RESEARCH ARTICLE

Screening, cultural and biochemical 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

Coffee (*Coffea arabica* L.) is one of the most important commodity crops and plays a vital role in cultural and socio economic life in Ethiopia. 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 and the characterization of the pathogen in this study area in Ethiopia. Therefore, this study was carried out to assess the disease intensity, cultural and biochemical characterization of BBC isolates from Sidama and Gedeo zones, SNNP Regional State, Ethiopia and the variations within *P. syringae pv garcae* isolates from six different districts viz. Wensho, Aletawondo, Aletachuko, Dara, Dilla and Wonago of the study zones were carried out. 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 distribution map was done by JPEG map created using Arc GIS 10.0 map data of GPS reading. 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 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%. The intensity of BBC disease also varied between different altitudinal categories; as altitude increases both incidence and severity were increased and at the lowest altitude

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ranges between 1625 and1725 m. a. s. l, both disease incidence and severity were recorded lowest. Symptomatic of 204 diseased samples were initially isolated and purified on nutrient agar (NA) and nutrient broth (NB) slants. The growth of bacterial colonies showed round colonies with 2-3mm in diameter and light cream with entire margins, dome shaped, shiny, smooth and mucoid on 5% NAS. A yellow supernatant fluid was observed when grown on 5% NAS media. The gram staining method revealed that the test isolates showed rod shaped and gram negative bacteria. Among 204 isolates, 37 BBC isolates were selected for biochemical tests based on similar morphological and in growth characteristics on selective media. All the BBC isolates were consistently gave similar positive results of catalase, KOH solubility, gelatin liquefaction, tween 80 hydrolysis and Levan production and negative in Arginine dihydrolase. The biochemical test results indicated that, 16 isolates showed negative reaction or failed to produce the dark colour to the hydrolysis of β-glucosidase activity. Hypersensitive and pathogenicity test response was conducted on tobacco leaves in which all the isolates induced the hypersensitive reaction and confirmed. The morphological, cultural and microscopic characters, gram stain, β-glucosidase activity and biochemical nature of the test isolates confirmed the organism identity as *Pseudomonas syringae pv. garcae.* The present study provided first information on in Gedeo and Sidama zones 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.

Keywords: Coffee, Assessment, Disease intensity, Cultural, Biochemical Characterization, Bacterial blight, *Pseudomonas syringae pv. Garcae.*

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). For centuries, Ethiopian coffee selections proved to be resistant or tolerant against many diseases and pests. However, Coffee production is constrained by a number of major diseases, including Coffee Leaf Rust (CLR) caused by *Hemileia vastatrix,* Coffee Berry Disease (CBD) caused by *Colletotrichum kahawae* and Bacterial Blight of Coffee (BBC) caused by *Pseudomonas syringae pv. garcae* (Mugiira *et al*., 2011).

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 dying branches or twigs. In some cases, as the infected branches and twigs dieback, the axial leaves or tips turn yellow and finally become brownish black.

The immature berries at pinhead and expanding stages are also very susceptible and become shrivelled (Ito *et al.,* 2008). The symptoms also include dark, water-soaked necrotic lesions on leaves, tips and nodes of vegetative and cropping branches culminating in a dieback (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 and the characterization of the pathogen in this study area in Ethiopia. Therefore, this study was carried out to assess the disease intensity and characterization of BBC isolates on the basis of cultural and biochemical characteristics from Sidama and Gedeo zones, SNNP Regional State, Ethiopia and the variations within *P. syringae pv garcae* isolates from different districts (Wensho, Aletawondo, Aletachuko, Dara, Dilla and Wonago) of the study zones were carried out.

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). 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. (Figs. 1 & 2). The zone has sub-humid tropical climate receives mean annual rainfall of 1500 mm with range of 1200 and 1800 mm. The rainfall pattern is bimodal, with short rain season between March and May, accounting for 30% of total rain fall and long rain season between July and October, accounting for more than 60% of total rainfall. The mean monthly temperature is 21.5°C with mean monthly maximum and minimum temperature of 25°C and 18°C, respectively (CSA, 2006).

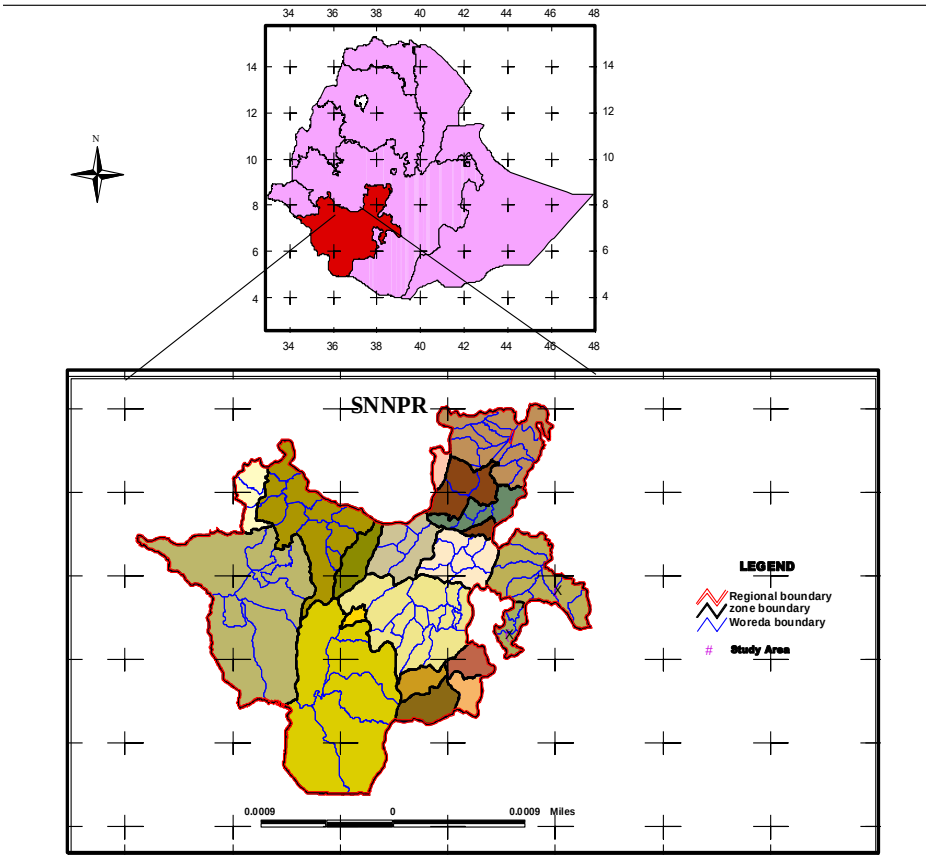


Fig. 1 Geographical location of the study areas, Ethiopia (Source: SNNP Region Rural Livelihood)

