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Isolation and identification of *Enset* wilt disease causing bacteria using 16S rRNA Gene Sequence samples collected from *Gurage* zone, Ethiopia

Belay TILAHUN[1], Degisew YINUR[2], Dejene ZENABU[2] and Flagot Mola MENESHA[2]

[1]*Institute of Biotechnology, University of Gondar, P. O. Box 196, Gondar, Ethiopia*
[2]*Department of Biotechnology, College of Natural and Computational Sciences, Wolkite University, P. O. Box 07, Wolkite, Ethiopia.*
Corresponding author's Email: belayt21@gmail.com

ABSTRACT

Introduction. *Xanthomonas campestris* is an important bacterium responsible for bacterial wilt disease, which causes predominantly a serious loss in enset production. In some enset-growing areas of Ethiopia, farmers are enforced to replace perennial enset plants with annual crops because of this disease devastates enset production. Aim. Therefore, the study aimed to identify the molecular diversity of bacterial wilt disease causing bacteria from infected enset plants that were collected from the Gurage Zone, using the 16S rRNA gene sequence. Methods. 60 infected enset samples were collected from infected enset plants. Presumptive identification of the bacterium was done through biochemical tests. 16S rRNA genes of bacterial isolates were amplified using the bacteria universal primers and the amplified products were sequenced at MRC-Holland, Amsterdam. Sequence analysis and comparison were conducted to identify the isolated microbes into species and strain levels. Results. Based on the biochemical tests, 18 bacterial isolates were motile, indole negative as well as citrate and catalase positive and they were hydrolyzed starch. The sequence analysis revealed that from 18 bacterial isolates 17 of them were identified as *Xanthomonas campestris* of different strains and one isolate was identified as an uncultured bacterium. In this study, different *Xanthomonas campestris* strains that have different virulence factors were identified in the study area. To effectively control and manage bacterial wilt disease of enset plant, it is important to examine antipathogenic agent or biological control mechanisms for all *Xanthomonas campestris* strains. Additionally, determining plant bacterial interaction using molecular tools and identify the virulence genes are also beneficial.

Original Article

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INTRODUCTION

Xanthomonas campestris is one of the most common bacteria responsible for bacterial wilt disease which attacks and kills enset plants at any developmental stage [1]. It causes predominantly a serious loss in enset production in which the farmer has already invested land, labor, and resources for several years [2]. Such situations have caused farmers to replace enset plants with annual crops in the southern region of the country (Ethiopia). Enset growing regions are densely populated with very small land coverage (average of 0.17 ha). However, such a replacement of enset by annual crops on such a small plot cannot fulfill the food demand of the region [2; 3].

Among the genus *Xanthomonas* bacteria *Xanthomonas campestris* which is rod-shaped, gram-negative bacteria and creamy and light yellow mucoid circular colonies on YDC agar [1] with growth temperature from 25-30 °C, causes the serious disease of enset and banana wilt in the most Africa countries [3]. The disease appears on any part of enset but mostly appears on youngest leaves that show greyish-brown at the tip the plant leaves [1]. In different agroecology, the disease is disseminated and the production of enset has been decreased in the areas where enset is growing and used as a staple food [3]. Thereof, it causes the problem of food insecurity in the enset growing regions of Ethiopia [4].

There are variations among the *Xanthomonas campestris* strains regarding to their pathogenicity which leads to great damage in crop production and makes it more complicated to manage the disease [3-5]. The ddiscrepancy among *Xanthomonas campestri* strains may have different virulent factors and difficult to screen resistance enset clones. Selections of resistant clones are non-reproducible when testing disease resistant enset clones by different *Xanthomonas campestri* isolates. Thus, to develop an effective controlling mechanism of wilt disease which causes by *Xanthomonas campestri* and to screen resistant enset cones, studying the diversity of

Xanthomonas campestris is needed. In order to overcome the bottle neck, this study was planned to assess the molecular diversity of available strain from infected enset plants which were collected in the study area.

