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In vitro Evaluation of Actinobacteria against Tomato Bacterial Wilt (*Ralstonia solanacearum* EF Smith) in West Showa, Ethiopia

Kassahun Sadessa Biratu[1], Thangavel Selvaraj[2]* and Tariku Hunduma[1]

[1]*Department of Bacteriology, Ambo Plant Protection Research Center, Ambo, Ethiopia*
[2]*Department of Plant Sciences and Horticulture, College Of Agriculture and Veterinary Sciences, Ambo University, Ambo, Post Box No-19, Ethiopia*

Abstract

Ralstonia solanacearum EF Smith is one of the most important and widespread pathogenic bacterium that causes a wilt disease in vegetable crops including tomato (*Lycopersicon esculentum* Mill.) in West Showa, Ethiopia, mainly in the off-cropping season. *In vitro* study was conducted to screen indigenous actinobacteria for their antibacterial activity against tomato bacterial wilt pathogen, *R. solanacearum*. 210 soil samples were collected from three districts of West Showa Zone, Ethiopia. Of all soil samples, 86 morphologically distinct actinobacterial isolates were isolated. In the preliminary screening test, 52 isolates were selected showing inhibitory activity against the pathogen and further tested for their antibacterial activity under secondary screening test. In the secondary screening test, all the isolates appeared significantly different ($P \leq 0.05$) in their inhibition against the target pathogen. Of these isolates, efficient 36 isolates were selected and subjected to the cell free suspension test. All the 36 isolates showed significantly different ($P \leq 0.05$) in their inhibition activity in the cell free suspension test. In both dual culture and cell free suspension test, totally 21 isolates showed inhibitory activity against the target pathogen. The actinobacterial isolate, Gosu-qoraS#196-1 had become superior antagonistic isolate against *R. solanacearum* and followed by Awaro S#174-2, Senkele S#132-5, Awaro S#183-1, Senkele S#133-3, Dhaga file S#113-1, Awaro S#176-4, Gabata S#21-1, Dhaga file S#128-2 and Awaro S#174-3 isolates. This study has concluded that the actinobacterial isolates having antibacterial activity against this target pathogen and also the investigated isolates can be used as one component of integrated disease management practice through detailed further test. Then, the further research on the actinobacterial isolates should be focused on species identification, *in vivo* evaluation of the isolates against tomato bacterial wilt and further testing of its host range.

Keywords: Actinobacteria; Screening; Tomato; Bacterial wilt; *Ralstonia solanacearum; In vitro*

Introduction

Tomato (*Lycopersicon esculentum Mill.*) is one of the most widely grown vegetables in the world and it is regarded as one of the top priority vegetable. Tomato contributes to healthy, because it is rich in minerals, essential amino acids, sugars, dietary fibers and it is considered to be fairly high in vitamins, vitamin C, lycopene and beta-carotene with potential for better quality processing [1]. In Ethiopia, tomato is one of the most important vegetable crop and its production has shown a marked increase since it became the most profitable crop providing a higher income to small scale farmers compared to other vegetable crops [2]. However, the national average of tomato fruit yield in Ethiopia was often low (125 kg/ha), when compared even to the neighboring African countries like Kenya (164 kg/ha) [2]. Current productivity under farmers' condition in Ethiopia is 190 kg /ha whereas yield up to 300 kg/ha recorded on research plots [1].

Farmers get lower yield mainly due to diseases, pests and sub-optimal fertilization [3]. However, there are a number of factors which limit tomato yield. These include: the lack of improved well performing varieties, poor fruit setting due to heavy rains and excessively high temperature, pests and diseases. In addition to these, fungal as well as bacterial diseases are considered to be the major constraints to tomato production [2]. Howard [4] reported that 10 to 50% production of tomato were affected by diseases annually. Fungal diseases such as damping-off, crown and root rot, gray mold, blight and powdery mildew and bacterial diseases of wilt and rots are some of the common problems in tomato production. Among those diseases, bacterial wilt (*Ralstonia solanacearum* EF Smith) of Solanaceous vegetables are the most destructive and widespread disease in tropical and subtropical regions of the world. It is one of the most important and widespread phytopathogenic bacterial wilt pathogen in Ethiopia, mainly in the off-cropping season and a destructive disease in vegetable crops including tomato. The percent incidence of tomato bacterial wilt was recorded as high as 55% [5] in major tomato producing areas of Ethiopia.

The management of tomato against this pathogen is important to maximize the crop's yield. Chemicals either not have direct bactericidal or fungicidal activities against the pathogen or converted into non-toxic derivatives by the pathogen. Management of this disease through chemicals and the use of resistant varieties are possible, but the hazardous impact of agrochemicals on the human health and environments, development of resistant mutants, escalating costs of fumigant pesticides and frequent breakdown of resistant varieties; strongly demand a sustainable and alternative disease management approaches [6].

Many bio-control agents (BCA) have been successfully identified for the control of various plant pathogens as alternative disease

***Corresponding author:** Thangavel Selvaraj, Professor of Pathology and Microbiology, Department of Plant Sciences and Horticulture, College of Agriculture and Veterinary Sciences, Ambo University, Ambo, P.O. Box 19, Ethiopia, Tel: +251-913073294; Fax: +251-112362037; E-mail: tselvaraj_1956@yahoo.com

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management approaches. Bio-control of pathogenic bacteria has gained considerable attention in the past two decades. Although several bacterial [7,8] bio-control agents for the control of pest and diseases developed and used in the production of vegetables including tomato crops [7]. Among different BCA, actinomycetes which is otherwise called as Actinobacteria or "higher Bacteria" [9] are known a very effective BCA and pesticide sources throughout the world. Actinobacteria play a quite important role in natural ecological system and they are also profile producers of antibiotics, antitumor agents, enzymes, enzyme inhibitors, vitamins, alkaloids and immune modifiers which have been applied in industry, agriculture, forestry and pharmaceutical industries [10,11].