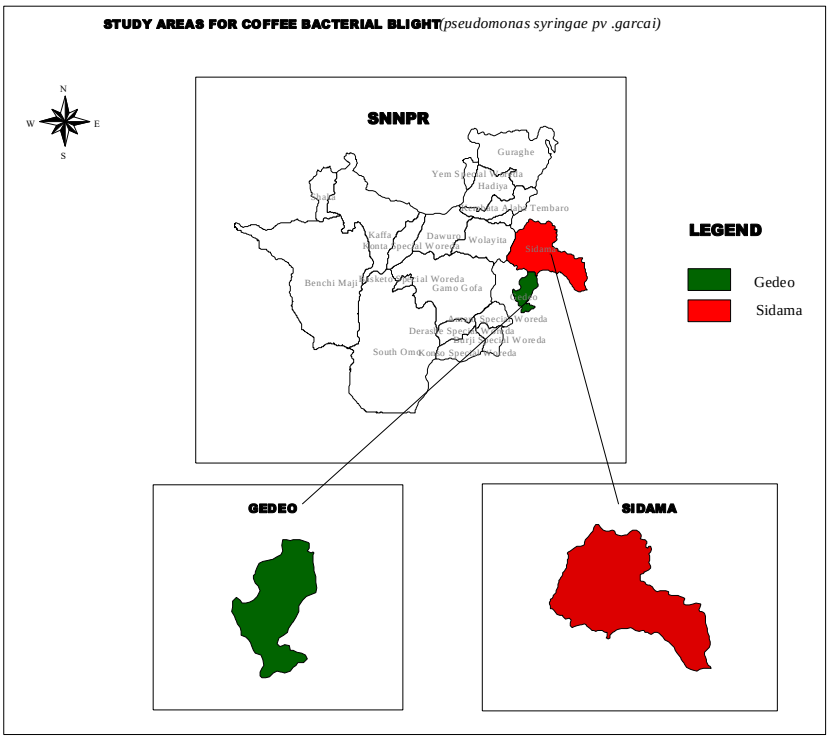


Fig. 2 Map of the study areas (Gedeo and Sidama zones)

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 km² 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. (Figs. 1& 2). The mean annual rainfall of the area varies between 1200 and 1599 mm, with 15°C to 19.9°C average annual temperature. A hot climatic zone, Kolla, covers 30% of the total area. Its elevation ranges from 500 to 1500 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. Cool climatic conditions known as Aliicho or Dega exist in the mountainous highlands. This covers 16% of the total area with an elevation between 2500 and 3500 m. a. s. l. This part gets the highest amount of rainfall, ranging from 1600 mm to 1999 mm. It has a mean annual temperature of 15 °C to 19.9 °C (Fig. 1and 2; 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. Systematic random sampling procedures were adopted and GPS data was recorded. 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. Alongside, a number of questionnaires on important factors such as any deviations in weather conditions that supposed to influence the disease occurrence noted in questionnaires and disease distribution was analysed according to CABI, (2007) and finally mapped.

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.

$$\text{Disease incidence \%} = \frac{\text{Number of Diseased plants}}{\text{Total number of plant inspected}} \text{ X } 100$$

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

$$\text{Disease severity \%} = \frac{\text{Number of diseased twigs, leaves, primary branches}}{\text{Total plant parts}} \text{ X } 100$$

Isolation and identification of the pathogen

Cultural 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). Small symptomatic pieces of samples taken from leaves, branches/ twigs and berries washed under running tap water for 2 minutes and rinsed in sterile distilled water. Approximately, 4x7 mm sized small tissue segments cut from active tissue margins of lesions and triturate in a drop of sterile distilled water. About 1g of samples were cut into pieces; surface sterilized by 1% sodium hypochlorite solution (laundry bleach) for one minute. The prepared samples were macerated in sterile distilled water and crushed using laboratory mortar and pestle. Finally, the filtrates were diluted using sterile distilled water and wait for 30 seconds and allowed the bacteria to float and then from each suspension of the plates were streaked with sterile wire loop on each of the agar media. The plates incubated at 28^{o}C for 24 to 48 hrs, while each plate was examined for culture growth. Those putative bacterial colonies were purified on the same media from a single cell colony and incubated for the same period. 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).

Morphological characterization

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^{o} 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. Shape of the isolate was identified by making simple staining method followed by its observation under light microscope. Bacterial smear was stained with methylene blue dye and examined. Microbial cells were observed for their shapes like rod or cocci or spiral. Gram staining was performed to look for the gram's nature of the isolates. Purple coloured cells remain Grams crystal violet and were called gram positive bacterium. Pink coloured cells lost primary stain and picked up safranin colour and were called s as gram negative bacterium.

Biochemical characterization

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 following biochemical characterization is essential to differentiate BBC isolates.

KOH solubility test

KOH solubility test was performed by the method of Fahy and Hayward, (1983) using 24 to 48 h old culture. Two drops of 3% KOH placed on to glass slide and the colonies of test pathogen were stirred into the solution clean loop for 5 to 10 seconds. When the solution was viscous enough to stick to the loop, causing a thin strand of thread like slime stretched up, the test recorded as positive.

Catalase test

Catalase test was performed by adding 1 ml of a 3% solution of hydrogen peroxide to glass slide, a loop of fresh culture grow on NA medium were added into the

solution by the method described by Sands, (1999). Released of bubble from the culture was recorded as catalase positive.

Cytochrome oxidase

A filter-paper saturated with 1% Kovac's oxidase reagent (tetra-methyl-p-phenylenediamine dihydro-chloride) and placed in a clean Petri dish to look for cytochrome enzymes. A suspected colony of bacteria from NA transferred with wooden stick to the filter paper and rubbed onto the reagent for 30 seconds. Isolates developed blue or deep purple colours within 30 seconds were considered as positive for Cytochrome oxidase (York *et al*., 2004).

Tween 80 hydrolysis test

Fatty acid esterase activity was tested by streaking the bacteria on to a nutrient agar medium containing calcium chloride and Tween 80, polymer consisting of polyoxy-ethylene-sorbitanmonoleate used by the method of Sands, (1999). The medium contains 10g peptone, 0.1gCaCldihydrochloride, 5g NaCl, and 15 g agar in 1litre of distilled water and with the pH adjusted to 7.4. Tween 80 was autoclaved separately; 10 ml/l added and mixed before plating. The plates incubated at 30^{o}C for up to 7 days as described by Fahy and Hayward, (1983). An opaque zone of crystals around colony recorded as positive reaction for hydrolysis of Tween 80 (Sands, 1999).

Starch hydrolysis

Nutrient agar plates containing 0.2% soluble starch (w/v) were streaked by the test isolates and incubated at 30^{o} C until heavy growth occur. Then plates were flooded with KI solution (Iodine, 1 g; Potassium iodide, 2 g; distilled water, 100 ml). A clear zone around a colony recorded as positive reaction (Sands, 1999).The results were recorded and used for identification of isolates.

Levan production

To test for the levan production of the isolates, a nutrient agar plates containing 5% sucrose were streaked by the test isolates and incubated for 3-5 days at 30^{o} C until heavy growth occur. Levan produced when colonies were convex, white, domed and mucoid (Fahy and Hayward, 1983).