MATERIAL AND METHODS

Isolation and selection of bacteria

A total of 60 infected enset samples was collected aseptically from infected enset plants. Those samples were obtained from different enset plant varieties that were found ten kebele of three woredas (Gumere Woreda, Cheha Woreda, and Ezia woreda). Then the samples were transported to Wolkite University Biotechnology Department Laboratory. Then after surface sterilization, 10 mg of tissues from each infected plant samples were transferred into 90 ml sterile 0.1% peptone water containing conical flask and homogenized by vortex for 10-15 min. From appropriate serial dilutions, 0.1 ml Aliquots were spread plated onYDC agar and incubated at 28°C for 48 h. The pure isolates were maintained on YDC agar slants at 4°C and sub-culture every four weeks was done until biochemical and molecular characterization was carried out [6].

Biochemical characterization of bacterial isolates

The morphological characterizations of bacterial isolates were determined according to their cultural characteristics using a microscope [7- 8]. Biochemical characterizations such as gram, oxidase, catalase, citrate, indole, H_2S, VP, citrate, catalase, and methyl red tests) were conducted for each bacterial isolates [9-11].

Starch hydrolysis test

Bacterial isolates were streak plated on nutrient agar plates containing 0.2% soluble starch (w/v) and incubated at 30°C until heavy growth occurred. Then plates were flooded with IKI solution (iodine, 1g; potassium iodide, 2g; distilled water, 100 ml) and the clear zone around a colony was recorded as positive reaction [12].

Amplification of 16S rRNA gene

The genomic DNA of bacterial isolates was extracted using DNeasy DNA Extraction kit, Qiagen [13- 14]. To amplify the 16S rRNA gene of each bacterial isolate, PCR reaction mixtures (50 µl) which contained 1 µl of the extracted DNA, 5 µl dNTPs, 1 µl of each of primers rD1 (5′-AGA GTT TGA TCC TGG CT C AG-3′) and fD1 (5′-AAG GAG GTG ATC CAG CC-3′) [15-16], 1 µl of *Taq* DNA polymerase (Fermentas, St. Leon- Rot, Germany), 5 µl PCR buffer and reverse osmosis purified water were used. The PCR reaction was programed as initial denaturation at 95°C for 60 sec, followed by 35 cycles of denaturation at 94°C for 60 sec, primer annealing at 51°C for 30sec and primer extension at 72°C for 60 sec with a final extension at 72°C for 60 sec. Then, the PCR products of the 16S rRNA gene were separated by gel electrophoresis using 1% agarose gel and 1µL loading dye with 5µL PCR products and stained with ethidium bromide for gel documentation [16-17].

Nucleotide sequencing and sequence analyses

The 16S rRNA gene PCR product of each bacterial isolate was sequenced by automated DNA sequencer (ABI model 377; Applied Biosystems), MRC-Holland, Amsterdam [16].

Phylogenetic analysis

After the raw DNA sequences were edited using the FinchTV package and consensus sequences were obtained. The sequences were compared with the NCBI DNA database using the BLAST search [16- 19]. Then sequences were then aligned using Clustalx 2.1 [19] and phylogenetic tree was constructed using MEGA 7 [16-19].

RESULT AND DISCUSSION

Bacterial isolation and morphological characteristics

From 60 infected enset samples, 18 bacterial isolates were obtained and purified. They were yellowish on nutrient agar, circular in shape, shiny, motile and all of them were also Gram-negative. Most of the isolates had slimy mucoid yellowish, therefore; results were in line with Tsehay [1] and Welde-Micheal et al. [2]. As indicated

in Aritua et al. [20] the bacterium is described as a motile, gram-negative rod, possessing a single polar flagellum and producing typical yellow, convex, mucoid colonies on nutrient agar and YDC media. Regarding on the morphological characteristics or their colony morphology results, 18 bacterial isolates which were resembled *Xanthomonas spp* were selected [20].

