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Isolation and Identification of Antagonistic Rhizobacteria and *In vitro* Evaluation of their Effectiveness against Postharvest Fungal Rot Pathogens of Apple (*Malus domestica*)

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Authors' contributions

This work was carried out in collaboration among all authors. Authors OJF, OKE and SS conceptualized the study. Authors OJF, OOE, AOF and KCJ contributed to the methodology. Authors OJF and OKE conducted the analysis and interpretation of the data. Authors OJF, OOE and OCC wrote the original manuscript. Author OKE reviewed and edited the manuscript. Funding and resources were provided by all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Isolation and identification of rhizospheric bacteria with antifungal potential against four postharvest rot fungi of apple fruits were investigated.
Study Design: Completely Randomized Design (CRD) laid out in 10 (rhizospheric bacteria**)** by 4 (rot fungi) factorial, replicated three times.
Place and Duration of Study: Microbiology Laboratory, Department of Biological Sciences, Bells University of Technology, Ota Ogun State, Nigeria, between 2022 and 2023.
Methodology: Rhizobacteria were isolated from soils using serial dilution and pour plate method. The bacteria were identified using morphological, biochemical, and molecular approaches targeting16S rRNA partial gene sequences. Antagonistic activity of the bacterial isolates were assessed *in vitro* against *Lasiodiplodia theobromae* (Lt)*, Fusarium equiseti* (Fe)*, Pennicillium* species (Ps) and *Curvularia aeria* (Ca) isolated from apple fruits using dual culture procedure. Data were collected on percentage inhibition of radial growth (PIRG). The PIRG data were analyzed using analysis of variance (ANOVA).
Results: Ten rhizobacteria viz: *Pseudomonas lactis*, *Neobacillus kokaensis*, *Paenibacillus pabuli*, *Cryseobacterium indologens*, *Calidifontibacillus erzurumensis*, *Rossellomorea marisflavi*, *Bacillus aerius*, *Brevibacillus reusreri*, *Stenotrophomonas pavanii* and *Cytobacillus firmus* were isolated. The inhibitory potential of these rhizobacteria against postharvest rot fungi varied (p =.01), with *Calidifontibacillus erzurumensis* producing highest inhibition of radial growth of Fe (74.5±16.2%), Lt (60.0±4.7%), Ps (60.6±5.2%) and Ca (52.9±5.9%).
Conclusion: *Calidifontibacillus erzurumensis* has the highest antifungal property against postharvest rot fungi of apple fruits indicating its potential for a sustainable postharvest pest management.

Keywords: Apple fruits; biocontrol; calidifontibacillus erzurumensis; molecular identification; postharvest diseases; rhizospheric bacteria.

1. INTRODUCTION

Apple (Malus domestica Borkh) is one of the most consumed fruits globally for their high nutritional and organoleptic properties. It is rich in important bioactive molecules, such as antioxidants, flavonoids, and dietary fibres, which can ensure human health benefits, reducing the risk of hypertension, diabetes, heart disease and chronic diseases (Bondonno, et al., 2017). Almost 71% of the fruit is consumed fresh while 20% is processed into different value-added products (Shalimi & Gupta, 2010).

Although not grown in Nigeria being a temperate crop, apple is one of the most important fruits with vast market and high consumption rate in almost every city of the country, especially among the elites. The growing middle class family, growing presence of large retail outlets and teeming Nigerians' population have enhanced its consumption and market (Balewu, et al., 2020). The fruit plays important roles in the agricultural and food markets due to its health benefits and is also of high social and economic benefits in Nigeria. Lack of good transport and storage systems, and perishability have been identified as important constraints to apple's marketing and availability in Nigeria (Ajibade, et al., 2023).

Harvested crops including vegetables and fruits face a significant threat from spoilage caused by diseases during their transportation from field to table. Postharvest disease management is key toward achieving high quality and productivity of crops. Postharvest pathogens currently pose the greatest challenge to the food production systems. They dramatically reduce shelf life and cause substantial deterioration of fruits during processing, distribution, and storage. Postharvest fruit diseases continue to have a large negative impact on the global economy with losses estimated to be 20% in industrialized countries and over 50% in developing areas with storage and transportation constraints (Kiaya, 2023).

Infections from bacteria and fungi cause postharvest rotting of fruits from farms to stores causing significant economic losses in agribusiness and adversely affect the market value of fruits in the developing countries (Tripathi, et al., 2008). Postharvest rot of apple fruits usually causes food safety problems and huge economic losses to marketers. Some of the

fungi associated with the postharvest apple rot include: *Botrytis cinerea*, *Venturia inequalis*, *Phacidiopycnis washingtonensis*, *Sphaeropsis phialophora*, and many species of Botryosphaeria, Penicillium, Fusarium, Mucor, Pezicula, Colletotrichum, Alternaria, Pyriputrescens, Rhizopus, Aspergillus, Trichoderma, and Cladosporium (Konstantinous, et al., 2011). Others are *Lasiodiplodia theobromae* and *Curvularia aeria* (Olumuyiwa, et al., 2024, Spadaro, 2024).