Arginine production and Gelatine liquefaction

Arginine medium was stab-inoculated by the test strains and incubated for 24-48 hrs. The colour was changed from yellow to red-pink recorded as positive reaction to Arginine production (York *et al*., 2004).

Gelatine liquefaction

Gelatine liquefaction was tested with 12% (w/v) gelatine containing test tube prepared and stab-inoculated by the test isolates and incubated at 20^{o}Cfor up to 15 days and then kept at 5^{o}Cfor 15 minutes before determining liquefaction. Positive reaction of liquefaction of gelatine was recorded when the test tube were tilted (Sands, 1999).

Aesculine hydrolysis

Aesculine medium containing plates streaked by the test isolates and incubated at 20^{o} C for 2-5 days. Dark colour developed indicates the presence of *β-glycosidase* activity then, recorded as positive and negative no dark colour developed.

Tartrate utilisation

Tartarate production test done on nutrient agar plates containing 2.0g sodium tartrate dissolved in 100ml of distilled water and bromothymol blue (1.5% in ethanol) added. The pH was adjusted to 7.2 with 40%NaOH and heated to dissolve and autoclaved and then the test isolates were streaked, then the plates were incubated at 30^{o}C for 1-2 days. Then the colour was changed to yellow indicated as positive reaction and no colour changed recorded as negative (Sands, 1999).

Fluorescent and non-fluorescent test

Finally, the above-mentioned Levan, Arginine, Aesculine, Gelatine liquefaction and Tartrate utilization characters were used to differentiate the test isolates into fluorescent and non-fluorescent *Pseudomonas.* In the biochemical tests, the positive reaction was lead indicated the fluorescent *Pseudomonas* isolates. Additionally, the fluorescent *Pseudomonas* produces a yellow–green to blue fluorescent pigments on iron-deficient media (KB media). All the test results were recorded and used for identification of isolates.

Hypersensitivity test

Tobacco plants inoculated with bacterial isolates provides defence mechanism in the plant triggered. Plant cells in the invaded area were dying off, restricting the invaded pathogen by preventing further spread to the rest of plant. The triggering of hypersensitive reaction in plants by phytopathogenic

bacterial isolates was used as a diagnostic tool; especially for fluorescent *Pseudomonas*. A bacterial suspension of 10^8 cfu/ml in sterile distilled water was prepared and then in the lower surface of the young tobacco leaves injected by sterile syringe. The distilled water was used as a control. The positive reaction in the filtered area was become dry and necrotic within 24 hours.

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.

RESULTS AND DISCUSSION

Descriptions 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. 3A & B). In some cases, as the infected branches and twigs dieback, the tips turn yellow and finally become brownish black (Fig. 3 A),

Figs. 3 (A) Typical symptoms of BBC disease on coffee, (B). BBC disease symptoms on new flash of suckers.

The disease usually begins on the upper or middle canopies of coffee trees with apparently succulent and actively growing vegetative parts of the plant that spread down the vertical heads with primary or secondary branches leading to complete death. All the attacked trees in the field appear as burnt by fire. When the rain stops or became erratic for some time (a week or more), the disease progress also cease and fresh symptoms may not be seen, instead new flash of suckers grow just below the cessation point that may be re-infected when the favourable conditions occurred again. In such a way, multiple infections are possible per season that finally deteriorated or debilitated the coffee trees although it did not entirely kill the plant. 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).

Distribution of Bacterial Blight of Coffee

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. The distribution map also showed that moderate branch and twig dieback level in Kumato, Metisho, Adame Gelawacho of and Dara district during the main rainy season of the year 2015 (Fig.4).

Additionally, Coffee wilt (CWD) and Coffee berry (CBD) diseases were also noticed with bacterial blight of coffee disease in forest coffee areas in Sidama and Gedeo zones. Coffee berry disease (*Colletotrichum kahawae*) and coffee wilt disease (*Giberella xylarioides*) were mainly observed in Gedeo zone at different intensity. Coffee leaf rust (*Hemileia vastatrix*) and leaf blight (*Ascochyta tarda*) were also observed in the forest coffee areas. 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 disease incidence and severity was observed during the first outbreak in 1997 which was about 80 and 70% and in the year 2008, which was about 90 and 68%, respectively. In subsequent years, the disease prevalence was recorded in already affected areas, fresh BBC outbreaks discovered on coffee trees in 11 localities of the neighbouring districts with mean severity that ranged from 6.4-38.4 and 2.6-45.7% in Aletawondo and Aletachuko, respectively and recorded considerable damages. 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. 5).The percentage of BBC disease incidence was recorded in and around Dilla, 72.2% and in and around Wonago areas, 47% was recorded (Fig.6). 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%.

The percentage of incidence and severity were also recorded difference among villages and farms. In some villages, the mean incidence and severity were very high because farmers used cutlasses (bushman knives) indiscriminately and without take care of coffee trees not to wound. The intensity of BBC also varied between different altitudinal categories; as altitude increases both incidence and severity were increased. At the lowest altitude ranges between 1625 and 1725 m. a. s. l, both disease incidence and severity were recorded lowest; but the high incidence and severity were recorded between the range of 2025-2125 and 2125-2225 m. a. s. l in Dilla areas. The M/Shakoha and M/Holayina villages, the incidence were recorded 90.0 and 91.7% at an altitude of 2148 m. a. s. l. and 2092 m a. s. l., respectively and in Aletawondo district, and Gobadamo village, the incidence was recorded 90% at an altitude of 2049m a. s. land 80.4% was noticed at an altitude of 2042 m a. s. l (Fig. 7).