Biochemical characterization of bacterial isolates

Motility is one of an important differentiating tools in bacteria, has long been recognized as a biological characteristic of microorganisms [4]. All 18 bacterial isolates were motile and indole negative which was unable to breakdown tryptophan to indole. Likewise, all isolates were citrate positive, which they change the color of the medium (deep green) to blue indicates the ability of to metabolize citrate. Besides, all isolates were catalase positive; produced bubbles when the colony of each isolate was dissolved in a few drops of 3% H_2O_2 which is supported by Haile et al. [4] (Table 1) report. After the plates were flooded with IKI solution clear zone around a colony was recorded as a positive reaction. The clear zone indicates the level of starch hydrolysis capacity of isolates (Figure 1). Potential isolates can produce large clear zone and indicate complete hydrolysis of starch. All isolates were able to hydrolyze starch and the clear zones were also observed.

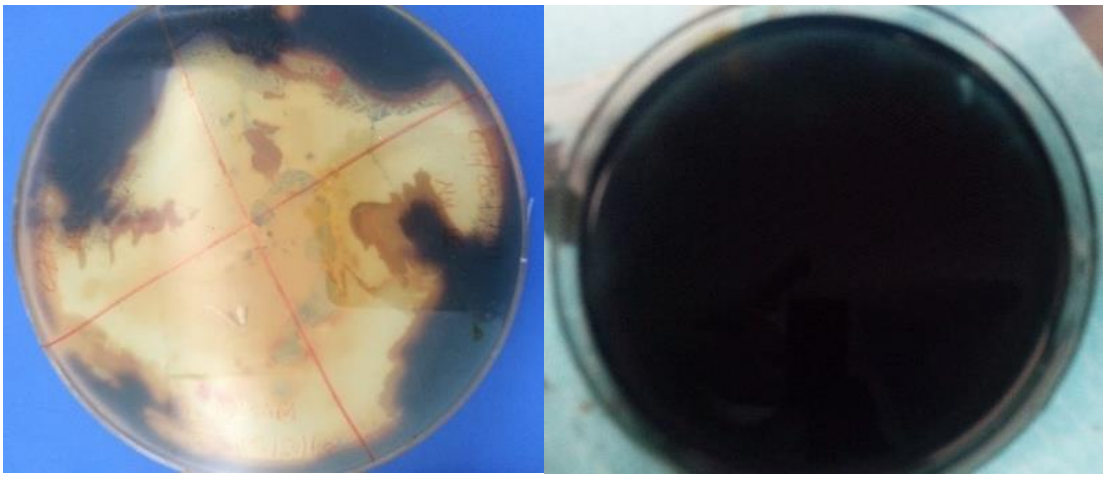

Figure 1. Clear zone observed around bacterial isolates

Molecular Characterization of bacterial isolates

Amplification of 16S rRNA gene of bacterial isolates. The PCR product of all bacterial isolates was shown in Figure 2 and all the isolates were shown to have PCR amplified fragments with around 1500bp size [16].

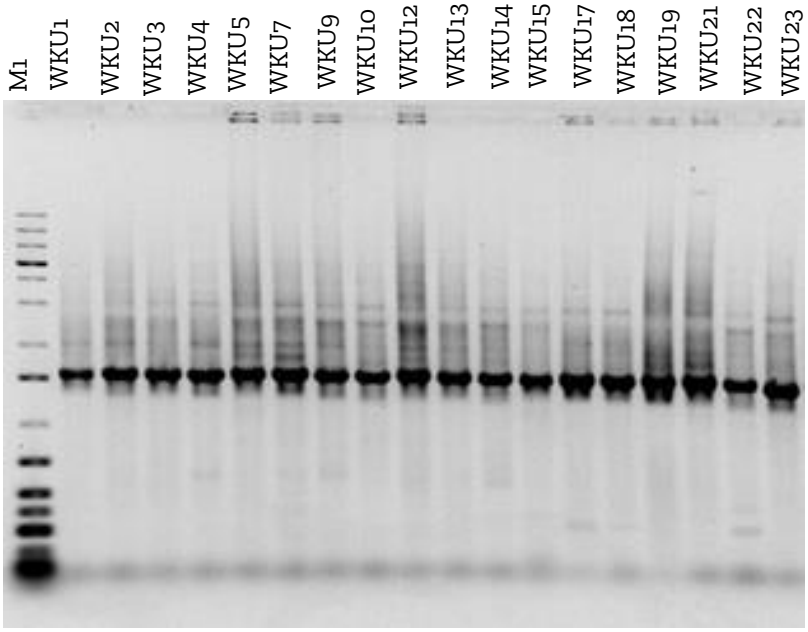

Figure 2. PCR amplification 16s rRNA using rD1 and fD1 bacterial universal primers, M1; 1kb DNA ladder, amplicon size; 1500bp.