The biodiversity of actinobacterial population density is less common in terrestrial and temperate soils. The actinobacteria are resources under extreme environments (including extreme high and low temperature, extreme high or low pH, high salt concentration etc.) and have received comparatively little attention from pathologists and microbiologists in Ethiopia. The difference in physical and chemical characteristics and the occurrences in the terrestrial and temperate environments of these actinobacteria may helps for their population density, diversity and potential which provide opportunities to a greater extent for the control of bacterial plant diseases [11]. Many studies have been attempted on the isolation of actinobacteria from terrestrial, tropical, sub-tropical and marine sediments [11-13] but not in temperate high mountain soils of Ethiopia.

The biological control of bacterial wilt using bio control agents like actinobacteria have shown a great potential and commercial preparations for the management of such diseases are in use [14]. However, much work still needs to be done in Ethiopia, especially with the bacterial wilt of tomatoes, since the disease is still causing much devastation on the crop. Hence, the present study was carried out to isolate and identify the potential actinobacterial isolates from soils of three different districts of West Showa, Ethiopia, to characterize and screen them to getting some actinobacterial isolates that produce antibiotics and active against tomato bacterial wilt as well as to determine their bio control potential against this test organism through *in vitro* condition.

Materials and Methods

Sample collection and isolation of tomato bacterial wilt pathogen

In this study, the target pathogen was obtained from preserved culture of Ambo Plant Protection Research Center (APPRC), Ambo and the samples of diseased tomato plants were collected from tomato growing areas of the country, mainly around Ziway area of West Showa. For isolation of the pathogen, about 1 g of the diseased sample was surface sterilized by 1% hypochlorite and macerated in sterile distilled water and the filtrate was further diluted using sterile distilled water. From the appropriate dilutions, 0.1 ml of an aliquot was spread-plated in duplicates on pre-dried surfaces of Triphenyl Tetrazolium Chloride (TTC) agar medium [15]. After 48 h of incubation at 30°C, the typical mummified fluid, slightly red-tinted colonies of *R. solanacearum* was easily distinguished, picked and further purified through repeated streak plating on the same medium. The biochemical characterization such as cytochrome oxidase, catalase [16], KOH solubility [17], Tween 80 hydrolysis, fatty acid esterase activity [18], starch hydrolysis [18] and pathogenicity (Koch's postulates test) of the target pathogen was tested.

Soil sample collection

The soil samples were collected from three different districts viz., Tikur Incini, Toke kutaye and Ambo of West Showa, Oromia, Ethiopia (Table 1). In each district, four different kebeles were selected. Nano, Bere, Gabata and Garmama kebeles were selected from Tikur Incini district, Malka Dera, Mutulu-Odobari, Kilinto and Gorfo kebeles were selected from Toke kutaye district and Sankele, Faris, Awaro and Gosu-kora Kebeles were selected from Ambo district. The soil samples were collected from forests, virgin lands, cultivated and grazing lands as stratified random sampling options. Soil samples were randomly collected from top 4 cm soil profile at an interval of about half a km between the sampling spots. Soil samples (approx. 200 g) were collected in sterile polythene bags, stored in iceboxes and transported to APPRC laboratory, Ambo for further analysis. From all sampling spots, totally about 210 soil samples were collected.

Isolation of actinobacteria from soil samples

For isolation of actinobacteria, Starch Casein Agar (SCA) medium [19] (soluble starch 10 g, K_2HPO_4 2 g , KNO_3 2 g, $NaCl_2$ g, casein 0.3 g , $MgSO_4$. $7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4$ 0.7 H_2O 0.01 g, agar 20 g, pH 7.2, distilled water 1000 ml) was used. Streptomycin (40 µl/ml) and griseofulvin (50 µl/ml) were used to prevent bacterial and fungal contamination, respectively. Soil samples were serially diluted up to 10^7 and 0.1 ml of aliquots were spreaded over the SCA plates. Actinobacterial colonies were identified by their cultural characteristics, after incubation at 28 to 32°C for about 7-14 days, actinobacterial colonies were purified by streak plate technique on cultivation medium.

Characterization of actinobacteria

Morphology: The actinobacteria were characterized morphologically by the following methods given in the International *Streptomyces* Project (ISP) [20]. The characters including colony morphology of the isolates such as the color of aerial mycelium (on the surface of agar), reverse side color, production of diffusible pigments [11,12], melanoid and soluble pigments [20] were observed after incubation at 28°C for 7-10 days on SCA. Gram staining [21] and KOH solubility test [17] were detected on the 7-day old cultures. The microscopic morphology of isolates such as formation of aerial and substrate mycelium and spore arrangement, which are highly characteristic and useful in the identification of actinobactera, were observed by cover slip technique [22] with light microscopy. The color of the colony was compared with standard color chart for identification [23].

Screening of antibacterial activity of actinobacteria

Preliminary screening: Antibacterial activity of actinobacteria was subjected to primary screening by cross streak plate technique [24]. The pure preserved actinobacterial isolates were reinitiated and used for anti-bacterial activity screening against tomato bacterial wilt. The

No.	Location/ District	Number of soil samples collected	Soil type	Soil color
1	Tikur Incini	60	Sandy loam Clay loam	Brown Black Light black
2	Toke kutaye	60	Silt-loam Clay-loam	Light red Brown to red
3	Ambo	90	Clay-loam Clay	Black Sticky silt
	Total	210		

Table 1: Soil samples collected from three different districts.

actinobacterial isolates were streaked on half part of pre-dried surfaces of modified nutrient agar plates and incubated at 30°C for 3-5 days. After observing good growth of the actinobacteria, the target pathogen was streaked at right angles to the streaked growing actinobacteria as close as possible and incubated at 30°C for about three days. This screening was done in triplicates for each isolate on Completely Randomized Design (CRD). The inhibitory activity was observed visually daily and actinobacterial isolates showing inhibition to the growth of the target pathogen was selected for further next secondary screening.

