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Isolation and Identification of Bacterial Soft-Rot-Causing Isolates from Cassava, Homa-Bay County, Kenya

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Cassava bacterial soft rot is of great importance to economically valuable crop varieties. Commonly, bacterial soft rot strains cause destructive vegetable and potato diseases worldwide. The strain act similar to other species of the family *Enterobacteriaceae* that cause soft rot in plants. However, the disease has spread in a wide range of environment with its prevalence and causative strains less known to most agroecological zones of Kenya. Generally, aim of this study was to isolate, characterize and identify bacterial soft rot causative strains in cassava, Homa-Bay County. At the Sub County level, 104 farms were identified through a simple random method and symptomatic cassava plants sampled along laid transect in each farm. Rotten cassava tubers were then randomly sampled, packed and taken to the laboratory for pathogen identification. The bacterial strains were isolated in Nutrient Agar at 37˚c for 24 hours to obtain pure cultures. Identification was done using morphological, biochemical and 16S rRNA analysis method. The study identified: two *Pectobacterium carotovorum*, three *Bacillus species: Bacillus amyloliquefaciens, Bacillus subtilis, and Bacillus tropicus,* and three *Achromobacter species: Achromobacter marplatensis, Verticiella sediminum, and Achromobacter xylosoxidans,* pathogenic

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strains causing cassava bacterial soft rot. In this study, characterization and identification of cassava bacterial soft rot in Homa Bay County was done for the first time. The results can not only be used in the characterization enzymes produced by cassava bacterial soft rot strains but also in the development of effective control strategy.

Keywords: 16S-23S rRNA intergenic transcribed spacer region (ITS); restriction fragment length polymorphism (RFLP); polymerase chain reaction; sequences.

1. INTRODUCTION

Diseases caused by *Pectobacterium* and *Bacillus* species are distributed worldwide i.e., occur in Europe, Latin America, North America, Australia, and Africa. Bacterial soft rot is well known in many crops, from potatoes and cassava to ornamental plants. Bacterial soft rot pathogens have been reported in 17 of 56 African countries [1]. Moreover, recent epidemiological studies have shown great spread of bacterial soft rot as it's caused by different species of bacterial pathogen [2]. In Kenya, Counties of Migori and Homa-Bay have recorded incidence of 0.00-54.7% of cassava bacterial diseases [3] [4]. However, it is noticeable that that bacterial diseases occur throughout the year with high incidence during the rainy seasons [4]. Nevertheless, the primary concern was on the identification of cassava bacterial soft rot strains in the cassava farms. *A report on cassava farmers in Homa- Bay County empowerment* highlighted cassava soft rot, among other diseases, as a major challenge affecting the commercialization of cassava production in the areas [5]. The report emphasized that "The diseases may wipe cassava out of production due to their severity and the fact that they cannot be eradicated. Therefore, there was need to establish programs on control to improve the cassava value chain". Contrarily, there was limited information on its distribution and incidences across different agroecological zones and farming systems.

Cassava bacterial soft rot is a bacterial disease majorly associated with *Erwinia carotovora* (*Pectobacterium*) and *Erwinia chrysanthemi* (*Dickey dadantii*) bacterial strains [6]. The disease can also be associated with some Bacillus species: (*B. subtulis, B. mycodes, B. pumilus, B. amyloliquefaciens*, and *B. lichenifomis*) *[7]. Clostridium manihotivorum sp*. Nov, an aerobic bacterium, produces cassava pulp-degrading enzymes that degrade tissues [8] and *Pseudomonas marginalis* [9]. Bacterial Soft rot pathogens infect cassava through fresh wounds of stem cutting then feed on the liquid from the damaged cells on the cut surface, grow and then release the pectolytic enzyme that hydrolyses pectic substances in the middle of the lamella [10]. This pectic enzyme causes maceration of the tissue, thus finding its way to the vascular system [11]. In the tubers, the lesion becomes sunken, water-soaked, swollen, and light to dark brown [12].

There is a wide distribution and variation of pathogen species in the agroecological zones of Kenya [7]. Pathogen characterization help determine the ideal cassava bacterial soft rot causative strains [13]. Consequently, morphological characterization explains bacterial physiology and antigenic features that influence its nutritional, developmental, ecological and heredity functions. However, the method is biased on the ability of bacteria to change their physiology based on changing environmental conditions. Morphological characterization may also underestimate viable organisms which are difficult to grow in *in vitro* cultures [14,15]. Biochemical characterization distinguishes pathogen strains on their nutritional and metabolic capabilities thus, provides an insight into the binding and action mechanism. However, the process may not be accurate, and possible contamination causes an heterogenous culture leading to bias false results [16]. Alternatively, molecular characterization uses molecular markers, including DNA, RNA, and proteins, to determine the genetic characteristics of bacterial cells. This helps in the identification of accessions and to discern genetic relationships among bacterial genotypes thus, help in detecting low amounts of infectious strains and thus effective in detecting pathogenic strains [17].

Therefore, the objective of this study was to characterize the cassava bacterial soft rot pathogens strains isolated from cassava tubers in the different cassava farms of the Rangwe, Kasipul and Ndhiwa Sub-Counties of Homa- Bay County.

