RESEARCH ARTICLE

Pathogenicity and molecular characterization of coffee bacterial blight (*Pseudomonas syringae pv. garcae* van Hall) isolates from Sidama and Gedeo Zones, SNNP Regional State, Ethiopia.

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ABSTRACT

Bacterial blight of coffee (BBC) caused by the phytopathogenic bacterium, *Pseudomonas syringae pv. garcae* van Hall is an important disease of Arabica coffee in Ethiopia due to its increasing incidence and severity. There is no information on the current status, pathogenicity and molecular characterization of the pathogen in this study area in Ethiopia. Therefore, this study was carried out to assess the disease intensity, pathogenicity and molecular characterization of BBC isolates from Sidama and Gedeo zones, SNNP Regional State, Ethiopia. A total 204 coffee trees, 96 coffee fields or peasant associations in 6 districts and in 2 coffee producing zones in SNNP Region was surveyed during the study time. The frequency and intensity of BBC disease was varied between the zones and districts of coffee producing surveyed areas. The percentage of disease incidence (70.0, 56.1, 44.6, 39.3%) and the severity (29.9, 15.7, 13.7, 12.5%) were recorded in Wensho, Dara, Aletachuko, Aletawondo districts of Sidama zone and the incidence (72.2 and 47%) and severity (21.6 and 13.1 %) were recorded in Dilla and Wonago areas of Gedeo zone, respectively. The prevalence of BBC disease was recorded in both Sidama and Gedeo zones, 87.5 and 93.8%, respectively while the mean BBC disease prevalence was 90.7%. Symptomatic of 204 diseased samples were initially isolated and purified on nutrient agar (NA) and nutrient broth (NB) slants. Among 204 isolates, 37 BBC isolates were selected for biochemical tests based on similar morphological and in growth characteristics on selective media. The pathogenicity test response was conducted on coffee leaves in which all the isolates induced the hypersensitive reaction and confirmed. Out of 37 isolates, 8 isolates were analysed for the molecular characterization through RAPD analysis. Out of five primers, OPAC 04 (5'- ACG GGA CCTG-3') primer revealed more number of RAPD patterns and polymorphism and hence differentiating all the isolates. The amplified fragments / bands ranged from 100 bp to above 1100 bp. Out of 8 isolates, the highest number of bands were observed in isolate 8

Gordahama FII T2. In other 6 isolates, the number of bands was ranged from 100 bp to above 950 bp and also uniform number of bands was noticed. The number of common bands was observed to be present in almost six isolates and the molecular weights of these ranged from 100 bp to 550 bp. This is clearly indicated that the genetic diversity of as *Pseudomonas syringae pv garcae.* The morphological and microscopic characters, β-glucosidase *activity,* biochemical nature and RAPD analysis of the test isolates confirmed the organism identity as *Pseudomonas syringae pv. garcae.* The present study provided first information on molecular epidemiological about BBC isolates from Sidama and Gedeo zones in Ethiopia. The coffee production in Gedeo and Sidama zones is threatened by BBC at present, appropriate measures new to be developed to solve this problem, otherwise, the country will be lost foreign currency as a result, the life standard of farmers will be collapsed and leading to lost foreign income.

Keywords: Coffee, Disease intensity, Pathogenecity, Molecular characterization, Bacterial blight, *Pseudomonas syringae pv. garcae,* RAPD-PCR

INTRODUCTION

Coffee **(*Coffea arabica* L.**) is one of the most important commodity crops, plays a vital role in cultural and socio economic life in Ethiopia and its contributing 60% of its foreign exchange earnings and employments in the production area (CSA, 2014/15). Ethiopia is the home and cradle of biodiversity of Arabica coffee seeds and also it is the centre for origin, diversification, and dissemination of the coffee plant (Bayetta, 2001). The total area coverage of coffee in Ethiopia is estimated to be around 800,000 ha of which and yet it is increasing, ninety five percent of Ethiopia's coffee is produced by small holder farmers on less than two hectares of land, while the remaining five percent is grown on modern commercial farms (Geiser *et al*., 2005). Bacterial blight of coffee is an important disease of Arabica coffee in Ethiopia due to its increasing incidence and severity. The first symptoms of the disease is blackening of nodes that progresses to internodes and darkening of petioles and basal parts of the leaves lamina attached on the same node of infected branches or twigs. During the rainy and wet season, most of the infected parts show clear water-soaked lesions. As the disease develops, these symptoms eventually cover the whole leaves turning dark brown rolling inward and often remain attached to the drying branches or twigs. The immature berries at pinhead and expanding stages are also very susceptible and become shrivelled (Ito *et al.,* 2008; Mugiira *et al.,* 2011). It can be a serious problem in high altitudes, where the plants are injured from heavy winds (Jansen, 2005) and have a protracted bimodal pattern of rainfall and often experience storms accompanied by hail (Kairu *et al*., 1985).

The occurrence and distribution of BBC was first reported by Korobko and Wondimageng (1997) from Wondo (Sidamo Zone) and later on by its distribution reported by Girma *et al.,* (2008). Yet the disease is recurring every year and spreading to the neighbouring zones. There is no information on the current status, pathogenicity and molecular characterization of the pathogen in this study area in Ethiopia. Therefore, this study was carried out to assess the disease intensity, pathogenicity and molecular characterization of BBC isolates on the basis of cultural and biochemical characteristics from Sidama and Gedeo zones, SNNP Regional State, Ethiopia.