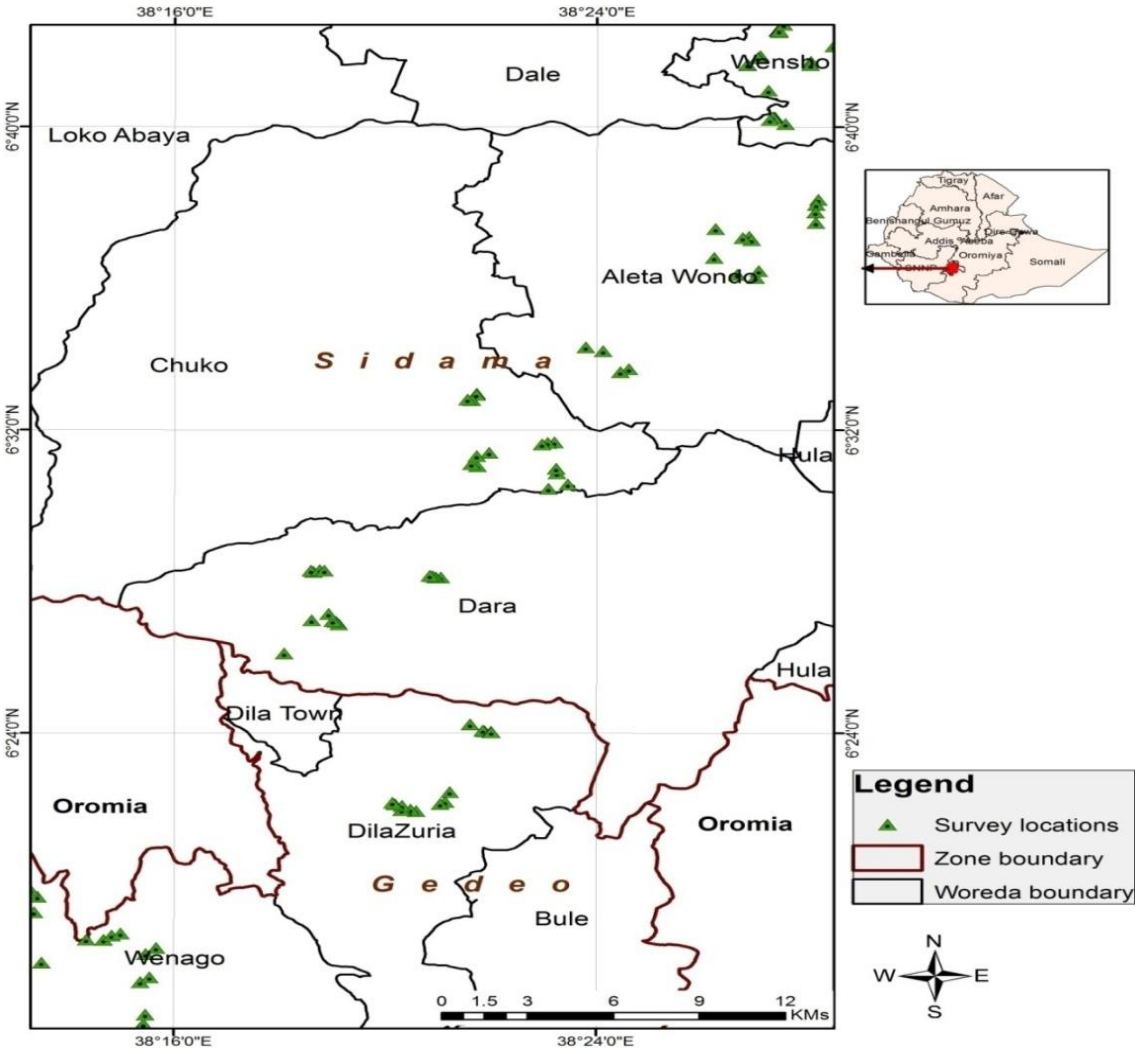


Fig. 4 Distribution map of bacterial blight of coffee in Sidama and Gedeo zones of SNNPR.

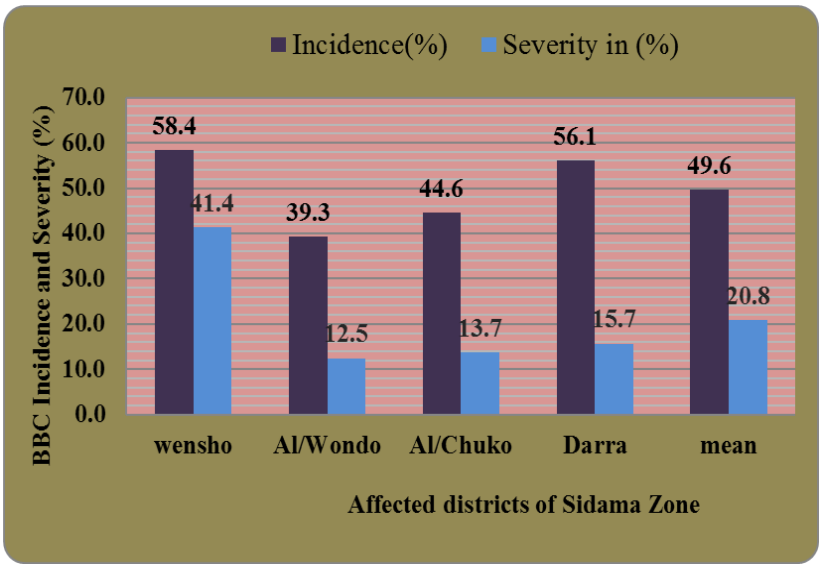


Fig. 5: Percentage of disease incidence and severity of bacterial blight of coffee in Sidama zone

Some farmers also agreed that the occurrence and outbreak of the disease followed the unusual torrential rainfall with heavy hailstorm destroying most of their crops including coffee. There have been hailstones injury observed on most of the infected branches/ succulent twigs during the disease assessments (Table 1). For example in Wensho and Dilla area districts, received high amount of rainfall and there was heavy frost destroying most of their coffee seedlings two months before this study survey.

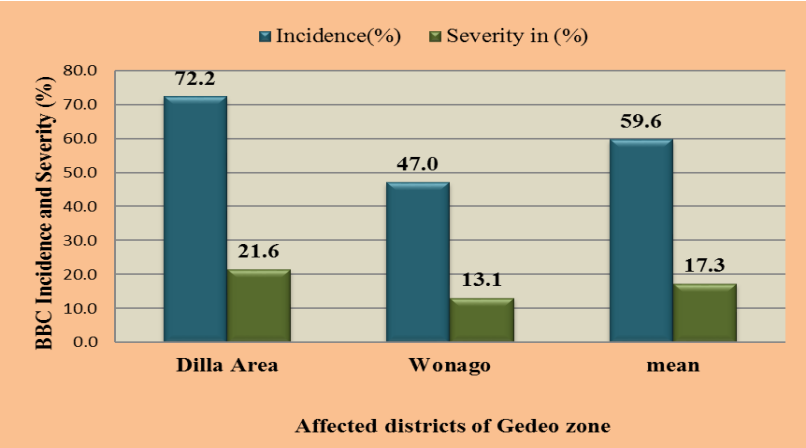


Fig. 6 : Percentage of disease incidence and severity of bacterial blight of coffee in Gedeo zone

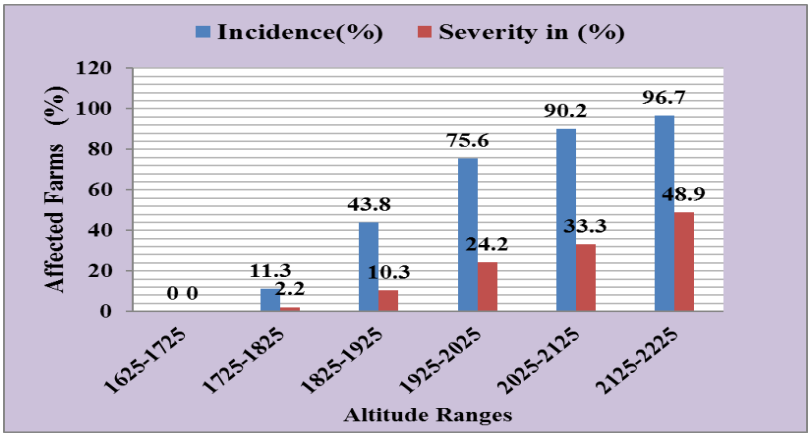


Fig. 7: Percentage of disease incidence and severity of bacterial blight of coffee related with altitudinal ranges in Sidama and Gedeo zones.