2. MATERIALS AND METHODS

2.1 Study Area

Sample collection was done in the cassava farms of Rangwe, Kasipul, and Ndhiwa Sub-Counties of Homa- bay County purposely to identify cassava bacterial soft rot strain in the cassava farms. The three Sub-Counties were selected based on their great contribution to cassava production in the County. Homa-Bay County is located in the former great Nyanza province, around Lake Victoria. The County falls South-West of Kenya and borders Kisii, Nyamira, Migori, Kericho, and Kisumu Counties.

2.2 Data Collection

The global positioning system was used to mark the locations, altitude and longitude of the sampled farms. Samples from cassava farms were taken between the months of April and May 2023 when there were rains and farm survey for cassava bacterial soft rot prevalence done. A simple random method of data collection involved walking along a transect fashion (X-like movement) as described by Curland *et al .* [18] 104 farms were picked with at least 32 farms per Sub-County based on symptomatic cassava plants. In the farms, five plots of approximately 0.5m x 0.5m were determined along the transect with distances between them not less than 5 meter. From each plot, all the plants were assessed for cassava bacteria soft rot disease. Symptomatic plants were uprooted and affected tubers collected and taken to the laboratory for further analysis.

2.3 Isolation of Soft Rot Pathogen Isolates

2.3.1 Media preparation

Nutrient agar was used for the isolation of the soft rot pathogen as used earlier by Abiodun & Abisola, [14].

2.3.2 Isolation of cassava soft rot pathogen strains

Cassava tuber samples affected with bacteria soft rot were prepared for pathogen isolation following the procedure used by Curland *et al .* [18]. Symptomatic soft rot parts were cut, samples washed with tap water to remove the soil debris, immersed in 1% sodium hypochloride (jik) for 1-2 minutes then dried on a sterile filter paper to remove the disinfectant. A piece of the rotted area was then cut and aseptically streaked on the nutrient media for growth. Sterile swabs were used to streak the agar plates and the nutrient agar media was allowed to dry. A total of three plates were inoculated for each sample. After one hour, the plates were incubated at 37˚c for 24 hours in an incubator (Memmert TYP INB200). Colonies characteristics such as smell, growth pattern, shape, and colony colour recorded. The characteristic colonies were reisolated in new plates to develop pure colonies. Purified colonies were then be stored at the temperatures of 4°C awaiting morphological characterization, biochemical and molecular analysis [19].

2.4 Characterization and Identification of Isolated Soft Rot Casual Agents

2.4.1 Morphological Characterization of Isolated Soft Rot Strains

Morphological characterization was exploited as the first level of bacterial soft rot identification. Colony features such as growth characteristics, colour, margin shape and appearance were evaluated. Each colony was compared to the structure for the bacterial strains about the existing taxa in a standard manual of determinative bacteriology as described by Abiodun & Abisola [14]. Microscopy was done using computer software (ImageFocus Plus V2, YM.2013) on laboratory microscope, (BlueBio: German Manufacturers). Lastly, Individual structures of the bacterial cells were noted as either rods, coccus, gram negative or gram negative.

2.4.2 Biochemical characterization of isolated soft rot strains

Biochemical characterization of isolated soft rot strains was done as described by Ganiyu *et al .* [16] and Oliveira *et al.* [20]. Standardized bacterial differential discs (HIMEDIA: REF; DD018-1VL) were used for oxidase test. Observations were then recorded as either positive (+) or negative (-). Positive represented colonies that were able to form either a purple ring around the disc or turn the disc purple and negative for no purple colour change on the discs after 60 seconds. For the catalase test, two drops of hydrogen peroxide were added to the surface of 48 hours-old culture slide smear of each isolate on

nutrient agar medium. Quick bubble formation was recorded as positive for catalase activity with no bubbles as negative.

Starch analysis was done as described by Ganiyu *et al .* [16] nutrient starch agar media was prepared as: nutrient agar (5.6 g), starch soluble (0.4 g), water (200 ml), and pH (7.0). The media was then dissolved and sterilized at 121^0C for 15 minutes at a pressure of 0.12 MPa and poured into sterilized Petri plates. The medium was allowed to solidify and inoculated with the test organism. The plates were incubated for 24 hours and tested for starch hydrolysis. The agar surface was flooded with Lugol's iodine and allowed to act for two minutes to develop a colourless zone around the bacterial growth. The formation of clear colourless zone was recorded as positive.

Lauryl Tryptose Broth (LTB. CM0451), a selective growth medium (broth) for coliforms was prepared for indole test and fermentation gas production. The test was done along The APHA[3] recommendations for detection of coliform in food samples. This followed the description by Oliveira *et al .* [20] . 10.7g of LTB was dissolved in 300ml of distilled water, sterilized by autoclaving at 121°C for 15 minutes, cooled and distributed in fermentation test tubes. The test tubes were inoculated with two loopful cultures then incubated at 37°C for 28 hours in an incubator (Memmert TYP INB200) and later the presence of indole was directly tested. Using a micropipette set at 0.5ml calibration, the Kovac's reagent was delivered directly into the aliquot. Observations were immediately made on the formation of a colour of the rings in the interface.