MATERIALS AND METHODS

Description of the study area

Gedeo zone is located at 369 km from Addis Ababa to Southern parts of the country and 90 km from Hawassa and Capital City of the Region, South Nation Nationality and People Regional State (SNNPRS), Ethiopia. Geographically, the zone is located

North of Equator from 5^0 53'N to 6^0 27'N Latitude and from 38^0 8' to 38^0 30' East, Longitude. The altitude ranges from 1500 to 3000 m. a. s. l. The zone has sub-humid tropical climate receives mean annual rainfall of 1500 mm with range of 1200 and 1800 mm. The mean monthly temperature is 21.5°C with mean monthly maximum and minimum temperature of 25°C and 18°C, respectively (CSA, 2006). Sidama zone has - geographic coordinates of latitude/North: 5° 45″ and 6′ 45″ and longitude/East, 38° and 39′. It has a total area of 10,000 km^2 in a variety of climatic conditions in which, warm conditions cover 54% of the area. Its altitude ranges from 1500 to 2500 m. a. s. l. It has a mean annual rainfall of 400 to 799 mm, and the mean annual temperature ranges from 20°C to 24.9°C (CSA, 2006).

Disease survey and sample collection

Road side survey was conducted between mid-July and August, 2015 and six districts from Sidama and Gedeo zones were randomly selected. From each district, 4 localities and 4 trees from each farm were randomly selected. Infected twigs or shoots were cut using sterilized pair of scissors. The samples were collected from diverse coffee agro-ecological zones of Sidama and Gedeo including Wensho, Aletawondo, Aletachuko, Dara, Wonago and Dilla districts. The disease symptoms were assessed in the fields during survey. The number of infected leaves, primary branches, twigs or damaged heads on each sampled trees counted and recorded. The samples were collected randomly in 'X' and 'W' patterns depending upon the land topography. About, 204 samples of leaves, branches /twigs and berries randomly collected from symptomatic blighted/ infected coffee trees in almost all the assessed fields. The samples were well labelled in perforated plastic bags and stored in an icebox**.** The samples were transported in paper bags to APPRC and the specimens were maintained in refrigerator at 4°C until isolation.

Disease incidence and severity rating

Incidence of BBC was assessed by counting the number of diseased plants per total number of plants inspected and expressed as percentage of total plants as described by CABI, (2006). Per cent disease incidence was competed according to the following equation.

$$\text{Disease incidence } \% = \frac{\text{Number of diseased plants}}{\text{Total number of plants inspected}} \text{x100}$$

The number of infected leaves per branch, number of infected branches and/or twigs per tree were used to rate the percentage of disease severity

$$\text{Disease severity } \% = \frac{\text{Number of diseased twigs, leaves, priymery branches}}{\text{Total plant part}} \text{x100}$$

Isolation and identification of the pathogen

Cultural, morphological and biochemical characterization

Isolation, cultural and biochemical characterization of BBC isolates were conducted at Ambo Plant Protection Research Centre, Ambo, Ethiopia. Nutrient agar and King's B medium were prepared and used to BBC pathogen isolates (Lelliott and Stead, 1987; Kairu 1997). After the incubation period, light cream to yellow and mucoid sheen colonies of suspected *P. syringae pv. garcae* appeared on Nutrient agar (NAS) and yellowish white supernatant fluid on NBS. The suspected colonies on both media agar plates were confirmed by streaking on King's B (KB) and identified morphologically as *P. syringae pv. garcae* described by Kairu, (1997)**.** Colony morphology on agar surface aids to identify the bacterial isolates. Each isolate from colonies of characteristic shape, size and appearance were observed. Characteristic features of the isolates were observed by macroscopic observations. A loopful of culture from overnight grown was streaked on the surface of nutrient agar and was incubated at 37° C for 24 hours. Colony morphology, colour and consistency were observed. Microscopic observations like shape and grams nature were revealed the availability of different morphological characters among isolates.

The characterization of phytopathogenic bacteria helps to know the target pathogen and its biological behaviours. Bacterial morphological and cultural features alone are of little taxonomic value; because they are too simple to provide enough taxonomic information (Kairu, 1997).The biochemical characterization is essential to differentiate BBC isolates. The KOH solubility (Fahy and Hayward, 1983), Catalase (Sands, 1999), Cytochrome oxidase (York *et al*., 2004), Tween 80 hydrolysis (Fahy and Hayward, 1983), Starch hydrolysis (Sands, 1999), Levan production (Fahy and Hayward, 1983), Arginine production and Gelatine liquefaction (York *et al*., 2004), Aesculine hydrolysis, Tartrate utilisation (Sands, 1999) and Fluorescent and non-fluorescent tests were carried out. All the test results were recorded and used for identification of isolates.

Pathogenicity test

The pathogenicity test was conducted on young coffee seedlings known coffee variety '971' as the method stated by Girma *et al.,* (2008). The seedlings were regularly watered by sterile water every two days. The 3 month old healthy seedlings were selected and used for further pathogenicity test. The seedlings grown on pots filled with sterilized soil, sandy and FYM in the ratio of 3:1:1 at Ambo Plant Protection Centre, Ambo. The coffee seedlings were inoculated with two groups of bacterial isolates at four to six pairs of true leaves stage. The two isolate groups were those bacterial colony showing typical yellowish green (fluorescent) pigment and the greyish-white (non-fluorescent) on *Pseudomonas* agar and King's B medium. Bacterial suspension was diluted in distilled sterile water to 10^8cfu/ml, and a pair of fully expanded young leaves of four seedlings (per coffee variety) were pricked and inoculated with one millilitre of the suspension using hypodermic syringe as described by Kairu, (1997). The coffee seedlings with the same number of leaves were pricked up with sterile water as a negative control. All the inoculated seedlings were immediately misted with sterile water, covered with transparent plastic sheet and maintained in air-conditioned growth room at 22^{o}C. The day and night temperatures varied between 25-35^{o}C with 12 hrs light and 12 hrs dark as similarly done by Klement *et al.,* (1990). Disease infection and appearance of symptoms were observed and the target pathogen was re-isolated.