Secondary screening: Actinobacterial isolates showing antibacterial activity during preliminary screening was used for the secondary screening step. The actinobacterial isolates were again further tested for their antibacterial activity using modified dual culture technique [25]. In dual culture method, the actinobacterial isolates were spot inoculated on pre-dried surfaces of nutrient agar plates and the plates were incubated at 30°C for 3-5 days. After good growth of actinobacteria, 1 ml (about10^7cfu/ml) of 24 h old culture of the test organism from nutrient broth was inoculated on the plates and carefully distributed by rocking. Excess inoculum was carefully removed by tilting the plate at about 45°C. Standard antibiotic disc, Chloramphenicol (30 µg/disc) was used for comparison. The plates were then incubated at 30°C and inhibitory activity of the actinobacteria and the standard antibiotic discs were continuously observed and recorded after 24-72 h. Visually observed zone of inhibition (both diameter and annular radii) was measured from the back side of the plates using calipers. All the plates were replicated three times in CRD. In the same manner as the preliminary screening, actinobacterial isolates showing better/equivalent inhibition zone as visually compared to the standard antibiotic discs were selected for further cell free suspension screening test.

Cell free suspension (CFS) test

Actinobacterial isolates selected during dual culture were inoculated into SCB and allowed to grow at ambient temperature on a shaker (120 rpm) for about a week until high turbidity was observed. After incubation, the broths were centrifuged twice at 6000 rpm for 30 min each and then the filtrates were again filtered using microbial filter of 0.22 µm. Finally, the extracted antimicrobial compounds were aseptically loaded onto 6 mm diameter sterile disc (50 µl/disc). Then the discs were placed on nutrient agar plates which were pre-seeded with the test organism. Standard Chloramphenicol discs (30 µl/disc) were used as comparison. The plates were then incubated at 30°C and inhibitory activity of the actinobacteria and the standard antibiotic discs were continuously observed and recorded after 24-72 h. Visually observed zone of inhibition (both diameter and annular radii) was measured from the back side of the plates using calipers.

Data analyses

Data were analyzed using SAS version 9.0 (Statistical Analysis System for Windows, 2001-2004, SAS Institute, USA). GLM procedure was followed for data analysis. Means were compared at 5% significance level, using Duncan's Multiple Range (DMR) test.

Results and Discussion

Isolation and characterization of the target pathogen

The target bacterial pathogen was isolated from freshly collected samples at farmers tomato cultivated fields and APPRC isolate culture was compared on the basis of their fluidity, color, and morphological structures. Both isolates produced mummified fluidal of irregular bacterial colonies with the pink color on the TTC agar medium after 48 h incubation at 30°C and were observed for rod shaped after examined under compound microscope. The cultures showed highly mummified fluidal viscous and slight creamy to white color on the TTC medium (Figure 1) and colonies color were showed brown color after 48 h of incubation at 30°C. All the isolates produced characteristic whitish on TTC medium and this observation was confirmed with the observation of Kelman [15].

Biochemical characterization of *R. solanacearum*

All the targeted bacterial pathogen isolates were catalase positive, oxidase positive and KOH solubility but none of the bacterial isolates showed clear zone during starch hydrolysis test. All the isolates were negative to starch hydrolysis test and were positive to Tween 80 hydrolysis test and also showed an opaque zone of crystals around the colony. The pathogenicity on the artificially inoculated test pieces and symptoms of the disease were also proved.

Isolation and characterization of actinobacteria

Among 210 soil samples, a total of 86 morphologically distinct and diverse actinobacterial isolates were isolated. Each isolate showed different morphological characteristics based on aerial mass color, reverse side pigments, melanoid and soluble pigments production (Table 2). The colors were recorded using standard color chart as recommended by ISP [20]. Among them, 8 isolates from Tikur Incini district soils which were 9.3%; 14 isolates from Toke Kutaye district soils which covers 16.28% and 64 isolates from Ambo district soils encompassing 74.42% of the total actinobacterial isolates. Common actinobacterial species at all the three different districts were not observed. The identified species (86) were morphologically distinct and were studied upon the aerial, reverse side color, diffusible pigment productions, melanoid and soluble pigments, formation of mycelium and sporophore morphology under microscopic examinations. Similar findings were also previously reported by Kim et al. [26] and Vijayakumar et al. [27]. The soil samples collected from the typical highland of Tikur Incini was almost covering sandy-loam soil types with black color; sandy soil with red to brown color which represents the low land soils collected from Toke kutaye and sand to clay-loam of black soils were stands for soil samples of the Ambo district mid high land i.e. more actinobacterial isolates were isolated from the soils collected from Ambo district. This indicated that actinobacterial isolates are very diverse and occur in diversified terrestrial habitats of cultivated, grazing and forest soils of all lowland, midland and high land of different soil types which were similarly stated by Okami and Hotta [10], as actinobacterial population density was less common in marine sediments relative to terrestrial soils. The soil samples were collected from forests, virgin land, cultivated and grazing lands as stratified

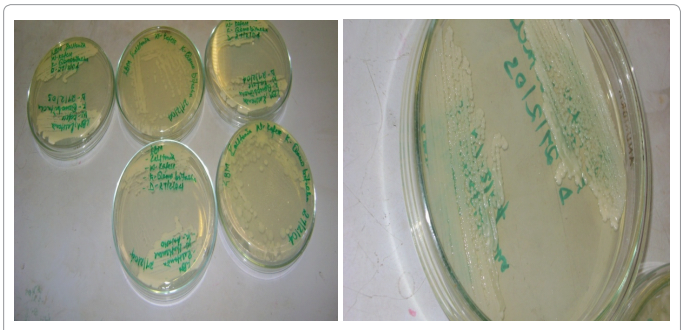

Figure 1: Cultural characteristics of R. solanacearum on TTC medium.