This observation indicates that such weather calamity might have predisposed the coffee plants to bacterial infections. Similar observations also reported by Kairu,(1994); that frost damage and provided the pathogen with infection entry avenues and in one case in Kenya frost injury was followed by severe BBC leaf and shoot infections.

The variable reactions were observed during the survey among different coffee varieties. The disease attacked the coffee trees at all stages, although there were variations among the trees that perhaps attributed to genetic differences among the coffee types and varieties under field conditions. It was more severe and caused extensive damage on some coffee varieties observed during the survey resulted in trees failure, and even destroyed coffee berries. The disease was serious in certain areas that forced some farmers' neglect their coffee fields while others cut down and uprooted those damaged trees and grown root crops and other vegetable crops.

Fig. 8 Coffee varietal differences in response to BBC disease in the same fields

Table 1: Incidence and severity of BBC disease in relation to rainfall and temperature in six districts of Sidama and Gedeo zones

Districts	RR (mm)	Temperature (°C)			Incidence%	Severity %
		max	min	average		
AletaWondo	4.4	23	13.6	18.3	39.3	12.5
Aletachuko	4.4	23	13.6	18.3	44.6	13.7
Dara	4.7	27.1	11	19.1	56.1	15.7
Wensho	4.9	29.7	17.5	23.6	58.4	41.4
Dilla area	4.6	26.8	13.1	20.0	72.2	21.6
Wonago	3.2	26.8	13.1	20.0	47.0	13.1

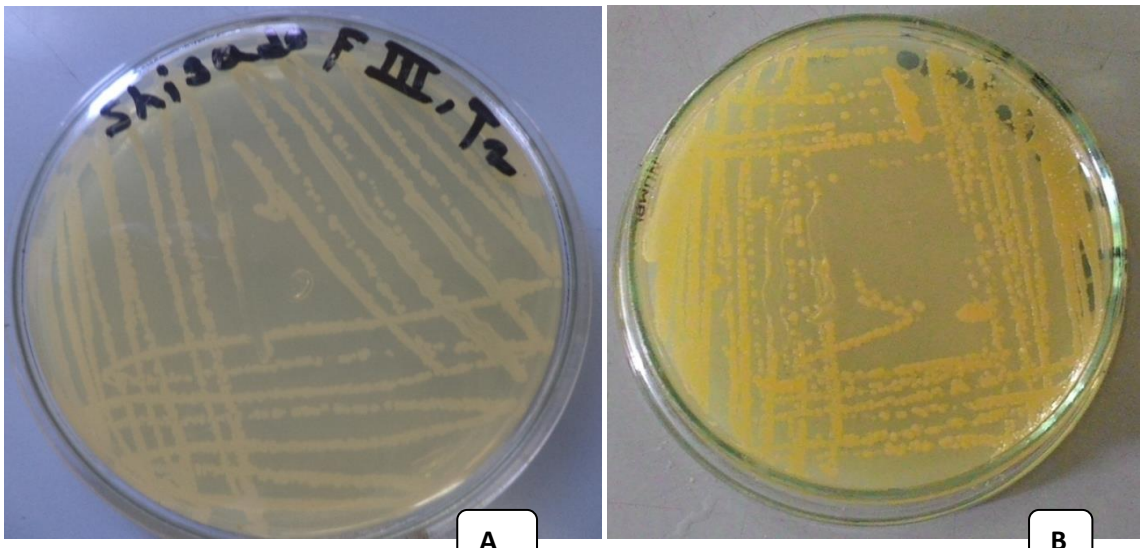
Table 2: Response of varieties evaluated on fields for their resistance to natural strains of *Pseudomonas syringae pv. garcae.*

S/N	District	Locality	Altitude (m. a. s. l)	Varieties	Incidence %	Severity %
1	Wensho	Shafina	1834	74110	0	0
2				74110	0	0
3				74112	0	0
4				74112	0	0
				Local	56.8	22.3
5	Aletawondo	Gobadamo	1920	74158	0	0
6				1375	0	0
7				74110	0	0
8				74112	0	0
				Local	59.2	22.6
9	'	Sheyicha	1966	74110	0	0
				Local	21.3	3.7
10		'		1377	0	0
11		'	1988	1377	0	0
12		Gordahama	2070	74110	0	0
				Local	40.3	10.6
13	Aletachuko	Lella Honcho	1854	1377	8.2	3.1
14				74110	0	0
15				744	0	0
				Local	62.2	21.7
16		Hallo	1884	1377	61.8	12.71
				Local	33.6	8.3
17	Dara	Gelawacho	1807	85238	12.5	0.2
				Local	25.0	5.2
18	Wonago	Kara	1870	74110	0	0
				Local	41.9	15.9
19				74112	0	0
20		B/Bukisa	1784	74112	0	0
				Local	17.7	3.2

Most local varieties of Sidama and Gedeo, including Sidama specialities showed high severity at fields to the BBC disease all inspected sites. However, the southwest varieties such as 74110, 74112, 744, and 74158, relatively not affected by BBC disease in different districts in the same climatic condition and the same fields, in many areas at the same field the local varieties were affected but the southwest (SW) materials were not affected at field conditions (Fig. 8). Finally, BBC disease intensity (prevalence, incidence and severity) was recorded across local coffee cultivars and released varieties (Table 2).

Isolation of bacterial blight of coffee pathogen

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. 9 A & B). 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 others biochemical tests conducted to identify the target pathogen.



Figs. 9 A & B Growth c athogen on NA and King's B media.

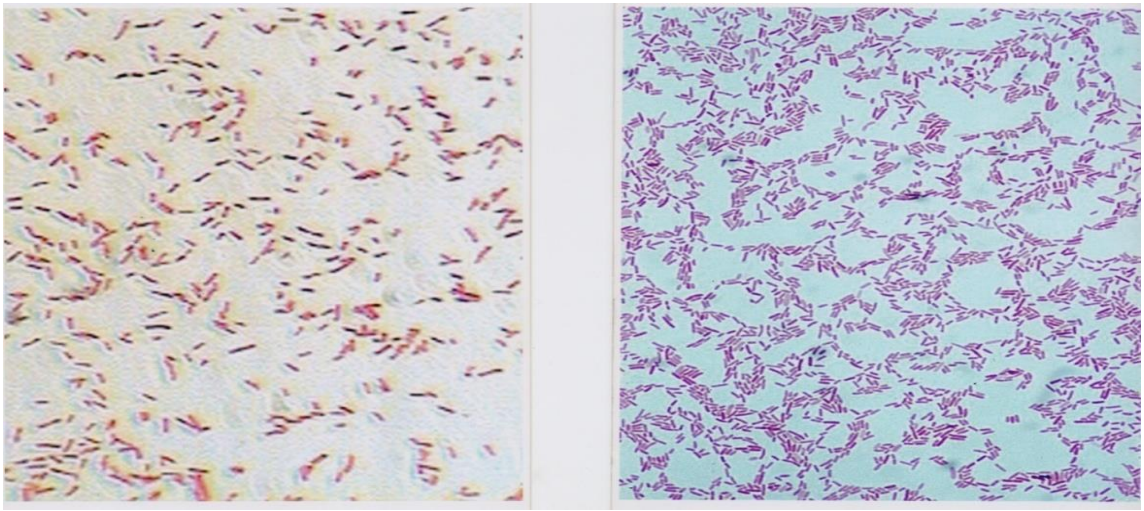


Fig. 10 Morphology and grams nature- Rod shaped and gram negative bacteria

Morphological and microscopic observations

Growth of bacterial colonies after 72hrs 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. A yellow supernatant fluid observed when grown on 5% NAS media. The morphological and microscopic characters, gram stain 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* (Figs. 9 A & B). All the cultural isolates were checked and identified through growth on selective media along with biochemical tests. The gram staining method revealed that test isolates showed rod shaped and gram negative bacteria (Fig. 10). Identification of BBC isolates based on standard culture, biochemical characterization and pathogenicity features were also supported by Barta and Willis, (2005).