Molecular characterization of BBC isolates

***Molecular characterizations of BBC isolates were conducted at Genei lab, Bangalore India*.**

Out of 204 bacterial isolates, 37 isolates were identified for the pathogen, *Pseudomanas* spp based on morphological, cultural and biochemical parameters. Out of 37 isolates, 8 isolates were further selected for the molecular characterization of this study. Molecular methods are used for the useful markers for complete characterization of BBC isolates. The following eight isolates viz., Manche FII T2, Boa FII T3, Hallo FIII T1, Hayilo FI T3, Sugale FIII T1, Shigado FI T2, Kara FII T1 and Gordahama FII T2 were selected for RAPD and plasmid profile analysis for confirmation of the species.

Isolation of Plasmid DNA

Isolation of plasmid DNA was carried out by alkaline lysis method with some modifications. One ml of an overnight culture was transferred into an eppendorf tube. The cells were sediment by centrifuging briefly (1000 rpm) in the microfuge and the supernatant was drain off. The pellet was re-suspended by adding 100 µl of solution A and the contents were mixed by vortex. Then 100 µl solutions B was added and mixed well by invert the content (4-5 times). To the above viscous content 100 µl of solution C was added and the content was inverted 4-5 times to get mixed, the bulk of genomic DNA and other cell debris will precipitate into a viscous clump. It was centrifuged at 12000 rpm in micro centrifuge and the clump was removed. The clear lysate (supernatant) was transferred to another eppendorf f tube. 150 µl of 100% isopropanal was added and mixed well and centrifuged the content at 12000 rpm for 30 minutes. The supernatant was drained off and 150 µl of absolute alcohol was added and centrifuged the content at 10000 rpm for 20 minutes. The supernatant was drained off and dissolved the DNA pellets with 20 µl TE buffer. The extracted Plasmid DNA was confirmed by running of Agarose gel.

Agarose gel electrophoresis

Agarose gel electrophoresis was carried out in a horizontal submarine electrophoresis unit. Sixty ml of 1 % Agarose gel was prepared with 1 x TBE buffer (do not mix) and heated to get up to clear solution for casting Agarose gel. After cooling the solution, 5 µl of staining dye solution was added into the casting system. The gel was allowed to solidify, and then carefully disassembled from the casting system without disturbing the wells and placed in 1 x TBE buffer filled electrophoresis tank (the buffer level should be above gel). 20 µl of Plasmid DNA was added and mixed with 2 µl of gel loading dye and then loaded to gel and simultaneously 3 µl of DNA marker was loaded in 1st well. The power card terminals were connected at respective positions, run the gel at 50 V, till the gel loading dye migrate more than half the length of gel. Then switched off the unit and visualized the isolated DNA under UV transilluminator.

RAPD analysis

It was performed by making use of the method given by Hong *et al*., (2008). According to this method, each polymerase chain reaction mixture consists of 2 µl of template DNA, 1 µl of 1.6 micromolar solution of primer (Sigma, USA), 10 µl 2 x PCR master mixes (Promega, USA) and make up to 20 µl with molecular grade water. Amplification was performed in a Bangalore Genei thermocycler, India. After initial denaturation at 95^{o}C for 15 min. the samples were subjected to 30 cycles of denaturation at 94^{o}C for 30 sec. annealing at 42^o C for 20 sec. and extention at 72^{o}C for 1.10 min. A final extension was performed at 72 ^{o} C

for 10 min. The PCR products were analyzed by electrophoresis on 1.5% Agarose gel at 80 V for 75 min. Gels were stained in a 0.5 µg/ml ethidium bromide solution for 15 min. and photographed by camera.

The following primers were used for RAPD analysis.
OPAC 01 5'- TCC CAG CAGT -3'
OPAC 02 5'- GTC GTC GTCT -3'
OPAC 04 5'- ACG GGA CCTG-3'
OPAC 05 5'- GTT AGT GCGG -3'
OPAC 07 5'- GTG GCC GATG -3'

Data analysis

The banding patterns obtained from the RAPD were scored. This binary data was fed to the online server Dendro UPGMA for generating a dendrogram. Unweighted Pair Group Method with Arithmetic Mean or UPGMA clustering method was used with Jaccuard coefficient. The resulting dendogram was drawn and viewed using Tree view (Roderic, 1996; Garcia –Vallve *et al.,* 1999). About 100 bootstraps were also done. Dendro UPGMA is available online at http://genomes.urv.cat/UPGMA/.