16S rRNA gene of bacterial sequence analyses

After all the raw the 16S rRNA gene sequences of 18 bacterial isolates were edited using the FinchTV package, consensus sequences (edited sequence) were blasted in Gen Bank of NCBI. Samples which showed from 92-100% percent of homology were identified as shown in Table 2. According to this study, the seventeen bacterial isolates were identified as *Xanthomonas campestris*, one isolate was identified as *Pseudomonas protegens*, one isolate was also identified as *Bacillus subtilis* and four isolates were also identified as uncultured bacteria.

The seventeen bacterial isolates were found to be the genus Xanthomonas, of which WKU1, WKU3, WKU17 and WKU18 were belongs to *Xanthomonas campestris pv. campestris* XCC-C7 with 100% of sequence homology. WKU2, WKU9, WKU10, WKU13, WKU14 and WKU15 were *Xanthomonas campestris* strain ATCC 33913 with 100% of homology. WKU4 and WKU21 were identified as *Xanthomonas campestris* strain Xan-E1with 92% and 100% of homology respectively.

WKU7 and WKU12 were identified as *Xanthomonas campestris* strain LMG 568; WKU19 and WKU22 were identified as *Xanthomonas campestris* strain PgBe189 and WKU23 were identified as *Xanthomonas campestris* strain with 100% of homology. As Young et al. [21] indicated that the isolates identified as *Xanthomonas* species by Biolog were confirmed as *Xanthomonas campestris* using the GspDm primers, sequence-based phylogenetic tree and pathogenicity tests. The diversity of *Xanthomonas* bacteria in banana (similar to a false banana, enset) which was studied by Studholme et al. [22] has concurred with this study. Hence, identified diversity of *Xanthomonas* within the enset plant, reveals that there has been an evolution of *Xanthomonas* species from time to time.

Maximum likelihood method of Tamura and Nei [19] was used to infer the evolutionary history of identified bacterial species. The highest log likelihood (-1679.15) is shown in the tree. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site as shown in Figure 3. There were a total of 994positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [23].

Molecular identification of *Xanthomonas spp.* from infected enset using 16s rRNA sequence was identified *Xanthomonas campestris* which is a more precise and accurate technique for identification of bacterial isolates. This is supported by Adriko et al., [24] the use of multiple tools for precise identification and characterization of *Xanthomonas* bacteria in bananas and elucidates the benefit of this microbial diversity in the management of bacterial wilt disease. Owing to the variable phenotypic and genotypic characteristics of bacteria, serving for their identification, their diagnosis often requires the use of complementary methods [25].

Table 1. Biochemical characteristics of the bacterial isolates

No.	Sample code	Bacterial isolates	Biochemical tests							
			Gram test	KOH Test	Starch hydrolysis	Citrate	Catalase test	H_2S test	Indole test	Motility
1.	BB9	WKU04	-	+	+	+	+	+	-	Motile
2.	YM18	WKU05	-	+	+	+	+	+	-	Motile
3.	XX1(A)	WKU07	-	+	+	+	+	+	-	Motile
4.	BN7	WKU12	-	+	+	+	+	+	-	Motile
5.	28	WKU02	-	+	+	+	+	+	-	Motile
6.	BB7SH	WKU09	-	+	+	+	+	+	-	Motile
7.	WY1	WKU10	-	+	+	+	+	+	-	Motile
8.	XY5	WKU13	-	+	+	+	+	+	-	Motile
9.	AY13	WKU14	-	+	+	+	+	+	-	Motile
10.	AV13(A)	WKU15	-	+	+	+	+	+	-	Motile
11.	WY3	WKU01	-	+	+	+	+	+	-	Motile
12.	BB10	WKU03	-	+	+	+	+	+	-	Motile
13.	TZ11	WKU17	-	+	+	+	+	+	-	Motile
14.	YN16	WKU18	-	+	+	+	+	+	-	Motile
15.	35	WKU22	-	+	+	+	+	+	-	Motile
16.	XX2	WKU19	-	+	+	+	+	+	-	Motile
17.	29	WKU21	-	+	+	+	+	+	-	Motile
18.	NM16SH	WKU23	-	+	+	+	+	+	-	Motile

Table 2. Identification of bacteria isolated from Enset as revealed by partial 16S rRNA gene sequence BLAST search.