No.	Isolate	Aerial mass color	Reverse side color	Diffusible pigments	Melanoid pigments	Soluble pigments
1	Bere S#08-1	Olive	Dark khaki	-	-	-
2	Bere S#08-2	Yellow green	Green yellow	-	+	-
3	Gabata S#21-1	Dark green	Chocolate	-	-	-
4	Gabata S#23-1	Pale green	Dark red	-	-	-
5	Gabata S#29-1	White	Floral white	White	-	-
6	Malika Dera S#33-1	Pale green	Firebrick	-	-	-
7	Caka malika dera S#43-1	Pale green	Firebrick	White	-	-
8	Caka malika dera S#54-1	Olive drab	Firebrick	White	-	-
9	Gorfo S#92-1	Antique white	Dark golden red	-	+	-
10	Gorfo S#104-1	Khaki	Beige	-	-	-
11	Gorfo S#106-1	Dark olive green	Golden red	-	-	-
12	Gorfo S#110-1	Deep yellow	Light green	-	-	-
13	Gorfo S#110-2	Yellow	Dark olive green	-	-	+
14	Dhaga file S#113-1	White	Dark blue	-	-	-
15	Dhaga file S#113-2	Gold	Dark red	-	-	-
16	Dhaga file S#121-1	Forest green	Gold	-	-	-
17	Dhaga file S#128-1	Yellow	Orange	Golden	-	+
18	Dhaga file S#128-2	Violet	Light green	-	-	+
19	Dhaga file S#128-3	Dark olive green	Dark red	Corn silk	-	-
20	Dhaga file S#128-4	Forest green	Red	-	-	+
21	Dhaga file S#129-1	Corn silk	Golden red	-	+	-
22	Dhaga file S#130-1	Dark olive green	Deep red	-	+	-
23	Dhaga file S#130-2	Peru	Firebrick	White	-	-
24	Senkele S#132-1	Dark sea green	Brown	Light white	-	-
25	Senkele S#132-2	Linen	Seashell	-	-	-
26	Senkele S#132-3	Linen	Sandy brown	-	-	-
27	Senkele S#132-4	Rough linon	Moccasin	Navajo white	-	-
28	Senkele S#132-5	Papaya whip	Peach puff	White	+	-
29	Senkele S#132-6	White	Navajo white	-	-	-
30	Senkele S#132-7	Navajo white	Burly wood	-	+	-
31	Senkele S#132-8	Burly wood	Bisque	-	-	-
32	Senkele S#132-9	Bisque	Antique white	-	-	-
33	Senkele S#132-10	Deep white	Wheat	-	-	-
34	Senkele S#132-11	Yellow green	Green	-	-	-
35	Senkele S#132-12	Dark yellow	Gray	-	-	-
36	Senkele S#132-13	Olive	Light salmon	White	-	+
37	Senkele S#132-14	Golden red	Dark golden rod	Burly wood	+	-
38	Senkele S#132-15	Floral white	Dark green	Ghost white	+	-
39	Senkele S#132-16	Ghost white	Red	-	-	+
40	Senkele S#133-1	Brown	Sea green	Ghost white	-	+
41	Senkele S#133-2	Golden red	Golden red	-	+	-
42	Senkele S#133-3	Steal blue	Rosy brown	-	+	-
43	Senkele S#142-1	Dark olive green	Dark orange	-	+	-
44	Senkele S# 142-2c	Sea green	Yellow green	-	+	-
45	Senkele S# 142-3	Teal	Tan	-	+	-
46	Senkele school S#147-1	Dark olive green	Brown	-	-	-
47	Senkele school S#147-2	Dark olive green	Dark golden red	-	+	-
48	Senkele school S#147-3	Dark-khaki	Dark golden red	Ghost white	+	-
49	Senkele school S#147-4	Blue violet	Brown	Red	-	+
50	Senkele school S#147-5	Dark olive green	Chocolate	Green	-	-
51	Senkele school S#147-6	Light green	Purple	-	-	+
52	Senkele school S#147-7	Golden red	Dark red	-	-	-
53	Senkele school S#147-8	Yellow	Khaki	-	-	+
54	Senkele school S#149-1	Olive drab	Brown	-	-	-
55	Senkele school S#149-2	Dark red	Firebrick	Red	-	-
56	Senkele school S#149-3	Lime green	Pale violet red	-	+	-
57	Senkele school S#149-4	Medium sea green	Medium violet red	-	+	-
58	Senkele school S#149-5	Olive-green	Red	-	-	+
59	Senkele school S#149-6	Firebrick	Floral white	-	-	-
60	Senkele school S#149-7	Tan	Ivory	-	-	-