RESULTS AND DISCUSSION

Descriptions and distribution of BBC disease symptoms

The first symptoms of coffee bacterial blight disease infected coffee trees were blacken of nodes that progressed to internodes, darkening of petioles and basal parts of the leaves lamina attached on the same node of infected branches or twigs. During the rainy and wet season, most of the infected parts showed clear water-soaked lesions. As the disease develops, these symptoms eventually cover the whole leaves turning dark brown rolling inward and often remain attached to the dying branches or twigs (Fig. 1 A & B). In some cases, as the infected branches and twigs dieback, the tips turn yellow and finally become brownish black (Fig. 1 A). The similar symptoms of bacterial blight of coffee, *Pseudomonas syringae* disease was known and documented by Ramos and Shavida, (1976) and Kairu, (1997) and subsequently confirmed by Girma, *et al.,* (2008). However, the coffee bacterial blight pathogen, *P. syringae* was recorded to be associated with coffee bean darkening or berry rot. The observed dieback symptom is different from branch dieback incited by thread blight (*Corticium koleroga*) and that of leaf blight (*Ascochyta terda*) which are commonly encountered during wet seasons and in highland coffee growing areas of Ethiopia as also reported by Girma *et al.*, (2008). Bacterial blight of coffee caused severely in branch dieback and the whole leaves turning dark brown rolling inward and often remains attached to the dying branches or twigs. Partial death of coffee trees during unusual torrential rainfall with heavy hailstorm at some localities (Wonsho, Dara, Aletachuko, Aletawondo, Dilla area and Wonago district) were recorded. Severe leaves defoliation was observed at M/Holayina, Sisota, and, M/Shakoha localities in districts around Dilla area and Hayilo, Bo'a, Shafina and Manche localities from Wensho district.

Fig. 1 (A) Typical symptoms of BBC disease on coffee, (B). BBC disease symptoms on new flash of suckers.

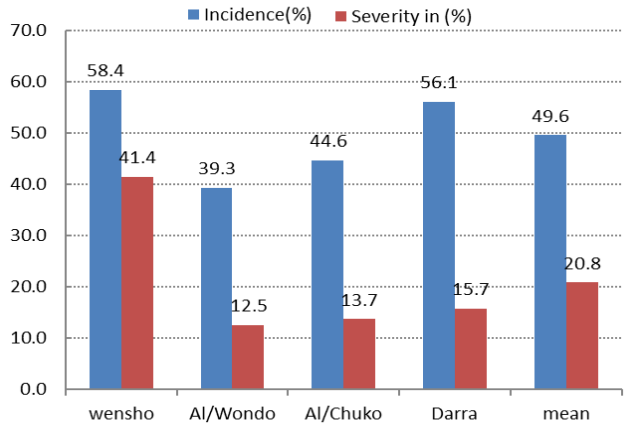


Fig. 2 Percentage of disease incidence and severity of bacterial blight of coffee in Sidama Zone

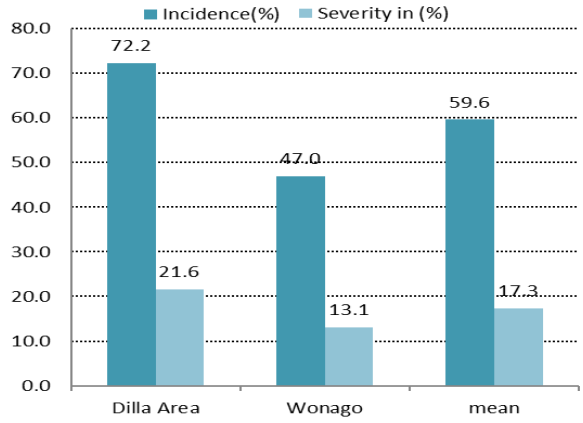


Fig. 3 Percentage of disease incidence and severity of bacterial blight of coffee in Gedeo zone

The frequency and intensity of BBC disease was varied between zones and the districts of coffee producing surveyed areas. Bacterial blight of coffee disease found in all assessed coffee localities that have been being posing considerable coffee tree losses. The survey results indicated that the disease incidence was 58.4, 56.1, 44.6, 39.3% and the percentage of severity were 41.4, 15.7, 13.7, 12.5% recorded in Wensho, Dara, Aletachuko, Aletawondo districts of Sidama zone respectively (Fig. 2).The percentage of BBC disease incidence was recorded in and around Dilla, 72.2% and in and around Wonago areas, 47% was recorded (Fig.3). The severity of the disease varied between 21.6 and 13.1 % in Dilla and Wonago areas, respectively. The prevalence of BBC disease was recorded in both Sidama and Gedeozones, 87.5 and 93.8%, respectively while the mean BBC disease prevalence was 90.7%.

Isolation, morphological and microscopic observations of BBC

Symptomatic of 204 diseased samples were initially isolated and purified on NAS and NBS. Growth of bacterial colonies after 72 hrs under aerobic conditions at 28°C, resulted round colonies with 2-3 mm in diameter and light cream with entire margins, dome shaped, shiny, smooth and mucoid on 5% NAS. A yellow supernatant fluid observed when grown on 5% NAS media (Figs. 4 A, B & C). The same descriptions were also reported by Mohammadi et *al.,* 2001 and Karimi-Kurdistani and Harighi, (2008). Representative strains of all the survey sites were further characterized using the methods described by Lelliott and Stead, (1987); Kairu (1997) on *Pseudomonas* base agar and King's medium. Finally, the isolates were identified and considered as *Pseudomonas syringe pv. garcae* on KB selective media in combination with biochemical tests conducted to identify the target pathogen. The morphological and microscopic characters, and β-glucosidase *activity*, biochemical nature of the *P. syringae pv. garcae* by the test isolates confirm the organism identity as *P. syringae pv. garcae*. All the cultural isolates were checked and identified through growth on selective media along with biochemical tests. Identification of BBC isolates based on standard culture, biochemical characterization and pathogenicity features were also supported by Barta and Willis, (2005).