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. The gram staining method revealed that test isolates showed rod shaped and gram negative bacteria (Fig. 10). KOH test was performed and confirmed that all the isolates showed negative reaction and were categorized as gram negative phytopathogenic bacteria (Fig. 11 A). All the coffee bacterial blight isolates were consistently gave similar positive results of catalase (Fig. 11 B), gelatine liquefaction (Fig. 12 A) Tween 80 hydrolysis (Fig. 12 B), starch hydrolysis (Fig 13 A) and levan production (Fig. 13 B). 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 (Fig. 14 A and B). 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 (Figs. 15 A & B).

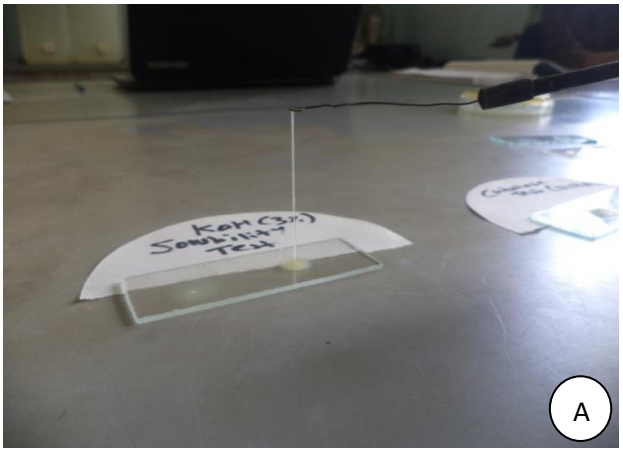


Figure 11: A Negative for KOH solubility

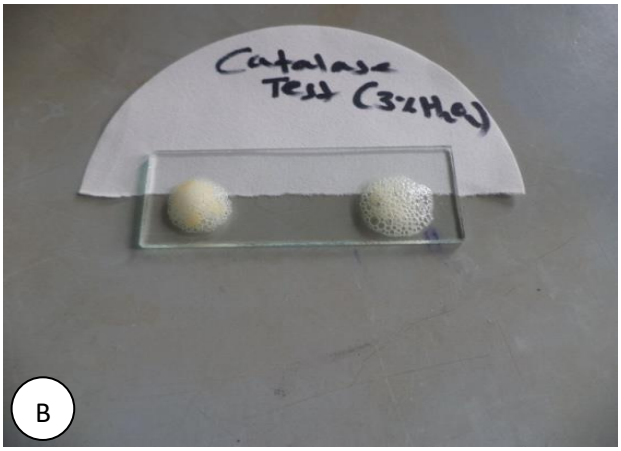
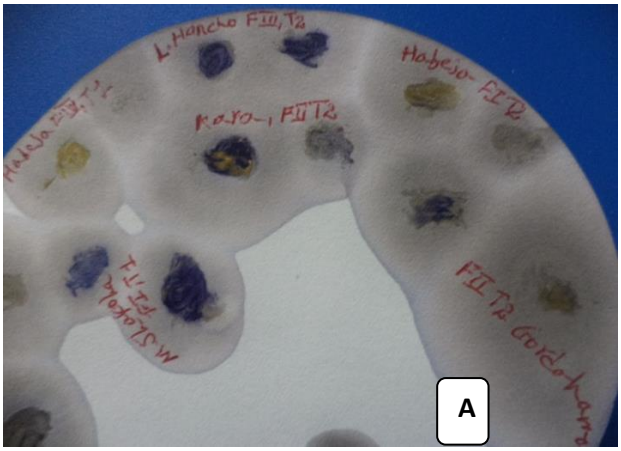


Fig. 11 B. Positive for Catalase test



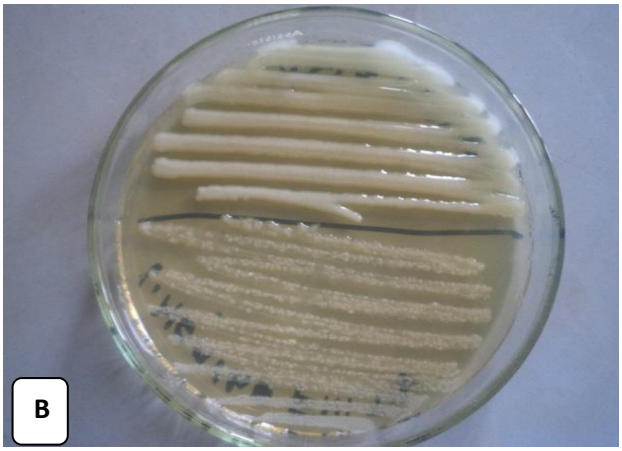


Fig. 12 A : Isolates changed the color ofblue **or deep purple colored - Positive for cytochrome oxidase.**
12 B: Opaque zone around colony recorded as positive reaction for hydrolysis of Tween 80.

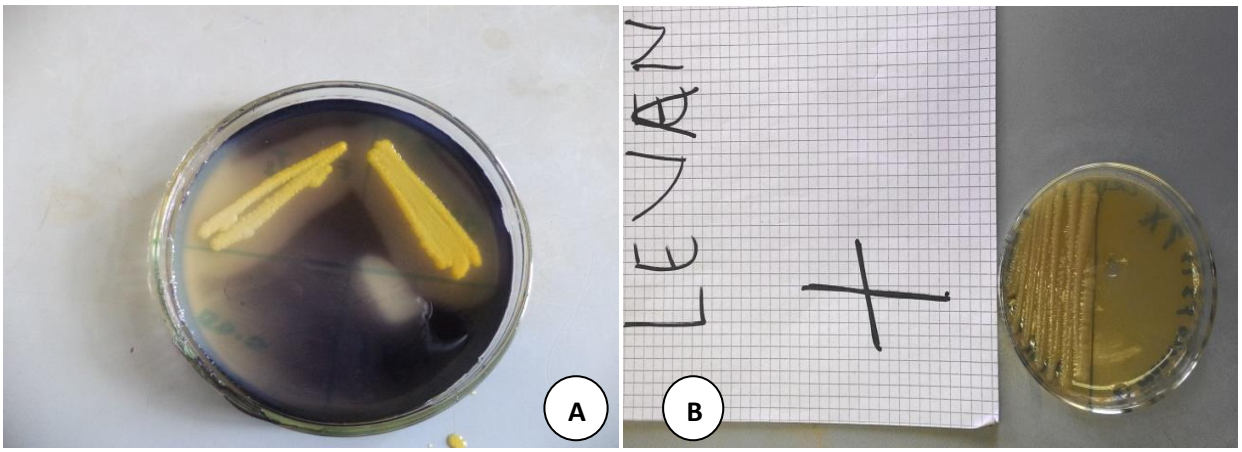


Fig. 13. A Clear zone around a colony recorded as positive reaction of starch hydrolysis
B. Convex, white, domed and mucoid on culture on levan test