No.	Sample code	Bacterial isolates	Infected Enset type (local name)	E value	% homology	NCBI Accession Number	ID based on NCBI BLAST
19.	BB9	WKU04	Agadi	0	92%	KT156667	*Xanthomonas campestris strain Xan-T1*
20.	YM18	WKU05	Ginbio	0	100%	GU272305	Uncultured bacterium clone CF12
21.	XX1(A)	WKU07	Agadi	0	100%	NR119219	*Xanthomonas campestris strain LMG 568*
22.	BN7	WKU12	Ameratye	0	100%	NR119219	*Xanthomonas campestris strain LMG 568*
23.	28	WKU02	Agadi	0	100%	NR074936	*Xanthomonas campestris strain ATCC 33913*
24.	BB7SH	WKU09	Ankafuye	0	100%	NR074936	*Xanthomonas campestris strain ATCC 33913*
25.	WY1	WKU10	Ankafuye	0	100%	NR074936	*Xanthomonas campestris strain ATCC 33913*
26.	XY5	WKU13	Yshrakinki	0	100%	NR074936	*Xanthomonas campestris strain ATCC 33913*
27.	AY13	WKU14	Yshrakinki	0	100%	NR074936	*Xanthomonas campestris strain ATCC 33913*
28.	AV13(A)	WKU15	Astara	0	100%	NR074936	*Xanthomonas campestris strain ATCC 33913*
29.	WY3	WKU01	Ankafuye	0	100%	MN108237	*Xanthomonas campestris pv. campestris XCC-C7*
30.	BB10	WKU03	Ginbio	0	100%	MN108237	*Xanthomonas campestris pv. campestris XCC-C7*
31.	TZ11	WKU17	Ameratye	0	100%	MN108237	*Xanthomonas campestris pv. campestris XCC-C7*
32.	YN16	WKU18	Astara	0	100%	MN108237	*Xanthomonas campestris pv. campestris XCC-C7*
33.	35	WKU22	Separa	0	100%	MH211280	*Xanthomonas campestris strain PgBe189*
34.	XX2	WKU19	Ameratye	0	100%	MH211280	*Xanthomonas campestris strain PgBe189*
35.	29	WKU21	Separa	0	100%	KT156666	*Xanthomonas campestris strain Xan-E1*
36.	NM16SH	WKU23	Separa	0	100%	EF059753	*Xanthomonas campestris*