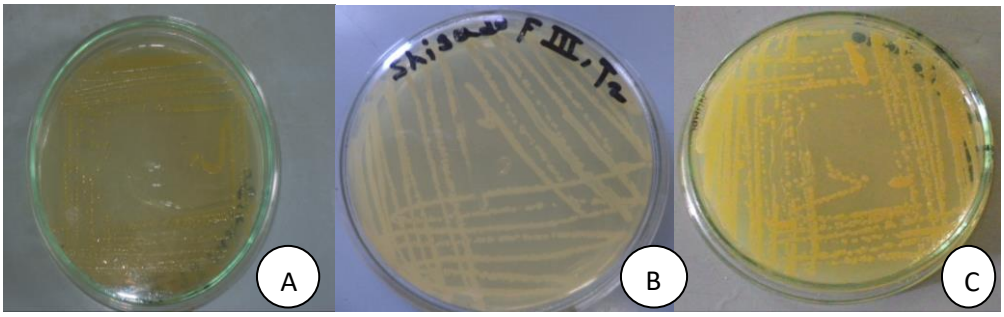


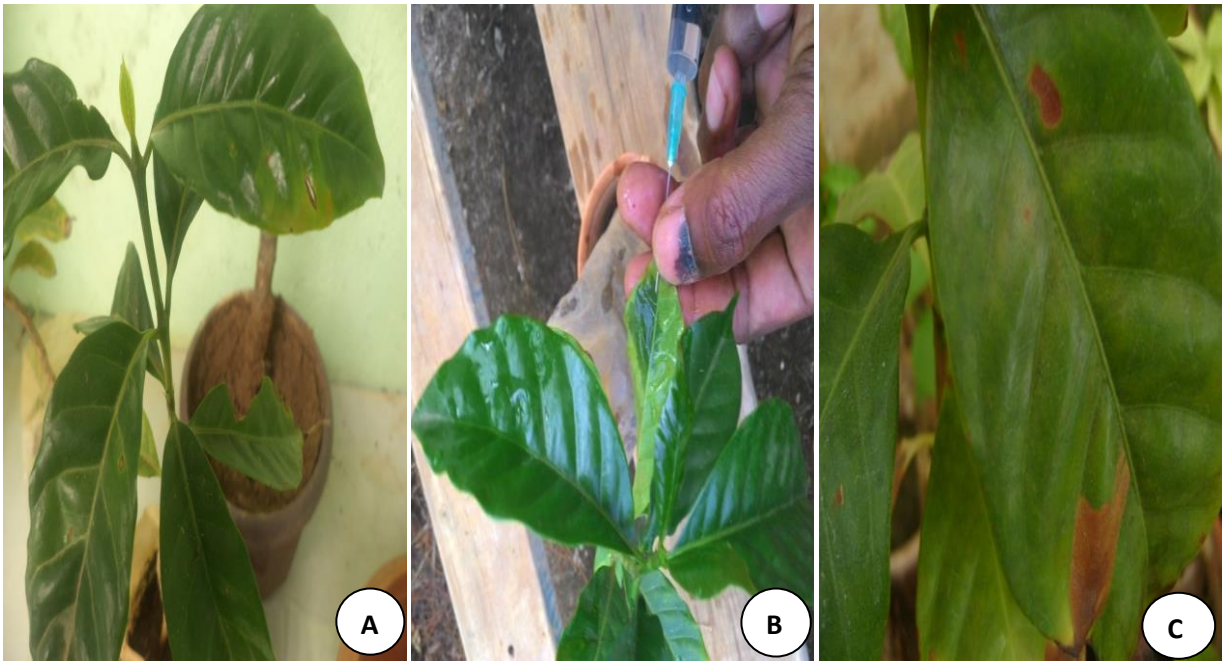
Fig. 4 A, B and C the bacterial suspensions were streaked on petriplates of the agar media for growth of bacteria B and C. Growth of BBC pathogen on NA and King's B media.

Biochemical test results

Thirty seven coffee bacterial blight isolates were selected for biochemical tests based on similar morphological and in growth characteristics on selective media. KOH test was performed and confirmed that all the isolates showed negative reaction and were categorized as gram negative phytopathogenic bacteria. *A*ll the coffee bacterial blight isolates were consistently gave similar positive results of catalase, gelatine liquefaction, Tween 80 hydrolysis, starch hydrolysis and levan production. All the BBC isolates were changed to black colour on aesculin test and also changed to yellow indicated as positive reaction to tartarate test and the negative reaction on Arginine dihydrolase tests. The positive reaction of the isolates were lead indicated the fluorescent *Pseudomonas* isolates. Additionally, the fluorescent *Pseudomonas* produces a yellow–green to blue fluorescent pigments on iron-deficient media. The biochemical test results indicated that, 16 isolates showed negative reaction or failed to produce the dark colour to the hydrolysis of β-glucosidase Aesculine test and 21 of them had developed dark colour that showed the presence of β-glucosidase activity. There was a variable reaction in the tartrate utilisation; 10 isolates produced yellow colour and 27 isolates failed to produce the desired colour. In the oxidase test, 15 isolates were proved negative reaction since they failed to produce the desired blue colour while 22 isolates produced within 30 seconds on the nitro-cellulose paper that were variable in reaction to the test. Similarly, 12 bacterial isolates showed positive reaction and the rest 25 isolates were negative to starch hydrolysis. Based on biochemical tests, the isolates showed similar positive test results in six tests while others were variable to three tests and one negative in their reaction to the tests.

