategy as these are apparently endophytes of coffee and show activity against *C. kahawae*. However, the fungi isolates can themselves be pathogenic to coffee under certain circumstances that would also need to take into account.

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