61	Farsi S#156-1	White	Rough brown	-	-	-
62	Farsi S#159-1	Orange	Purple	Light salmon	-	+
63	Awaro S#174-1	Light white	Navajo white	-	+	-
64	Awaro S#174-2	Olive drab	Maroon	Green	-	-
65	Awaro S#174-3	Yellow green	Sienna	Snow	-	-
66	Awaro S#174-4	Red	Golden yellow	-	-	+
67	Awaro S#176-1	Navajo white	Mint cream	-	+	-
68	Awaro S#176-2	Snow	White	Light white	-	-
69	Awaro S#176-3	Ghost white	Floral white	-	+	-
70	Awaro S#176-4	Sandy brown	Sienna	Red	-	-
71	Awaro S#177-1	White	Purple	Olive-green	-	+
72	Awaro S#178-1	Purple	Golden yellow	Light-yellow	-	-
73	Awaro S#178-2	Golden red	Dim gray	White	+	-
74	Awaro S#178-3	Green	Purple	-	-	+
75	Awaro S#183-1	Tan	Violet red	Green	-	-
76	Awaro S#184-1	Khaki	Orange	-	-	+
77	Awaro S#184-2	Golden rod	Orange	Yellow	-	+
78	Awaro S#188-1	Dark olive green	Dark red	-	-	-
79	Gosu Qora S#196-1	Goldenred	Yellow	White	-	-
80	Gosu Qora S#196-2	Dark olive green	Coral	White	-	-
81	Gosu Qora S#201-1	Deep white	Lemon chiffon	-	+	-
82	Gosu Qora S#205-1	Pale green	Deep red	-	-	-
83	Gosu Qora S#205-2	Ghosh white	Orange	-	-	+
84	Gosu Qora S#205-3	Tan	Yellow	-	-	+
85	Gosu Qora S#205-4	Gray	Moccasin	White	-	-
86	Ambo Univer. S#210-1	Yellow green	Orange	-	-	+

+ Present; - Absent

Table 2: Morphological characteristics of the actinobacterial isolates.

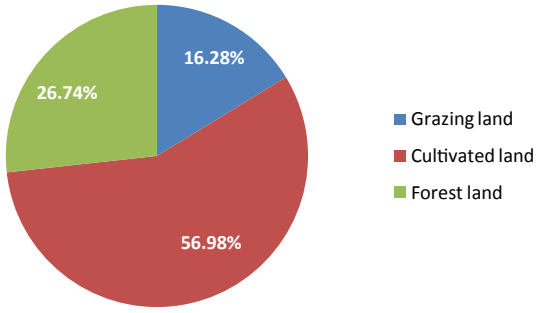

Figure 2: Percentage of actinobacterial isolates isolated from cultivated, forest and grazing lands.

random sampling options simply to extend the chance of success of obtaining very distinct actinobacterial isolates. Actinobacteria isolated from forest soil samples were covered 26.74%, cultivated land for 56.98% and grazing land for 16.28% (Figure 2), in which 23, 49 and 14 isolates were isolated from forest, cultivated and grazing lands, respectively.

Among 86 actinobacterial isolates, 32 isolates have produced reverse side diffusible pigments, 20 isolates have produced melanoid pigments and 19 isolates produced soluble pigments whereas, 27 isolates produced no reverse side, melanoid and soluble pigments (Figure 3; Table 2) which were similarly reported by Sivakumar [12]; Oskay et al. [14] and Vijayakumar et al. [27]. Morphological characters of the isolates were observed by smears from colonies of 7 day old cultures, stained by Grams' method. Under microscopic examination, all actinobacterial isolates have retained the violet color or not changed to red and were remained Gram-positive bacteria. Likewise, all the actinobacterial isolates were gram positive under KOH solubility test which is similar with work done by Fahy and Hayward [17].

Screening for antibacterial activity

Preliminary screening: The preliminary screening was conducted for all 86 actinobacterial isolates against the test pathogen, *R. solanacearum*. Of all the tested isolates, 52 isolates showed inhibition effect to *R. solanacearum* (Figure 4) and were selected for further screening in the secondary test as recommended by Martin and French [28].

Secondary screening: The 52 actinobacterial isolates showing inhibition to the target pathogen during the preliminary screening were further tested for their inhibition using dual culture technique. From the actinobacterial isolates screened by dual culture method, 36 of them were performed effective against tested pathogen in their inhibition activity (Figure 5 and Table 3). Both diameter and annular radius of the inhibition zone were measured and analyzed. All the isolates appeared significantly different ($P \leq 0.05$) in their inhibition against *R. solanacearum* pathogen. According to the analysis made, there was very highly significance difference among actinobacterial isolates; Gosu qoras#196-1 which was the superior isolate under this experiment, Awaros#174-2, Senkeles#132-5 and Awaros#183-1 were second, third and fourth antagonistic isolates exhibited more inhibition activity against *R. solanacearum* in their order of inhibition importance (Table 3). Finally, 36 isolates having relatively higher/equal mean of annular radii inhibition zone than control or standard antibiotic disc of Chloramphenicol were selected for their supernatant activity test.

Cell free suspension test screening: From the top performing isolates, 36 of them were again tested using their cell free suspension (Figure 6). Cell free suspension from all the 36 isolates showed significantly different inhibition activity. The actinobacterial isolate,

Figure 3: Distinct morphological characteristics of reverse and aerial mycelium color of some actinobacterial Isolates (A) Isolate S# 176-1 -Reverse side color (B) Isolate S# 176-1 Aerial mycelium color C) Isolate S# 130-1 Reverse side color (D) Isolate S#130-1- Aerial mycelium color (E) Isolate S#133-1 Reverse side color (F) Isolate S#133-1- Aerial mycelium color (G) Isolate S#178-1- Reverse side color (H) Isolate S#178-1-Aerial mycelium color (I) Isolate S#174-1- Reverse side color (J) Isolate S#174-1-Aerial mycelium color (K) Different actinobacterial isolates- Reverse side color (L) Aerial mycelium color.