Pathogenicity test

Pathogenicity test response was observed after tobacco coffee seedlings inoculated with the isolates of *Pseudomonas syringae* at bacterial concentrations of $1x10^8$ cfu/mL induced colour changed from green to yellow and collapse of host tissue in the inoculated region within 16 hrs of inoculation. After 24 to 48 hrs of injection, the injected leaf area became necrotic and in 3-4days, the treated tissue was entirely dry and yellow. This test study was confirmed through various studies done by Klement and Goodman (1967). Klement et al. (1990) reported that in many plant-pathogen interactions, the resistant reactions characterized by localized death of host cells in the region of infection showed the hypersensitive response.



Figs. 5 (A, B & C) Coffee seedling inoculation with *Pseudomonas syringae* and (B) Chlorosis and induced necrotic blight symptomson leaves.

After two weeks of inoculation with bacterial blight pathogens, the coffee seedlings showed exactly similar with the original blight disease symptoms, caused necrotic lesions at the inoculation site and showed a light-brownish, enlarging area surrounding the inoculation site (Figs. 5A, B &C). Re-isolations from symptomatic plants yielded bacterial colonies on NA and NBA that were identical to those used for the inoculations and yielded the same responses and the control plants did not showed any symptoms. Therefore, in this artificial inoculation studies, the pathogen was able to incite symptoms in growth room similar to those observed in coffee orchards, as well as very mild symptoms on some other plant species and confirmed that pathogenic to coffee plants which is in agreement with the bacterial blight of coffee in Kenya mentioned by Kairu, (1997) and also reported in Ethiopia by Girma et al. (2008).

Molecular characterization of BBC isolates

Molecular methods are useful markers for the complete characterization of BBC isolates. Out of 37 isolates, 8 isolates were completely identified based on the morphological and microscopic characters, gram stain and β-glucosidase *activity***,** biochemical nature of the *Pseudomonas syringae pv. garcae* by the test isolates confirm the organism identity as *Pseudomonas syringae pv. garcae.* All the cultural isolates were checked and identified through growth on selective media along with biochemical tests. Based on the characters, the molecular characterization of the 8 BBC isolates was analysed through RAPD analysis.

Determination of genetic diversity by RAPD

The following BBC isolates viz. Manche FII T2, Boa FII T3, Hallo FIII T1, Hayilo FI T3, Sugale FIII T1, Shigado FI T2, Kara FII T1 and Gordahama FII T2 were used for RAPD analysis. The RAPD patterns of most isolates (out of 8, six isolates) were performed as to determine the genetic diversity among by them by PCR amplification. In this study, the random primers (OPAC 01 5 '- TCC CAG CAGT -3 '; OPAC 02 5 '- GTC GTC GTCT 3 '| OPAC 04 5 '- ACG GGA CCTG-3' **;** OPAC 05 5 '- GTT AGT GCGG -3 '; OPAC 07 5 '- GTG GCC GATG -3 ') were used, of which OPAC04 5 '- ACG GGA CCTG-3' primer was revealed more numbers of RAPD patterns and polymorphism and hence differentiating all the isolates (Figs. 6 & 7). The amplified fragments / bands ranged from 50bp to above 1100 bp. Out of 8 isolates, the highest number of bands were observed in isolate 8 Gordahama FII T2. The bands were not observed in isolate 7 Kara FII T1. In other 6 isolates, the number of bands was ranged from 100 bp to above 550 bp and also uniform number of bands noticed. The number of common bands was observed to be present in almost six isolates and the molecular weights of these ranged from 100bp to 550 bp which were observed in most of the isolates. The bands with highest molecular weights more than 1000 bp were observed in Gordahama FII T2. The low molecular weight 100 bp was observed in two isolates. This is clearly indicated that genetic diversity of as *Pseudomonas syringae pv. garcae.* The 6 isolates viz. Manche FII T2, Boa FII T3, Hallo FIII T1, Hayilo FI T3, Sugale FIII T1, Shigado FI T2 were identified and confirmed *as Pseudomonas syringae pv. garcae.* The other two isolate*s,* Kara FII T1; and Gordahama FII T2 may be confirmed as gram positive bacterium.

A total of different RAPD patterns were noted and are shown in (Fig. 7). The isolates, viz. Manche FII T2, Boa FII T3, Hallo FIII T1, Hayilo FI T3, Sugale FIII T1, Shigado FI T2 showed similar RAPD pattern, which indicated that the pathogens could be from a single source. RAPD is a simple and widely used method for strain and isolate differentiation, since it does not require and specific knowledge of the DNA sequences in the target organism. Though, RAPD technique has certain limitations, still it is being used as a molecular typing method due to its simplicity, sensitivity, flexibility and relatively low cost (Abou-dobara *et al.,* 2010). Haryani *et al*. (2008) determined that four RAPD profiles among the seven *Enterobacter cloacae* studied whereas Trautmann *et al.,* (2006) reported that isolates of *Pseudomonas syringae pv garcae* banding patterns commutability of their genotypes. This study was provided that molecular epidemiological information about BBC isolates from Sidama and Gedeo zones. The banding patterns obtained from the RAPD were scored. This binary data was fed to the online server Dendro UPGMA for generating a dendrogram. Unweighted Pair Group Method with Arithmetic Mean or UPGMA clustering method was used with Jaccuard coefficient. The resulting dendogram was drawn and viewed using Tree view (Fig. 8) (Garcia Vallve *et al.,* 1999). About 100 bootstraps were also done. Dendro UPGMA is available online at http://genomes.urv.cat/UPGMA/.