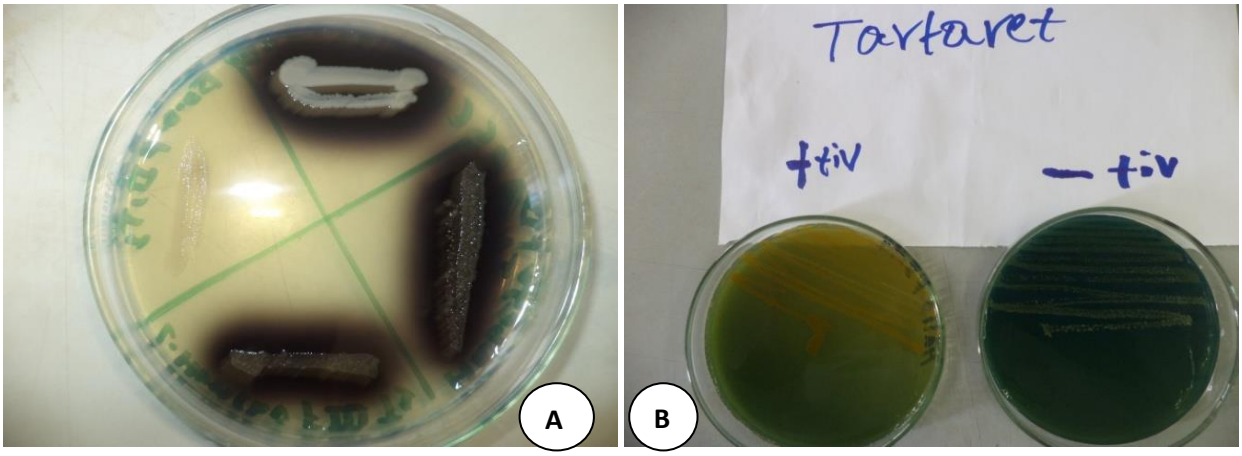
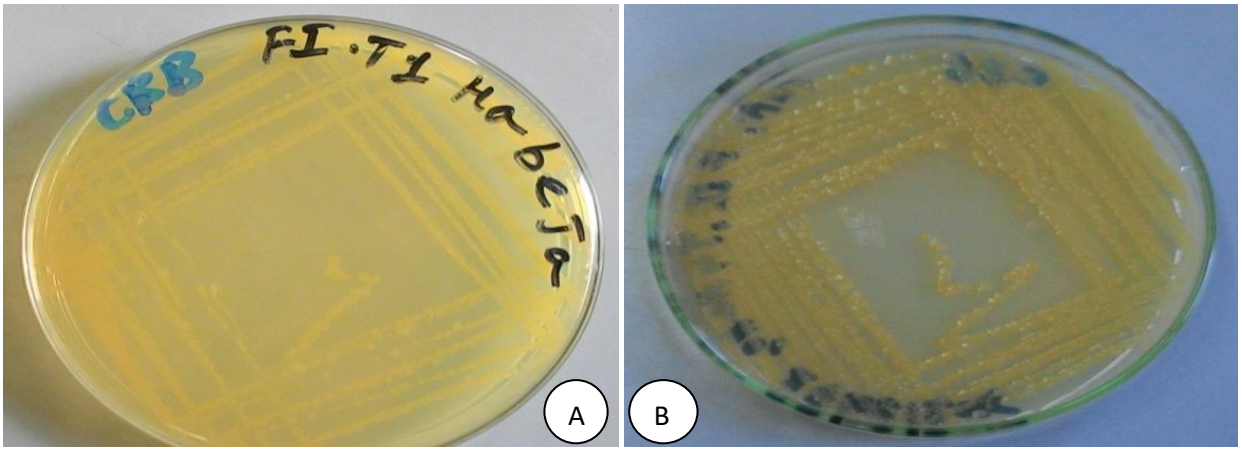


Fig. 14 (A) BBC isolates changed to black colour on aesculin test and
14 (B) BBC isolates changed to yellow indicated as positive reaction to tartarate test.



Figs.15 A and **B:** BBC isolates Fluorescent *Pseudomonas* on king's B medium.

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 isolate sproduced 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

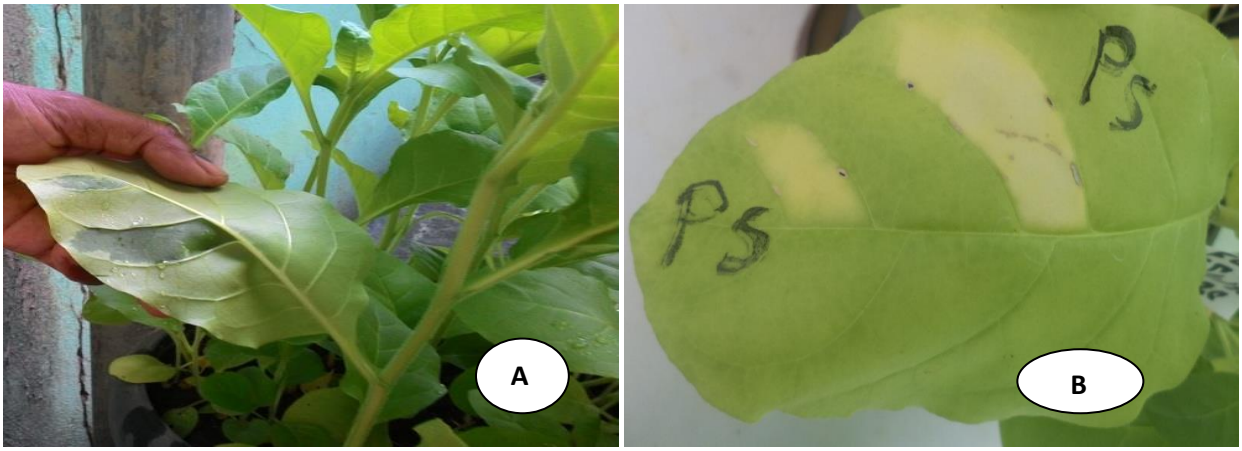
while others were variable to three tests and one negative in their reaction to the tests (Table 3). Kazempour et al.(2007) and Bultreys and Kaluzna, (2010) reported that the similar biochemical test results and those tests considered as diagnostic biochemical characters for identification and confirmation of coffee bacterial blight pathogen as *Psuedomonas syringae pv.garcae* to distinguishing pathovars within *P. syringae,* gelatine hydrolysis, Aesculin hydrolysis, and tartrate utilization were used as common tests as done by Lelliot and Stead, (1987) in addition to more differences in the assimilation reactions. In the present study, these reactions were suggested that *Pseudomonas* spp is *Pseudomonas syringae* pv. *garcae.* This result was similar with characteristics of *Pseudomonas syringae* pv. *garcae* reported earlier by Ramos and Shavdia (1976) and Ramos, (1979).