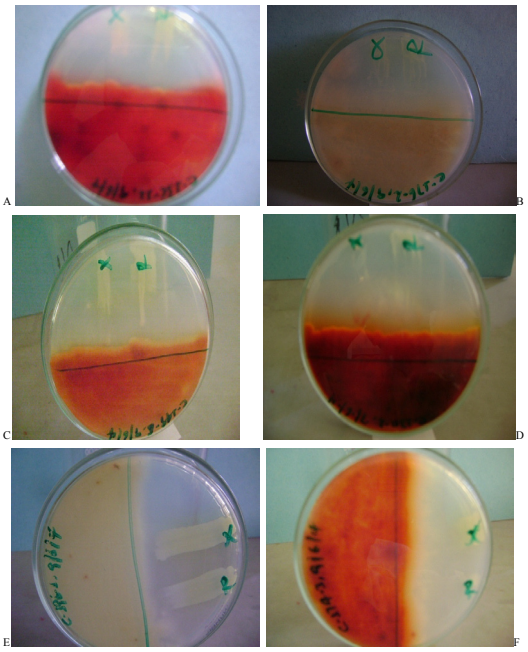

Figure 4: Some actinobacterial Isolates showing inhibition to the target pathogen during Preliminary screening (A) IsolateS#132-11 (B) IsolateS#176-2 (C) IsolateS#149-6 (D) IsolateS#130-2 (E) IsolateS#196-1 (F) Isolate S#174-3, R=*Ralstonia solanacearum*.

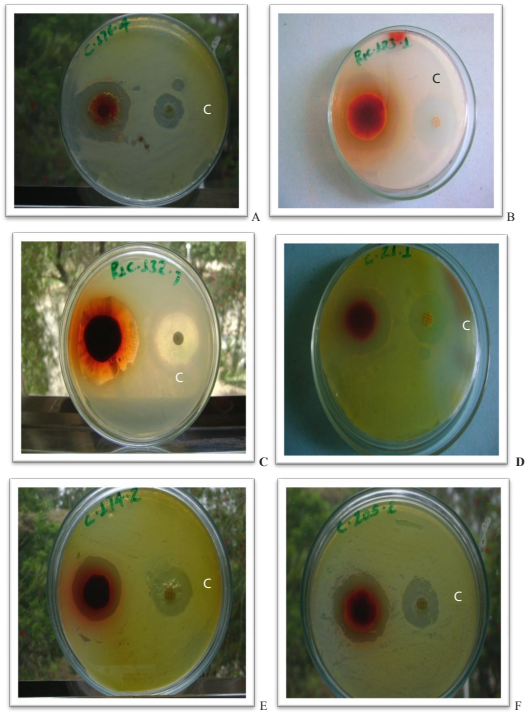

Figure 5: Some Actinobacterial Isolates showing inhibition against R. solanacearum using dual culture (A) Isolate S# 176-4 (B) Isolate S#183-1 (C) Isolate S#1132-5 (D) Isolate S#21-1 (E) Isolate S#174-2 (F) Isolate S#205-2, C= Chloramphenicol antibiotic standard disc.

Gosu qoras#196-1 became superior antagonist isolate against *R. solanacearum* and it was very highly significant within and between isolates in mean inter-comparison at $P \leq 0.05$. There were very highly significant differences between and among very effective and attractive actinobacterial isolates i.e. Awaros#174-2, Senkeles#132-5, Awaros#183-1, Senkeles#133-3, Dhaga files#113-1, Awaros#176-4, Gabatas#21-1, Dhaga files#128-2 and Awaros#174-3; which was 2nd, 3rd, 4th, 5th, 6th, 7th, 8th, 9th and 10th, respectively in their sequential orders of decreasing inhibition zone observed during mean separation against the test organism (Table 4) and the results obtained were exactly the