Barta and Willis (2005) reported that the biological and molecular evidence that *Pseudomonas syringae* pathovars *coronafaciens, striafaciens*and*garcae*are

likely the same pathovar. A group of strains designated as *Pseudomonas syringae pvgarcae* causes a disease on coffee plants (*Coffea arabica* L.) that was reported first in Brazil and later in Kenya (Ramos and Shavdia, 1976). Sands *et al.,* (1970) conducted a survey of phytopathogenic *Pseudomonads* and grouped them on the basis of nutritional and physiological properties; the strain of *P. syringae* pv. *garcae* that was included in the study was placed in the same subgroup as strains of *P. coronafaciens.* More isolates need to be examined and their pathogenicity on coffee plants needs to be ascertained. Additional techniques such as DNA–DNA hybridization, sequencing of ribosomal 16SRNA genes and ITS regions, These techniques are useful in distinguishing bacteria at the species level but the term pathovar is an infra sub-specific designation based on only partially characterized host range because it is rare that a given pathovar is tested on all of the world's plant species (Krieg, 1984).

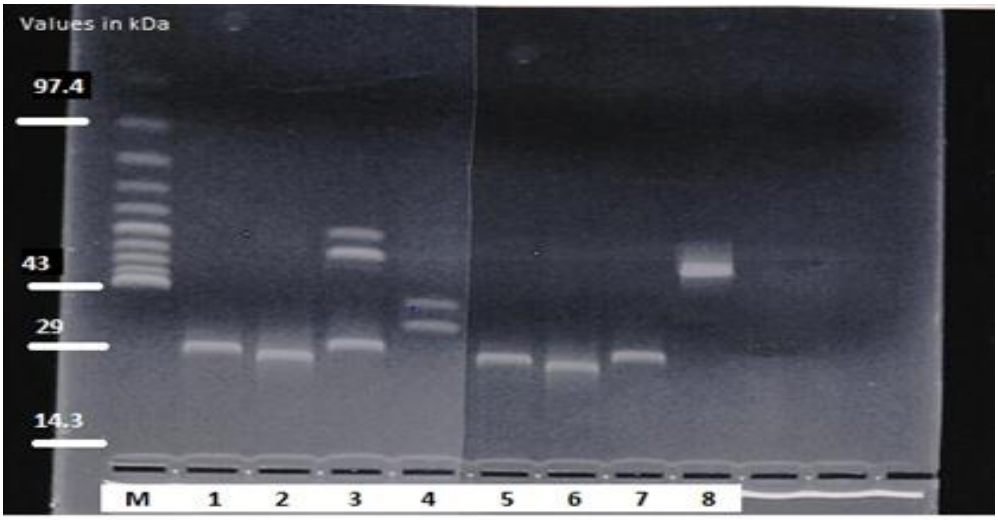


Fig. 6. Plasmid DNA isolated from 8 different BBC isolates using Agarsose gel electrophoresis M- Standard DNA marker; isolate 1 Manche FII T2; isolate 2. Boa FII T3, isolate 3 Hallo FIII T1 ; isolate 4 isolate Hayilo FI T3; 5 Sugale FIII T1, isolate 6 Shigado FI T2; isolate 7 Kara FII T1; and isolate 8 Gordahama FIIT2.

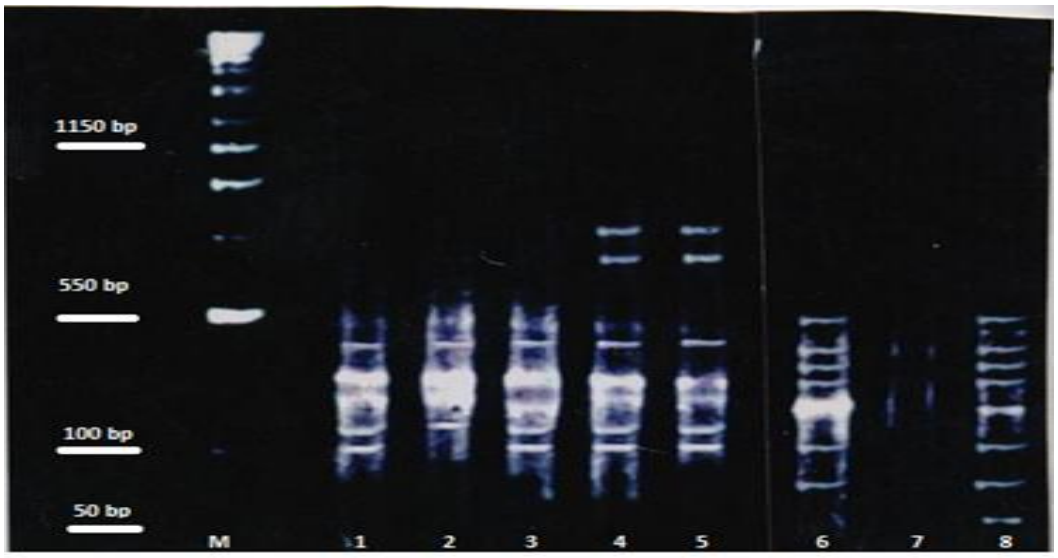


Fig. 7. BBC isolates using analysis of RAPD
M- Primer OPAC 04 5'- ACG GGA CCTG-3'; isolate 1 Manche FII T2; isolate 2. Boa FII T3, isolate 3 Hallo FIII T1, isolate 4 Hayilo FI T3; isolate 5 Sugale FIII T1, isolate 6 Shigado FI T2; isolate 7 Kara FII T1; and isolate 8 Gordahama FII T2.