MR-VP Broth (Glucose Phosphate Broth) media was used for the performance of IMViC test, methyl red test. 14.28 g of MR-VP Broth (HIMEDIA) was dissolved in 400ml of distilled water, autoclaved at 121°C for 15 minutes, cooled and distributed in test tubes. The tests tubes were then inoculated with two loopful of cultured bacteria, and reincubated at 37°C for 48 hours in an incubator (Memmert TYP INB200). 0.02% methyl red solution was prepared as: 0.033g of methyl red solution was dissolves with 100 ml of ethyl alcohol (95%) and distilled water used to make 200 ml mark. 2-3 drops of methyl red solution were added to the aliquot and the change in colour observed immediately.

2.4.3 Molecular Identification of Isolate Groups

2.5 DNA Extraction

A total of six isolates, a representative from each group of isolates identified based on morphological and biochemical markers, were used for molecular identification as described by Domitila *et al .* (2022). A 10 mL of mid-to late-log-phase cultures of the six isolates (0.5 – 0.7 at OD600) were transferred to a falcon tube and the cells pelleted through centrifugation at 7,500 rpm for 10 minutes. Thereafter, the supernatant was discarded. The pellets were then resuspended with 467 µL RNase A in Buffer P1 and transfer to a 1.5-mL microcentrifuge tube, 8 µL lysozyme and 5 µL achromopeptidase were added, gently mixed and incubated at 37°C for 60 minutes. This was followed by addition of 30 µL 10% SDS (sodium dodecyl sulfate) and 3 µL proteinase K, then gently inverted and incubated at 50°C for 60 minutes. Thereafter, 525 µL PCI (Phenol:Chloroform:Isoamyl) solution was added and mixed for 10 minutes by gentle inversion followed by centrifugation at 12,000 rpm for 15 minutes. The upper aqueous phase was then transferred to a sterile 1.5-mL microcentrifuge tube, with care taken not to disturb the bilayer. After which, an equal volume of −20°C 100% ethanol was added, gently mixed by inversion and afterwards centrifuged at 12,000 rpm for 20 minutes. Lastly, the supernatant was carefully decanted and the pellets thoroughly dried in a 50°C incubator for 3 hours. The pellets were then resuspended in 50 µL TE (Tris-EDTA) buffer and allowed to sit overnight at 4°C. Confirmation of the presence and concentration of bacterial DNA was done by running 5 µL of product on a 1% agarose gel.

2.6 Polymerase Chain Reaction

Characterization of the soft rot strains was based on the 16S-23S rRNA intergenic transcribed spacer region (ITS) and the restriction fragment length polymorphism (RFLP) profiles. The DNA genomes extracted were amplified using universal primers [Forward primer (5' AGA GTT TGA TCC TGG CTC AG 3')] which amplify the pectate lyase gene in the soft rot strains. Polymerase Chain Reaction amplifications were done using a Thermal-cycler (Bio-Rad: Model 680 XR reader, Made in China). The reactions were done in a total volume of 50µl with 0.4µM for the forward primer, 200 µM dNTPs (Fermentas) and 0.5 U Dream Taq DNA

polymerase (Fermentas) and 1 × Taq DNA polymerase reaction buffer (Fermentas). The PCR reaction was performed following the thermal profile; initial denaturation at 94ºC for 2 minutes, 35 amplification cycles at 94ºC for 45 ºC, 55 ºC for 1 minute, 72 ºC for 1 minute, and the final extension at 72 ºC for 10 minutes. All the PCR products were separated using 1% agarose gel and further digested *Cfoi* and *RsaI* restriction enzymes (Promega, Corp.) to generate the characteristic RFLP profiles. Purified DNA appeared as a defined band when visualized under UV light as described by Domitila *et al* . (2022).

2.6.1 Sequencing

For the sequence analysis, primers targeting the amplification of the soft rot strains genes, glyceraldehydes-3-phosphate dehydrogenase A (*gapA*) and malate dehydrogenase (*mdh*) were used. The total DNA genome previously extracted, *gapA326F* and *mdh 2* were together used to perform a PCR reaction in a final mixture of 50µl with 0.4µM for the forward primer, 200 µM dNTPs (Fermentas) and 0.5 U Dream Taq DNA polymerase (Fermentas) and 1 × Taq DNA polymerase reaction buffer (Fermentas) targeting the *gapA* and *mdh* regions. The thermos cycler conditions were: initial denaturation at 94ºC for 2 minutes, 35 amplification cycles at 94ºC for 45 ºC, 55 ºC for 1 minute, 72 ºC for 1 minute, and the final extension at 72 ºC for 10 minutes. All the PCR amplicons were size separated using 1% agarose gel in 1 × TAE buffer. Thereafter, The PCR DNA amplicons were cleaned using a commercial kit (Canvax, P.E. buffer). Direct unidirectional sequencing was done using ABI 3730xl Genetic analyzer at ILRI then the raw sequences edited using MEGA X version 11 software.

2.6.2 Sequences analysis

The new sequences obtained together with the blasted strain sequences in the NCBI GenBank were aligned using the MUSCLE program in MEGA X version 11 software before fitting them into the jModel test for a suitable model. The Clustal W. Distances were generated using Neighbour-Joining (Balanced Minimum Evolution Criterion) for multiple alignment. Finally, bootstrapping was done to 1000 replications to bring consistency to the data collected to assess support for each clade.