No	Isolates/Treatments	Annular Radius of Inhibition Zone Measured (mm)			Mean of Inhibition Zone of annular radius (mm)
		R1	R2	R3	
1	Gosu Qora S#196-1	14.95	14.91	14.97	14.94333a
2	Awaro S#174-2	14.53	14.47	14.5	14.50000b
3	Senkele S#132-5	13.9	13.9	13.9	13.90000c
4	Awaro S#183-1	13.56	13.58	13.52	13.55333d
5	Senkele S#133-3	13.52	13.49	13.56	13.52333de
6	Awaro S#176-4	13.46	13.49	13.45	13.46667de
7	Gabata S#21-1	13.42	13.39	13.4	13.40333e
8	Awaro S#174-3	13.93	13.14	13.14	13.40333e
9	Dhaga file S#128-2	13.37	13.41	13.38	13.38667e
10	Dhaga file S#113-1	13.1	13.02	13.07	13.06333f
11	Gabata S#23-1	12.94	12.93	12.98	12.95000gf
12	Dhaga file S#128-1	12.83	12.83	12.83	12.83000gh
13	Senkele S#132-2	12.84	12.83	12.81	12.82667gh
14	Dhaga file S#130-1	12.7	12.8	12.77	12.75667h
15	Senkele school S#149-6	12.6	12.61	12.64	12.61667i
16	Awaro S#184-1	12.44	12.46	12.49	12.46333j
17	Ambo UniverS#210-1	10.01	12.35	12.29	12.31667k
18	Dhaga file S#113-2	12.23	12.24	12.24	12.23667lk
19	Senkele S#133-1	12.12	12.1	12.18	12.13333lm
20	Gosu Qora S#205-2	12.11	12.11	12.14	12.12000lm
21	Senkele school S#147-3	12.07	12.09	12.12	12.09333m
22	Awaro S#184-2	11.92	11.97	11.94	11.94333n
23	Awaro S#178-1	11.92	11.9	11.96	11.92667n
24	Awaro S#174-4	11.75	11.75	11.72	11.74000o
25	Senkele school S#149-3	11.63	11.72	11.69	11.68000po
26	Senkele school S#147-5	11.55	11.56	11.55	11.55333p
27	Senkele school S#147-7	11.44	11.28	11.46	11.39333q
28	Bere S#08-2	11.41	11.42	11.33	11.38667q
29	Malika DeraS#33-1	11.17	11.2	11.19	11.18667r
30	Senkele school S#149-2	11.13	11.11	11.14	11.12667r
31	Senkele S#132-6	11.12	11.13	11.11	11.12000r
32	Caka Malika De-raS#54-1	10.72	10.69	10.71	10.70667s
33	Senkele school S#149-1	10.48	10.44	10.46	10.46000t
34	Senkele school S#149-5	10.44	10.37	10.44	10.41667t
35	Gosu qoraS#201-1	12.31	10.09	10.06	10.05333u
36	Gosu qoraS#110-1	9.95	9.94	9.89	9.92667u
37	Senkele school S#149-4	9.29	9.26	9.24	9.26333v
38	Senkele school S#147-6	9.11	9.13	9.12	9.12000w
39	Farsi S#156-1	8.91	8.89	8.93	8.91000x
40	Dhaga file S#130-2	8.72	8.74	8.76	8.74000y
41	Dhaga file S#128-3	8.52	8.56	8.51	8.53000z
42	Senkele school S#149-7	8.31	8.28	8.3	8.29667A
43	Senkele S#133-2	7.98	8.06	8.01	8.01667B
44	Awaro S#176-1	7.97	7.98	7.99	7.98000B
45	Caka malika dera S#43-1	7.91	8.05	7.93	7.96333B
46	Dhaga file S#128-4	7.07	7.13	7.08	7.09333C
47	Senkele S#132-1	7.04	7.02	7	7.02000C
48	Awaro S#176-3	4.64	4.65	4.68	4.65667D
49	Senkele S#132-13	4.47	4.41	4.45	4.44333E
50	Awaro S#188-1	3.53	3.87	4.09	3.83000F
51	Senkele S#132-12	3.8	3.76	3.77	3.77667G
52	Senkele S#132-3	2.41	2.38	2.41	2.40000G
53	Standard disc	10.01	9.99	10	10.000u

Key: R=replication, Means with the same letter are not significantly different

Table 3: Mean comparison of inhibition zone of actinobacterial isolates against *R. solanacearum* using dual culture method.

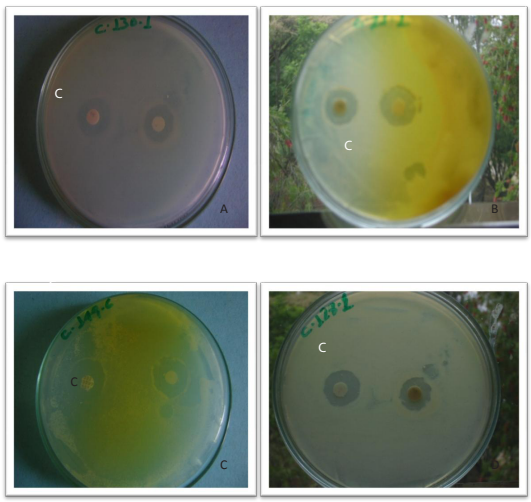

Figure 6: Few Actinobacterial isolates showed best inhibition to the target pathogen under CFS test. A=Isolate S#130-1, B= Isolate S#21-1, C Isolate S#149-6 and D= Isolate S#128-2 C= Chloramphenicol antibiotic standard disc.

same with the results of the dual culture experiment and compared (Table 3).

In both dual culture and cell free suspension test, 21 of isolates such as isolate Gosu qoras#196-1, Awaros#174-2, Senkeles#132-5, Awaros#183-1 and others showed significant differences between actinobacterial isolates (Table 5) and have showed inhibitory activity against the target pathogen and this shows that their inhibitory activity comes from secretion of extracellular antimicrobial compounds as stated by Brock [29]. However, 15 isolates showed very minimal inhibitory activity against the target pathogen and this may indicate that the inhibitory activity during the dual culture test might have come from the competition effect than the secretion of extracellular antimicrobial compounds as the similar work of Brock [29].

Conclusions

This study concluded that the actinobacterial isolate, Gosu-qoraS#196-1 had become superior antagonistic isolate against *R. solanacearum* followed by Awaro S#174-2, Senkele S#132-5, Awaro S#183-1, Senkele S#133-3, Dhaga file S#113-1, Awaro S#176-4, Gabata S#21-1, Dhaga file S#128-2 and Awaro S#174-3 isolates. Therefore, *R. solanacearum* has been controlled successfully by actinobacterial isolates under *in vitro* condition. In this study, the levels of bio control achieved in laboratory condition by the various isolates of actinobacteria has provide reliable, effective, and alternative management approach to the *R. solanacearum*. This study has investigated actinobacterial isolates having antibacterial activity against this target pathogen, from these very few samples indicating that actinobacteria from Ethiopian ecology can be a good potential for investigation of bioactive metabolites for