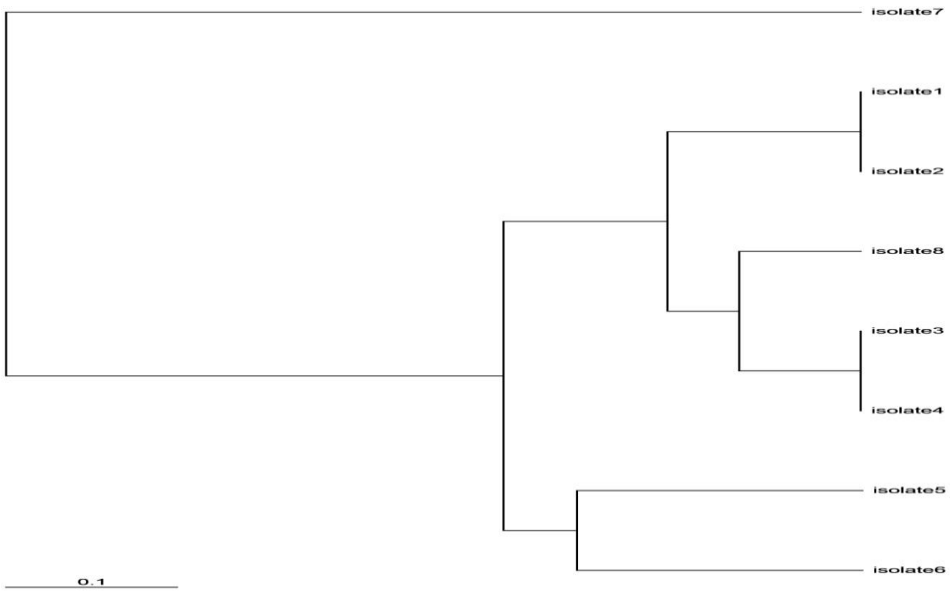


Fig. 8. Dendrogram of *Pseudomonas syringae* pv.*garcae* isolates from *Sidama and Gedeo zones* viewed using Tree view.

CONCLUSION

Bacterial blight of coffee caused by the phytopathogenic bacterium, *Pseudomonas syringae pv. garcae* is an important disease of Arabica coffee in Ethiopia due to its increasing incidence and severity. In this study, the frequency and intensity of BBC disease was varied between zones and the districts of coffee producing surveyed areas. Bacterial blight of coffee disease found in all assessed coffee localities that have been being posing considerable coffee tree losses. Growth of bacterial colonies after 72 hrs under aerobic conditions at 28°C, resulted round colonies with 2-3 mm in diameter and light cream with entire margins, dome shaped, shiny, smooth and mucoid on 5% NAS. A yellow supernatant fluid observed when grown on 5% NAS media. All the coffee bacterial blight isolates were consistently gave similar positive results of catalase, gelatine liquefaction, tween 80 hydrolysis and Levan production and negative KOH solubility and Arginine dihydrolase. Hypersensitive and pathogenicity test response *was* observed after coffee seedlings inoculated with the isolates of *Pseudomonas syringae* at bacterial concentrations of $1x10^8$ cfu/ml induced colour changed from green to yellow and collapse of host tissue in the inoculated region within 16hrs of inoculation. Eight isolates were identified based on the morphological, microscopic characters and biochemical nature of the *Pseudomonas* syringae pv. garcae and selected for molecular characterization through the use of RAPD analysis. The RAPD patterns of most isolates (out of 8, six isolates) were performed as to determine the genetic variations among by them by PCR amplification. The random primers (OPAC 01 5'- TCC CAG CAGT -3'; OPAC 02 5'- GTC GTC GTCT -3'; OPAC 04 5'- ACG GGA CCTG-3'; OPAC 05 5'- GTT AGT GCGG -3'; OPAC 07 5'- GTG GCC GATG -3') were used, of which OPAC 04 5'- ACG GGA CCTG-3'primer revealed more numbers of RAPD patterns and polymorphism and hence differentiating all the isolates. This study clearly indicated that genetic diversity of as *Pseudomonas syringae pv garcae*. The 6 isolates viz. Manche FII T2, Boa FII T3, Hallo FIII T1, Hayilo FI T3, Sugale FIII T1, Shigado FI T2 were identified and confirmed *Pseudomonas syringae pv. garcae.* The isolate, Kara FII T1 was confirmed as Gram positive bacterium. The isolates, viz. Manche FII T2, Boa FII T3, Hallo FIII T1, Hayilo FI T3, Sugale FIII T1, Shigado FI T2 showed similar RAPD pattern, which indicated that pathogens could be from a single source. This study provided first information on disease intensity and molecular characterization of BBC isolates from Gedeo and Sidama zones in SNNP Regional State of Ethiopia and useful for researchers, which is on threatening at present, unless otherwise taking appropriate measurement which solves this problem, otherwise, the country will be lost foreign currency as a result, the life standard of farmers will be collapsed and leading to lost foreign income.

Conflicts of interest: The authors stated that no conflicts of interest.

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