3. RESULTS

3.1 Morphological Characterization of Cassava Bacterial Soft Rot Pathogen Strains

All the isolates were able to grow on nutrient agar at 37°C after 24 hours but only thirty-one isolates had positive cultures for cassava bacterial soft rot. In the initial growth, the cassava bacterial colonies would appear whitish before turning cream or yellowish with foul smell. Colonies had either rapid, medium or slow growth with a fine or course texture. Majority of the cultures had rapid growth with smooth colony texture. Again, the variation in texture, growth rate and margin appearance was not much evident on Nutrient Agar. In addition, there was no significant variation in color (cream- white) of the colonies except for the ND11 and R06 which appeared cream yellow (Table 1, Plate 1). The individual bacteria had rod- shape peripheral flagellates which were either gram-positive or gram- negative.

Plate 1. Morphological structures of the isolated bacterial strains: A. smooth shinning white strain. b and c. cream-coloured strains

Table 1. Morphological characterization of isolated strain of cassava bacterial soft rot from samples collected

Isolate	Growth characteristics	Colony texture	Colony color	Colony Margin
[1]RG-01	Medium	Smooth shinny	Cream-white	Irregular
[1]RG-02	Rapid	Smooth shinny	Cream-white	Irregular
[1]RG-03	Medium	Smooth shinny	Cream-white	Irregular
[1]RG-04	Rapid	Smooth shinny	Cream-white	Irregular
[1]RG-05	Rapid	Smooth shinny	Cream-white	Irregular
[1]RG-06	Medium	Rough shinny	Cream-white	Smooth
[1]RG-07	Rapid	smooth shinny	Cream-white	Irregular
[1]RG-08	Medium	smooth shinny	Cream-white	Smooth
[1]RG-09	Rapid	Smooth shinny	Cream-white	Irregular
[1]RG-10	Rapid	Smooth shinny	Cream-white	Irregular
[2]ND01	Medium	smooth shinny	Cream-white	Irregular
[2]ND02	Rapid	Smooth shinny	Cream-white	Irregular
[2]ND03	Medium	Smooth shinny	Cream-white	Irregular
[2]ND04	Rapid	Smooth shinny	Cream-white	Irregular
[2]ND05	Medium	Smooth shinny	Cream-white	Irregular
[2]ND06	Rapid	Smooth shinny	Cream/yellow	Irregular
[2]ND07	Rapid	Smooth shinny	Cream-white	Irregular
[2]ND08	Medium	Course shinny	Cream-white	Irregular
[2]ND09	Medium	Smooth shinny	Cream-white	Irregular
[2]ND10	Medium	Smooth shinny	Cream-white	Irregular
[2]ND11	Medium	Smooth shinny	Cream yellow	Irregular
[3]KA01	Rapid	Smooth shinny	Cream-white	Irregular
[3]KA02	Rapid	Smooth shinny	Cream-white	Irregular
[3]KA03	Rapid	Smooth shinny	Cream-white	Irregular
[3]KA04	Slow	Smooth shinny	Cream-white	Irregular
[3]KA05	Medium	Smooth shinny	Cream-white	Irregular
[3]KA06	Rapid	Smooth shinny	Cream-white	Irregular
[3]KA07	Medium	Smooth shinny	Cream-white	Irregular
[3]KA08	Rapid	Smooth shinny	Cream-white	Irregular
[3]KA09	Rapid	Smooth shinny	Cream-white	Irregular
[3]KA10	Medium	Smooth shinny	Cream-white	Irregular

*Individual morphological features observed on pure cultures made on nutrient agar after 24hours of incubation. [1]Rangwe, [2]Ndhiwa, [3]Kasipul. *Growth: Rapid= formation of thick colony, Slow= thin colony. Smooth= wet surface, Rough= dry surface. Irregular= serrated.*

3.2 Biochemical Characterization of Cassava Bacterial Soft Rot Pathogen Strains

To support further identification using molecular studies, biochemical tests done shown precise nutritional description of the isolated pathogens (Table 1).

3.3 Molecular Characterization of Cassava Bacterial Soft Rot Pathogen Strains

The representative isolates were selected from each group based on the similar phenotypic characteristics preliminarily identify using Bergey's Manual of Systematic Bacteriology (Ninth edition), Enterobacteriaceae IDflow chart. The isolate B1, B2, B3 B4 and B5 formed bands between 1000 and 1500bp bands from 16S rRNA amplification (Plate 2). It was observed that the DNA isolated from the six representative strains had good intensity and purity. The quality was checked by running in 1% agarose gel and observation under UV transilluminator. Thus, the protocol optimized for bacterial DNA isolation from all the six samples was effective.

Percentage similarity of the cassava soft rot pathogen isolates and the isolated published in the NCBI data base ranged 80 – 95% (Table 3).