Many strategies such as low-temperature refrigeration, physical treatments and chemical control have been used to manage postharvest diseases of crops while the most common control strategy is the application of chemical fungicide in large quantities (Yang, et al., 2016). Although agrochemicals are widely used to control pest and diseases, they harm consumers' health, the environment as well as the quality of fruits. Considering the dangers synthetic fungicides pose to the handlers, consumers and the environment, development of a more sustainable eco-friendly methods for disease control is crucial (Neubeck, et al., 2017).

In recent years, biological control has received extensive attention due to its safety, efficiency and environmental protection (Ogunsola & Ogunsola, 2023). Due to its importance to human health, ensuring the quality and safety of fruits is paramount, given their consumption in an unprocessed state. This necessitates the use of naturally occurring, or exogenously introduced epiphytic antagonistic microbial flora on the surfaces of these crops. Most of these beneficial microbes, often endemic to the fruit and vegetables, serves to mitigate postharvest spoilage. Some of them that were recovered in potatoes, tomatoes, citrus roots, mangos, and bananas and are already used to control plant pathogens include: *Bacillus subtilis*, *Rhodotorula glutinis* Y-44, *Lactobacillus acidophilus*, and *Trichoderma harzianum* (Fenta & Kibret, 2021).

Many potential biocontrol agents have already been identified and available for possible deployment. For example, Bacillus and Pseudomonas species have become a significant biocontrol agents for disease suppression under field conditions (Panpatte, et al., 2016). *Pseudomonas syringae* has been reported against *Botrytis cinerea*, *Monilinia fructicola*, *Penicilium digitatum*, *P. expansum*, and *P. italicum* pathogens in apple, citrus, potato and sweet potato (Spadaro, 2024). A new species, *P. lactis*, first isolated from bovine raw milk in Germany (Spadaro, 2024), and later from chicken faeces at poultry farms in Ogun and other states of South West Nigeria (Olofinsae, et al., 2022), has also produced antimicrobial property against *Calonectria pseudonaviculata* causing boxwood blight (Kong, 2019).

The active ingredients obtained from biotic stress-tolerant microbes have shown potential antagonistic behavior toward postharvest pathogens (Kumari et al., 2021). The multifaceted mechanisms by the biocontrol agents, including mycoparasitism, antibiosis, competition for iron and nutrients, secretion of antimicrobial compounds, and induction of induced systemic resistance in crops, provide them an edge over their chemical counterparts (Kumari et al., 2021). Raio and Puopolo (2021) reported that Bacillus, Pseudomonas, and Serratia genera secrete various secondary metabolites with potent inhibitory effects on plant pathogens. Meanwhile, despite the vast commercialization of apple fruits in Nigeria (Balewu, et al., 2020), there are limited reports on the use of microbial biocontrol agents in the management of postharvest rot diseases of the fruits which suffer a high level of loss due to the infections (Tripathi, et al., 2008). Thus, this study was undertaken to identify antifungal potential of rhizobacterial isolates against the postharvest rot pathogens of apple fruits.

2. MATERIALS AND METHODS

2.1 Source of Postharvest Rot Fungi of Apple

Four Postharvest rot-fungi viz: *Lasiodiplodia theobromae* (accession number: MT7 OR501385) *Fusarium equiseti* (previously identified as *Fusarium roseum graminearum*) (MT3 OR501381) *Pennicillium* species (MT6 OR501384) and *Curvularia aeria* (MT8 OR501386) earlier isolated from apple fruits from markets at the Ado-odo Otta Local Government area of Ogun State, were obtained from the Microbiology laboratory of the Bells University of Technology, Ota Ogun State, Nigeria (Olumuyiwa, et al., 2024). The isolates were sub-cultured to further confirm their respective identities morphologically.

2.2 Isolation of Rhizosphere Bacteria from Soil

Ten grams rhizosphere soil samples were collected from each of three different locations on maize and vegetable cultivated farms of the

Centre for Agricultural Technology and Entrepreneur Studies (CATES), Bells University of Technology Ota, Nigeria. Each sample was thoroughly mixed and 1 gram of the rhizospheric soil was homogenized in 9 ml sterile distilled water inside a 20 ml test tube. The suspension was vortexed and serial dilutions of 10^{-3}, 10^{-5} and 10^{-7} were made. Each dilution (0.1 ml) was then spread on nutrient agar (NA) medium inside Petri plates in triplicates. These spread-plate cultures were subsequently incubated at 37 °C for 24 h. Representative colonies with different morphological appearances were selected from the countable plates and re-streaked on a new plate to obtain pure colonies.