No.	Isolates/Treatments	Annular Radius of Inhibition Zone Measured (mm)			Mean of Inhibition Zone of annular radius (mm)
		R1	R2	R3	
1	Gosu qoraS#196-1	8.44	8.4	8.41	8.41667a
2	Awaro S#174-2	8.36	8.35	8.37	8.36000b
3	Senkele S#132-5	8.13	8.12	8.12	8.12333c
4	Awaro S#183-1	7.45	7.46	7.47	7.46000d
5	Senkele S#133-3	7.41	7.43	7.44	7.42667e
6	Dhaga file S#113-1	7.38	7.4	7.41	7.39667e
7	Awaro S#176-4	7.12	7.12	7.1	7.11333f
8	Gabata S#21-1	7.08	7.05	7.07	7.06667g
9	Dhaga file S#128-2	6.99	6.98	6.97	6.98000h
10	Awaro S#174-3	6.89	6.9	6.93	6.90667i
11	Gabata S#23-1	6.89	6.9	6.88	6.89000i
12	Dhaga file S#128-1	6.77	6.76	6.76	6.76333j
13	Senkele S#132-2	6.55	6.58	6.53	6.55333k
14	Dhaga file S#130-1	6.36	6.37	6.34	6.35667m
15	Senkele school S#149-6	6.33	6.33	6.3	6.32000n
16	Awaro S#184-1	6.28	6.26	6.29	6.27667o
17	Ambo UniverS#210-1	6.27	6.29	6.26	6.27333o
18	Dhaga file S#113-2	6.25	6.25	6.27	6.25667o
19	Senkele S#133-1	6.11	6.1	6.12	6.11000p
20	Gosu qoraS#205-2	6.09	6.1	6.11	6.10000p
21	Senkele school S#147-3	5.4	5.41	5.43	5.41333q
22	Standard disc	6.5	6.51	6.49	6.50000l
23	Senkele school S#147-7	5.39	5.38	5.4	5.39000q
24	Awaro S#178-1	5.31	5.32	5.34	5.32333r
25	Awaro S#174-4	5.2	5.25	5.18	5.21000s
26	Senkele school S#149-3	5.04	5.09	5.11	5.08000t
27	Bere S#08-2	4.87	4.90	4.92	4.89667u
28	Senkele school S#147-5	4.78	4.77	4.82	4.79000v
29	Malika DeraS#33-1	4.2	4.23	4.27	4.23333w
30	Senkele school S#149-5	3.89	3.94	3.91	3.91333x
31	Senkele school S#149-1	3.22	3.2	3.28	3.23333y
32	Senkele school S#149-2	3.01	2.98	2.99	2.99333z
33	Caka Malika DeraS#54-1	2.42	2.4	2.44	2.42000A
34	Senkele S#132-6	1.2	1.26	1.25	1.23667B
35	Gosu qoraS#201-1	1.02	1.03	1.05	1.03333C
36	Gosu qoraS#110-1	0	0	0	0.00000D
37	Awaro S#184-2	0	0	0	0.00000D

Key: R=replication, Means with the same letter are not significantly different

Table 4: Inhibition effect of actinobacterial isolates against test organism by CFS test.

No	Isolates	Mean of Annular radius of Dual Culture Inhibition Zone (mm)	Mean of Annular radius of CFS Inhibition Zone (mm)
1	Gosu qoraS#196-1	14.94333a	8.41667a
2	Awaro S#174-2	14.50000b	8.36000b
3	Senkele S#132-5	13.90000c	8.12333c
4	Awaro S#183-1	13.55333d	7.46000d
5	Senkele S#133-3	13.52333de	7.42667e
6	Dhaga file S#113-1	13.06333f	7.39667e
7	Awaro S#176-4	13.46667de	7.11333f
8	Gabata S#21-1	13.40333e	7.06667g
9	Dhaga file S#128-2	13.38667e	6.98000h
10	Awaro S#174-3	13.40333e	6.90667i
11	Gabata S#23-1	12.95000gf	6.89000i
12	Dhaga file S#128-1	12.83000gh	6.76333j
13	Senkele S#132-2	12.82667gh	6.55333k
14	Dhaga file S#130-1	12.75667h	6.35667m
15	Senkele school S#149-6	12.61667i	6.32000n
16	Awaro S#184-1	12.46333j	6.27667o
17	Ambo UniverS#210-1	12.31667k	6.27333o
18	Dhaga file S#113-2	12.23667lk	6.25667o
19	Senkele S#133-1	12.13333lm	6.11000p
20	Gosu qoraS#205-2	12.12000lm	6.10000p
21	Senkele school S#147-3	12.09333m	5.41333q
22	Standard disc	10.000u	6.50000l
23	Senkele school S#147-7	11.39333q	5.39000q
24	Awaro S#178-1	11.92667n	5.32333r
25	Awaro S#174-4	11.74000o	5.21000s
26	Senkele school S#149-3	11.68000po	5.08000t
27	Bere S#08-2	11.38667q	4.89667u
28	Senkele school S#147-5	11.55333p	4.79000v
29	Malika DeraS#33-1	11.18667r	4.23333w
30	Senkele school S#149-5	10.41667t	3.91333x
31	Senkele school S#149-1	10.46000t	3.23333y
32	Senkele school S#149-2	11.12667r	2.99333z
33	Caka Malika DeraS#51	10.70667s	2.42000a
34	Senkele S#132-6	11.12000r	1.23667b
35	Gosu qoraS#201-1	10.05333u	1.03333c
36	Gosu qoraS#110-1	9.92667u	0.00000d
37	Awaro S#184-2	11.94333n	0.000000d

Means with the same letters are not significantly different

Table 5: Mean of inhibition zone comparison for actinobacterial isolates showing inhibitory activity both during dual culture and cell free suspension test.

further use. The investigated isolates can be used as one component of integrated disease management practice through detailed further test. More research is also needed on the formulation and delivery of the actinobacteria bio control agent's preparations. Further research on the actinobacterial isolates should be focused on species identification, *in vivo* evaluation and further testing of its host range and disease controlling capabilities in other pathogens. The strong inhibition of actinobacteria to bacterial pathogen, *Ralstonia solanacearum* justifies the need for evaluating its potential as bio control agents for controlling a wider range of pathogens.

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