4. DISCUSSION

In the field, affected cassava plants appeared as premature wilting and drying of the uppermost leaves moving down to the lower leaves. In most farms, the symptoms advance to appearance of a line of watery decay that extended to the stem and appearance of a slimy soft rot around the plant base whorl. There were no external symptoms in the soil for bacterial soft rot and the symptoms prevailed upon uprooting the tubers. Affected tubers had foul smell and darkening of the affected areas. Brown or grey strikes formed around the vascular tissues of the root tubers.

Table 1. Biochemical characterization on the iMVC tests results

Isolate	Gram[a]	Grow[b]	SHT[c]		CT[d]	OT[e]	MRT[f]	IT[g]	GP[h]
RG-01	-	+	+	+	+	-	-	+	+
RG-02	-	+	+	+	+	-	+	-	-
RG-03	-	+	+	+	+	-	-	+	+
RG-04	-	+	+	+	+	-	-	+	+
RG-05	-	+	+	+	+	-	+	-	-
RG-06	+	+	+	+	+	-	+	+	+
RG-07	-	+	+	+	+	-	+	-	-
RG-08	-	+	+	+	+	-	+	-	-
RG-09	-	+	+	+	+	-	-	+	+
RG-10	-	+	+	+	+	-	+	+	+
ND-01	-	+	+	+	+	+	-	-	-
ND-02	-	+	+	+	+	-	-	+	+
ND-03	-	+	+	+	+	-	-	+	+
ND-04	-	+	+	+	+	-	+	+	+
ND-05	-	+	+	+	+	+	-	-	-
ND-06	+	+	+	+	+	-	-	+	+
ND-07	-	+	+	+	+	+	-	-	-
ND-08	-	+	+	+	+	+	-	-	-
ND-09	-	+	+	+	+	-	-	+	+
KA-01	-	+	+	+	+	+	-	-	-
KA-02	-	+	+	+	+	+	-	-	-
KA-03	-	+	+	+	+	-	+	+	+
KA-04	+	+	+	+	-	-	+	+	+
KA-05	+	+	+	-	-	+	+	-	-
KA-06	-	+	+	+	+	-	+	+	+
KA-07	-	+	+	+	+	-	+	+	+
KA-08	-	+	+	+	+	-	+	-	-
KA-09	-	+	+	+	+	-	+	-	-
KA-10	-	+	+	+	+	-	+	-	-
Co1	-	+	+	+	+	-	+	+	+
Co3	-	+	+	+	+	-	+	+	+
No	-	+	+	+	+	-	+	-	-
Gr 1 a.	-	+	+	+	+	-	+	-	-
Gr 1 b.	-	+	+	+	+	-	-	+	+
Gr 2a	-	+	+	+	+	+	-	-	-
Gr 2b		+	+	+	-	-	-	-	-
Gr3a	+	+	+	+	+	+	+	*	*
Gr 3b	+	+	+	-	-	+	+	*	*
Gr 4	-	+	+	+	+	-	-	+	+

Biochemical Characterization on the iMVC tests and growth on starch medium. Positive test (+). Negative test (-).
[a]Gram reaction, [b] Growth at 37^{0}C, [c] Starch hydrolysis test, [d] Catalase test, [e] Oxidase test, [f] Methyl red test, [g] Indole test, [h] Gas production. Referenced isolates; Gr-Group.
** Results given according to Kreigh and Holt (1984) from Bergey's Manual of Systematic Bacteriology (Ninth edition), Enterobacteriaceae IDflow chart.*

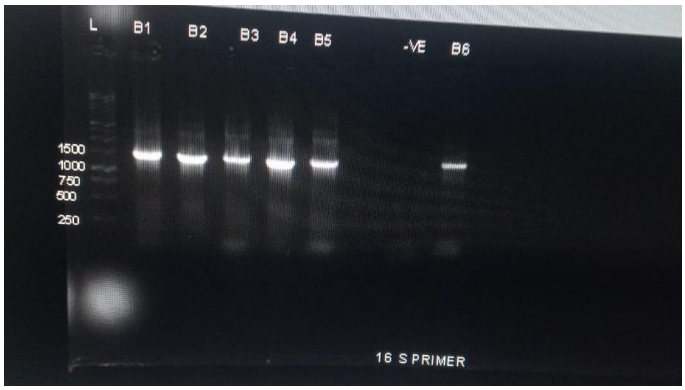

Plate 2. Amplification from all cassava soft rot pathogens isolated from cassava samples obtained from Homabay county in Kenya using 1500bp ladder. Referenced isolates; B1=K03, B2=ND01, B3=R06, B4=ND11, B5=ND06, B6=R01

Table 3. Percentage similarity of the cassava soft rot pathogen isolates and the isolated published in the NCBI data base

Pathogen Isolates	Organisms in the NCBI	Similarity (%)	Origin
B2-16S-F09	[1]NR_119367.1	96.89	Germany
	[2] NR_125539.1	98.74	USA
	[3] NR_041971.1	97.24	Korea
	[4] NR_118855.1	97.81	Germany
B6-16S-F09	[5] NR_116022.1	84.78	Taiwan
	[6] NR_118950.1	84.87	U.K
	[7] NR_102783.2	84.87	France
	8 NR_116017.1	85.20	Taiwan
	[9] NR_113265.1	85.20	Japan
	[10] NR_113265.1	85.20	Japan
	[11] NR_112116.2	85.20	Japan
	[12] NR_118972.1	85.20	U.K
	[12] NR_157736.1	91.07	China
B5-16SF-09	[13] NR_133951.1	92.88	China
	[14] NR_117614.1	94.99	Belgium
	[15] NR_044925.1	93.89	Germany
	[17] NR_117706.1	95.78	Belgium
	[19] NR_113733.1	94.13	Japan
	[20] NR_113732.1	94.13	Japan