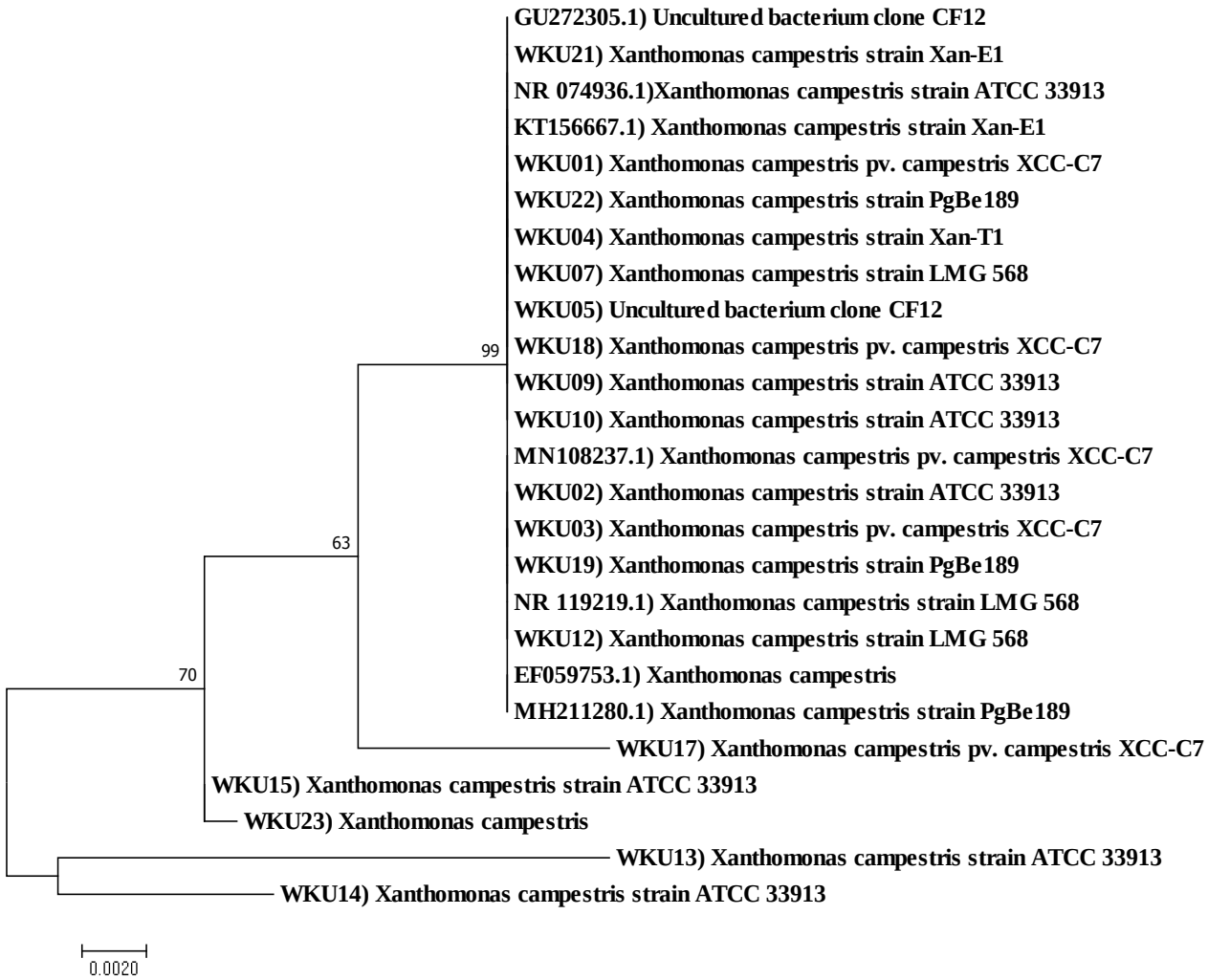

Figure 3. Phylogenetic analysis based 16s rRNA gene sequences of bacterial strains isolated from *Enset*; coded as WKU followed by numbers and reference bacterial strains using the Maximum Likelihood method.

CONCLUSION

In this study, important strains of *Xanthomonas spp* which were found in the study area were identified. Sequencing based phylogenetic highlight relationships and possible interactions among the diverse bacteria populations in a plant system were also recognized. Therefore, the result of this study indicated that various *Xanthomonas campestris* strains are responsible for bacterial wilt disease of *enset* plant. Identification of these isolates could contribute valuable information for the understanding of enset plant pathogenic interaction and to develop controlling mechanisms of the pathogen. Moreover, Understanding the dynamics of *Xanthomonas spp* in enset and could provide insight into the effect of microbial interactions on BXW disease development as well possibly using some of these endophytic bacteria as bio control agents.

DECLARATIONS

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Authors' contributions

B.T. performed all the laboratory works, collected samples and materials, analyzed the data and wrote the manuscript. D.Y. performed molecular laboratory works, wrote and edited the manuscript. D.Z. and F.M.

collected samples, performed some part of laboratory work and involved in data records. All Authors read and agreed on the first manuscript.

Conflicts of interest

The Authors declare no conflict of interest.

Data availability statement

All the data of study are available from the corresponding author.

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