Table 3 Biochemical characterization of bacterial blight of coffee isolates

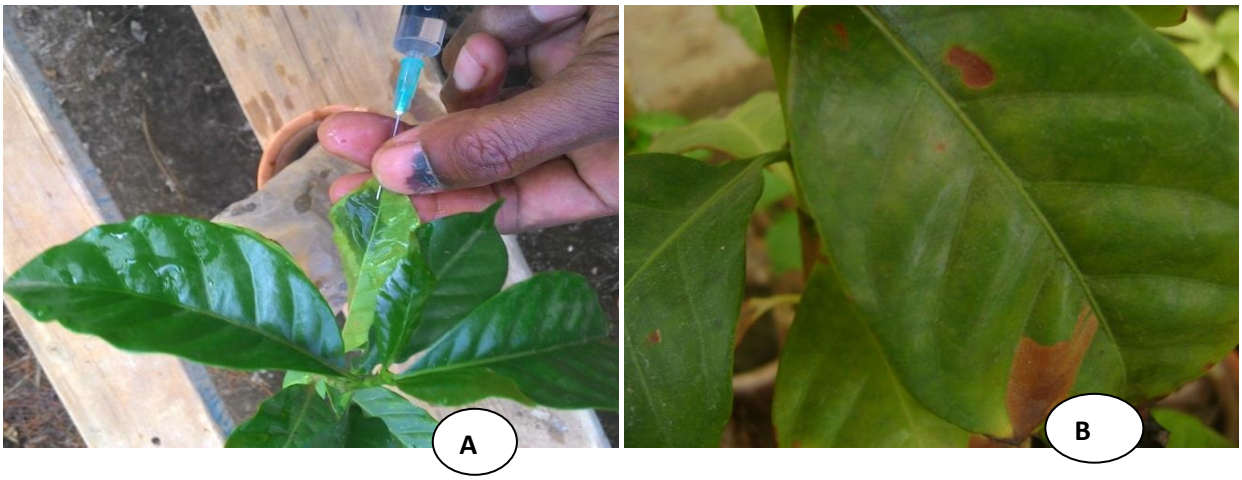
No.	Selected isolates	Biochemical Test									
		Catales	KOH	Oxidase	Gelatine	Tween 80	Aesculin	Starch hydrolysis	Levan	Tartrate	Argenine
1	Hayilo - FIT3	+	-	-	+	+	-	+	+	+	-
2	Hayilo -FIT4	+	-	-	+	+	-	+	+	+	-
3	Manche - FIT4	+	-	-	+	+	-	-	+	-	-
4	Manche - FIIT2	+	-	-	+	+	-	-	+	-	-
5	Manche - FIIIT1	+	-	-	+	+	-	-	+	-	-
6	Gordahama- FIT2	+	-	-	+	+	+	-	+	+	-
7	Shafina - FIVT4	+	-	-	+	+	+	-	+	+	-
8	Bo'a - FIIIT3	+	-	+	+	+	-	-	+	-	-
9	Bo'a - FIT3	+	-	+	+	+	+	+	+	-	-
10	Bo'a - FIIT1	+	-	+	+	+	-	+	+	-	-
11	Gordahama - FIIT2	+	-	-	+	+	+	-	+	-	-
12	Gordahama -FIVT3	+	-	-	+	+	+	+	+	+	-
13	Gobadamo - FIT2	+	-	+	+	+	+	-	+	-	-
14	Gobadamo - FIIIT1	+	-	+	+	+	+	-	+	-	-
15	Gobadamo -FIVT3	+	-	+	+	+	+	+	+	-	-
16	Abeja - FIT2	+	-	-	+	+	+	+	+	-	-
17	Abeja -FIIIT1	+	-	-	+	+	+	+	+	-	-
18	Abeja - FIVT1	+	-	+	+	+	+	-	+	-	-
19	L/ honcho -FIIIT1	+	-	+	+	+	+	-	+	-	-
20	L/ Honcho- FIT1	+	-	+	+	+	+	-	+	-	-
21	L/ Honcho - FIIIT2	+	-	+	+	+	-	-	+	-	-
22	L/ Honcho - FIVT4	+	-	+	+	+	+	-	+	+	-
23	Hallo - FI T4	+	-	+	+	+	-	-	+	-	-
24	Hallo -FIII T1	+	-	+	+	+	-	-	+	-	-
25	Futaye - FI T4	+	-	-	+	+	-	-	+	-	-
26	Futaye - FII T3	+	-	-	+	+	-	+	+	-	-
27	Futaye -FIV T2	+	-	+	+	+	+	-	+	+	-
28	Kumato - FI T3	+	-	-	+	+	-	-	+	-	-
29	Gelawacho -FI T4	+	-	-	+	+	-	+	+	-	-
30	Metisho -FII T3	+	-	-	+	+	+	-	+	-	-
31	Sokicha -FIII T3	+	-	-	+	+	+	+	+	+	-
32	Kara -FIIT1	+	+	+	+	+	+	-	+	+	-
33	sugale -FIIIT1	+	-	+	+	+	+	-	+	-	-
34	M/Holayna -FIT1	+	-	-	+	+	+	+	+	+	-
35	Shigado -FIT2	+	-	-	+	+	-	-	+	-	-
36	Shigado -FIIIT2	+	-	-	+	+	+	-	+	-	-
37	M/Shakoha -FI T1	+	-	-	+	+	-	-	+	-	-

Hypersensitivity and pathogenicity test

Hypersensitive and pathogenicity test response was observed after tobacco 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. All strains induced the hypersensitive reaction in tobacco leaves (Figs. 16A and B).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. 16 (A) Inoculation of *Pseudomonas syringae* on tobacco leaves (B) Hypersensitive response observed



Figs. 17 (A) 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. 17 A and B). 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).

CONCLUSIONS

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. 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%. The intensity of BBC disease also varied between different altitudinal categories; as altitude increases both incidence and severity were increased and at the lowest altitude ranges between 1625 and1725 m. a. s. l, both disease incidence and severity were recorded lowest. 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 2-3mm 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 tobacco 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. Finally, the isolates were identified and considered as *Pseudomonas syringae pv. garcae* on KB selective media in combination with others biochemical tests conducted to identify the target pathogen. The morphological, cultural, microscopic characters and biochemical tests of isolates as confirmed that the organism identity as *Pseudomonas syringae pv. garcae.* This study provided first information on disease intensity and 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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