[1]Pectobacterium carotovorum strain LMG 2404, [2]Pectobacterium actinidiae strain KKH3, [3]Pectobacterium carotovorum strain DSM 30168, [4]Pectobacterium carotovorum strain ATCC 15713, [5] Bacillus amyloliquefaciens strain BCRC, [6] Bacillus amyloliquefaciens DSM 7 = ATCC 23350, [7] Bacillus subtilis subsp. subtilis strain 168, [8] Bacillus subtilis strain BCRC 10255, [9] [10]Bacillus subtilis strain JCM 1465, [11]Bacillus subtilis strain IAM 12118, [12] Bacillus subtilis strain NCDO 1769, [12] Bacillus tropicus strain MCCC 1A01406, [13] Verticiella sediminum strain XH089, [14] Achromobacter marplatensis strain R-46660, [15] Achromobacter xylosoxidans strain Hugh 2838, [17] Achromobacter insuavis strain LMG 26845, [19] Achromobacter xylosoxidans strain NBRC 15126, [20] Achromobacter denitrificans strain NBRC 15125

Studies by da Cunha Ferreira *et al .,* [17] found that cassava bacterial symptoms occur at various developmental stage of the disease. Similarly, some of the observed symptoms were previously reported by da Cunha Ferreira *et al .,* [17] and Ikeogu *et al* ., [11] but these studies reported other symptoms like blackening of the bark, yellowing of the lower leaves and as the plant grows to maturity the symptoms become more destructive. Studies by Veena *et al* ., [21] Osdaghi, (2023) and Huang *et al* ., [22] also found that in early stages of development, the central pith darken and soften causing disintegration of tissues. Laemchiab, [23] and Thakur & Shyam [24] also report similar results but found that root rot may produce soft rot with

no foul smell. Therefore, findings of the current study supports the current literature since the symptoms in the farms were almost similar to those reported in other regions and crops affected by bacterial soft rot. Moreover, it was important for the farmers to know the symptoms of cassava soft rot to help develop proper management and communicate such symptoms to help them get solution to the disease.

Enterobacteriaceae members use organic molecules from the rhizosphere to improve their growth character and colonize either plant surfaces or intercellular space of the root cortex. The current study was able isolated cassava bacterial soft rot strains from the surface of infected cassava tuber tissues. Similarly, Veena *et al* ., [21] found that bacterial genera *Erwinia , Bacillus, Pseudomonas, Serratia, Azotobacter, Azospirillum, Micrococcus and Arthrobacter* can be isolated from surface-disinfected plant tissues. The study also suggested that Enterobacteria reside inside plant almost in their entire lifecycle causing detrimental effects to the host plant. However, some endophytes have no impacts to the host plant.

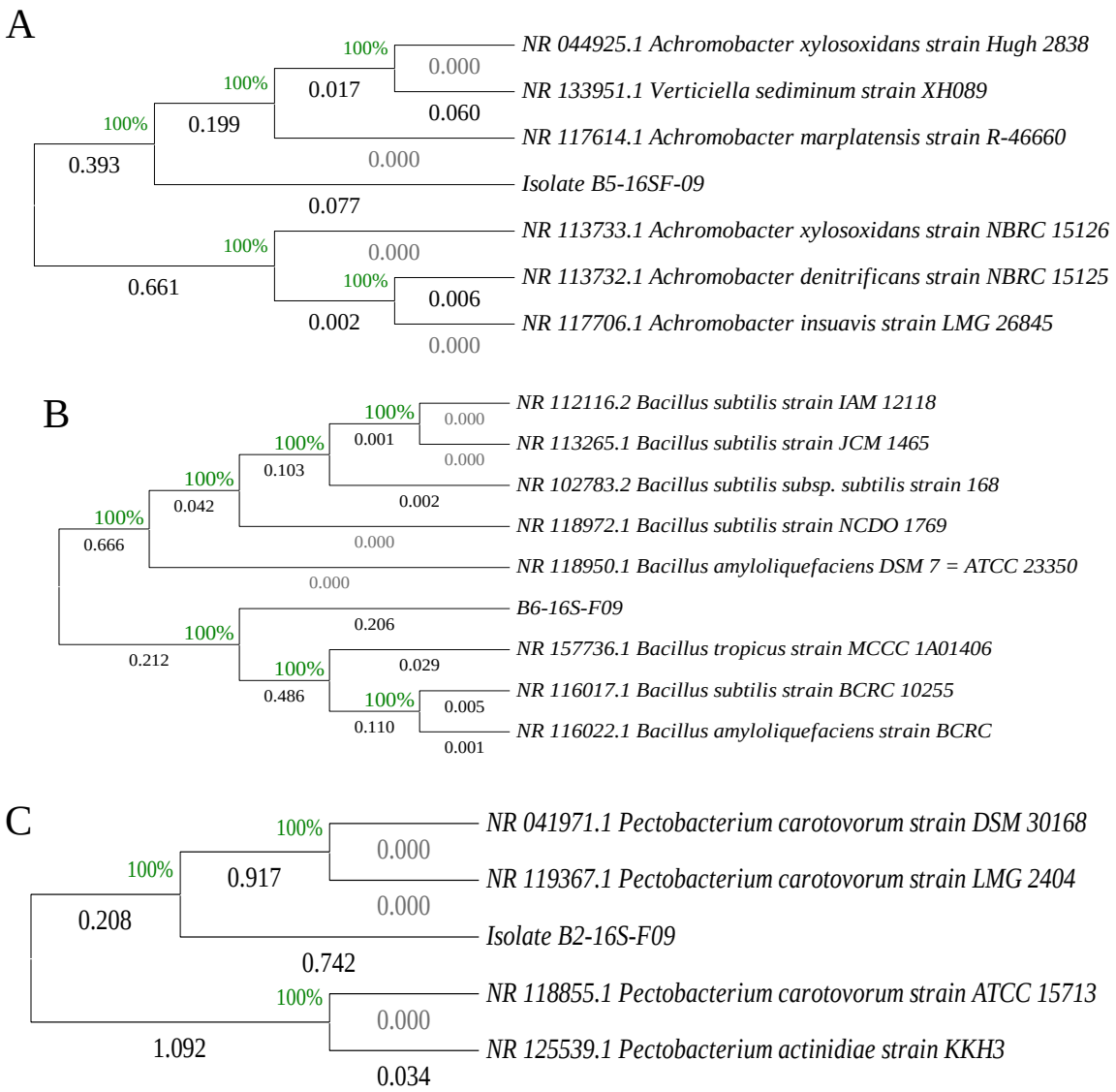

Fig. 1. Phylogenetic analysis of nucleotide sequences of soft rot bacteria pathogen of cassava
isolate B5-16S-F09 (A), B6-16S-F09 (B) and isolate B2-16S-F09 (C) diseased cassava collected from Homabaya County in Kenya. The branching pattern was generated by the Neighbor-Joining method with 1000 bootstrap replication in MEGAX version 11 software.

Report by Etesami & Adl, [25] also found that the pathogens indirectly increase the phytopathogenic effects to the host plants and resulting directly to impaired plant metabolism and reduced defences-related enzymes such as chitinase and β-1,3-glucanase, an increase in endogenous stress-related ethylene (ET). In addition, Nazli, *et al* ., [26] found that the reduction of phytohormones such as Indole-3-acetic acid reduce absorption of water in the xylem tissues and enhance the development of the phytopathogens thus leading to plant stress and tissue maceration. Therefore, bacterial soft rot strains can be isolated both in the xylem tissues of symptomatic plants and well as macerated areas in tubers but it is best evident in soft rot tissues of plant since the pathogen would be at its mass developmental stage.

After 24 hours of inoculation and incubation in nutrient agar, colonies appeared a small pale yellow or cream-white coloured with irregular margins and different diameters. The findings showed the development of bacterial colonies of different growth patterns and rates. However, the variability was hardly noticeable. Microscopic analysis showed twenty- seven, gram negative, rod-shaped peripheral flagellates and only four-gram positive rods .The finding of the study by Huang *et al* ., [22] were consistent with the morphological results in the current study. The study reported on the adaptative nature of the bacterial strains and gave small variability in the colonies and individual cells were hardly noticeable, furthermore, these changes were associated with the changes in the climatic conditions. In addition, García-Bonillo *et al* ., [27] and Tsai *et al* ., [28] found bacterial morphological similarities and related it to the increased immobilization of bacterial colony film upon the chain in conditions of growth.

The microscopic pink/red rods were concurrent with that of [29] identification of *Pectobacterium.* In addition, the evidence of the gram positive, green rods form a tandem with results by Dhahir & Ahmed, [30] who reported on Enterobacter cloacae causing soft rot disease on potato in Iraq. However, Jana *et al* ., [31] found that a single species of soft rot strain can only change gram stain characteristic when exposed to adverse conditions. Cassava bacterial soft rot is caused by a number of both gram positive and gram-negative bacteria and there is a large morphological similarity among the bacterial strains. Heterogeneity of a single phytopathogen attribute to deviation in the colour of the individual bacterial cell due to adaptability needs. Thus, bacterial identification is not much evident through morphological characterization and further characterization should follow such identification.

Majority of the cassava bacterial isolates had positive catalase test; thus, all the isolates were aerobic except KA04 and KA05. However, all the isolates had the ability to hydrolyse amylose and amylopectin leading to the formation of a colourless zone around the bacterial cultures. Isolates ND01, ND05, ND07, ND08, KA01 , KA02 and KA05 had positive oxidase result. Isolates R01, R03, R04, R06, R09, R10, ND02, ND03, ND04, ND06, ND09, KA03, KA04, KA06 and KA07 produced the red ring for indole test. Isolates R02, R05, R06, R07, R08, R10, ND04, KA03, KA04, KA05, KA06, KA07, KA08, KA09, KA10 had positive MR results. Hydrolases and catalases are major enzymes that play a significant role in enhancing pathogen virulence. This create the need to test for the presence of enzymes that can lead to tissue invasion and rot as evident in cassava bacterial soft rot.

Studies by Pontes *et al* ., [32] Verma *et al* ., (2022) and Osdaghi, (2023) found that pathogenic strains have numerous hydrolases and catalase that react affecting central metabolism of the plant and tuber tissues. The current study is in tandem with other publications by Pontes *et al* ., [32] Verma *et al* ., (2022) and Peivastegan, [33] indicating that the presence of a- amylase and oligo-1,6-glucosides breakdown starch into maltose In addition, these studies found that cytochrome- C containing enzymes contain intercellular oxidase that help the strains to transport electrons from NADH to usual oxygen. This was tested with , N, N, N', N'-tetramethyl-p-phenylenediamine dihydrochloride discs receptors and the test and results aligned with procedure and finding of the current study. Pontes *et al* ., [32] also found that virulence ability of the bacterial isolates are highly dependent on the ability of molecules to induce signal that could coordinate bacterial behaviour and this explained ability of the bacteria to colonise and cause effect on the host tissue by production of indole. In addition, possibility of glucose fermentation help in detecting fermentation pathway used by a bacterium isolates. The change in the chemical of starch caused a change in colour from yellow to red resulting from change from glucose, pyruvic acid, a mixed acid pathway, produced lactic acid [34]. Thus, the current study associated the

biochemical characteristics of the isolated strains to the ability of the pathogens to produce soft rot. This would help in the identification of the strains responsible for soft rot.

The molecular analysis revealed that the isolated cassava soft rot bacteria pathogen, labeled as B6-16S-F09, exhibited a striking resemblance to various strains within the *Bacillus* genus, particularly *Bacillus amyloliquefaciens, Bacillus subtilis*, and *Bacillus tropicus*. Surprisingly, a contrasting observation was made for another isolate, B5-16SF-09, which shared genetic similarities with distinct species including *Achromobacter marplatensis, Verticiella sediminum,* and *Achromobacter xylosoxidans*. B2-16SF-09 had great resembles to *Pectobacterium carotovorum* strain DSM 30168 and *Pectobacterium carotovorum* strain LMG 2404.

This intriguing association of *Bacillus* spp. with plant diseases contradicts the typical trend where they have demonstrated potential as biocontrol agents [35,36,37]. In fact, previous studies have documented several of these species as being phytopathogenic [38,39,40]. Li *et al* ., 2009). For instance, *B. macerans* was found to cause decay in flax and wheat roots [38] while *B. pumilus, B. subtilis*, and *B. polymyxa* were linked to postharvest soft rot in vegetables [41]. Additionally, *B. pumilus* was identified as a causal agent of postharvest decay in garlic cloves [42]. potato tuber soft rot during storage [43,44] and even the soft rot disease in pine seedlings and wetwood disorder in young Scot's pine trees [45]. Notably, *B. amyloliquefaciens* has been associated with bacterial rot in onions [46] black rot in arrowhead (Zhong, *et al* ., 2015), and soft rot in potato tubers within agricultural fields [47]. However, no earlier report was available that links some of the pathogen identified here to soft rot of cassava.

Bacteria, *Achromobacter xylosoxidans* that was isolated in cassava in this study have been reported to associated with soft rot diseases like that of *Amorphophallus konjac* in China (Wei, *et al* ., 2023). Consequently, *Achromobacter xylosoxidans* was isolated from edible mushroom *Coprinus comatus* with serious rot disease on its stipe in Japan [48]. However, most of the *Achromobacter* plant phytopathogens occur a soil and environment opportunistic pathogens found on the sample surfaces [49] and again literature linking *Achromobacter spp* to soft rot of cassava is scanty.

Largely, *Pectobacterium carotovorum* have been associated with bacterial soft as a primary pathogen. *Pectobacterium carotovorum* isolated in the current study have similarities and reported similar phytopathogenic activities. However, this has been isolated and identified in other crops but not cassava. For instance, *Pectobacterium carotovorum* has been isolated from lettuce, (Cinisli, *et al* .., [50] and as soft rot in potato [51,52,53] while *Pectobacterium actinidiae* have been reported to cause devastating effect on kiwifruit in Jiangxi, Eastern China (Yan, *et al* .., [54]. Therefore, molecular characterization provided an ideal method of pathogen strains identification and also showed the phylogenetic relationship and clustal distance between related strains [55,56].

5. CONCLUSION

Cultural and biochemical characterization could not precisely identify the causative strains of cassava bacterial soft rot in the regions of study. Upon molecular analysis *Bacillus amyloliquefaciens, Bacillus subtilis*, and *Bacillus tropicus. Achromobacter marplatensis, Verticiella sediminum,* and *Achromobacter xylosoxidans. Pectobacterium carotovorum strain* DSM 30168 and *Pectobacterium carotovorum* strain LMG 2404 strains were identified to be associated with cassava bacterial soft rot. However, the variation was not significant at the Sub-Counties of study.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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