2.3 Identification of Rhizobacterial Isolate Strains

Bacterial isolates were identified using macroscopic and microscopic (Euromax Microscopen, Spain) examination of the growth, biochemical reaction tests and molecular diagnostics. The morphological and biochemical tests conducted were as described by Okigbo (2003) below:

2.3.1 Gram stain

Bacteria smear were heat-fixed on a grease free glass slide and flooded with crystal violet for 1 min, after which the primary stain was washed off with sterile distilled water (SDW). Following heat fixation, the smear was flooded in iodine solution for a minute and subsequently rinsed with SDW. After that, a 95% alcohol rinse was employed for ten seconds, followed by safranin for a minute. The slides were thoroughly washed with SDW after each staining step. Microscopic examination then revealed the presence of either Gram-positive bacteria (appearing purple) or Gram-negative bacteria (appearing pink).

2.3.2 Catalase test

A small amount of bacterial colony was placed on the surface of a clean dry glass slide using a sterile wire loop. Then, a drop of 3% H_2O_2 was added. The catalase test becomes positive when addition of H_2O_2 produces evolution of oxygen bubbles.

2.3.3 Oxidase test

This test involved picking a bacterial colony with a sterile wire loop and smearing onto a filter paper soaked with the substrate (tetramethyl-p-phenylene diamine dihydrochloride) which was immediately moistened with SDW. A colour change of the inoculated area to deep blue or purple within 10-30 seconds indicates a positive result.

2.3.4 Triple sugar iron (TSI) test

A bacterial isolate was streaked onto a slant of TSI agar and incubated at 37 ^oC 18 to 24 hours. Following incubation, the slant aerobic zone and the deeper portion (anaerobic zone) of the medium were examined for red or yellow colour changes, gas bubble and hydrogen sulfide production.

2.3.5 Sulphide-indole-motility (SIM) test

The motility**,** indole, and hydrogen sulfide production of the bacterial isolates were tested using SIM. The isolates were aseptically stabbed into the semi-solid SIM medium and incubated at 28 ^oC for 24 hours. Motility was indicated by the spreading of the organism outside the line of stab, indole production was by the presence of a red pink ring at the interphase after Kovac's reagent was added and sulfide production by the changing of the original yellow colour media to black (Cheesbrough, 2000).

2.4 Molecular identification of Rhizospheric Bacteria Isolate Strains

2.4.1 DNA extraction and PCR amplification

The isolated bacterial strains were grown overnight (24 hours) at 37 ^oC on Luria Bertani (LB) agar plates. Genomic DNA was extracted from the overnight bacterial culture using a commercially available "Quick-DNA Fungal/Bacterial Miniprep kit" (Zymo Research, USA). The extraction procedure followed the manufacturer's instructions.

Polymerase chain reaction (PCR) was performed to amplify the 16S ribosomal RNA (rRNA) partial gene sequence using universal bacterial primers; 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-CGGTTACCTTGTTACGACTT-3′). The PCR reaction mix consisted 25µl total volume, containing12.5 µl of 2X ready master mix (containing the dNTPs, Taq DNA polymerase, $MgCl_2$, and the reaction buffer); 2 µl of template genomic DNA; 1 µl each of forward and reverse primers (5 µM), and 8.5 µl of nuclease free water. The thermal cycling programme used comprised of initial denaturation at 95 ^oC for 5min; 30 cycles of denaturation, annealing and extension at 94 ^oC, 52 ^oC and 72 ^oC for 30s, 30s and 1min: 25s

respectively, followed by a final extension at 72°C for 10 min and kept at a hold temperature of 4 °C (Chikere & Ekwuabu, 2014).

2.4.2 Sequencing and analysis

The amplified PCR products were purified and sequenced using a dye terminator sequencing kit. The sequencing reactions were run on an ABI Prism DNA sequencer at Inqaba Biotech Ltd (Pretoria, South Africa). The obtained chromatograms of the 16S rRNA partial gene sequences were analyzed and edited using BioEdit sequence alignment software. The final consensus sequences generated from the edited sequences were compared to existing sequences in the National Center for Biotechnology Information (NCBI) GenBank database using Basic Local Alignment Search Tool (BLASTn) algorithm. The comparison identified the closest relatives and putative species of the isolated bacteria. The identified 16S rRNA gene sequences of the isolates were submitted to the GenBank database for accession number assignment.

2.4.3 Phylogenetic analysis

The evolutionary relationship between the identified bacteria and their closest relatives were analyzed using MEGA 7.0 software (Version X) (Tamura, et al. 2013). The neighbor-joining method was used to construct phylogenetic tree. Bootstrap analysis with 1000 replicates was used to evaluate the robustness and statistical support of the branching patterns in the neighbor-joining tree (Letunic & Bork, 2019).

2.5 Antagonism Assay of Rhizospheric Bacteria against Apple Fruit Rot Causing Fungi

Antagonist test was carried out to determine the percentage inhibition of radial growth (PIRG) of test pathogens (Fadhilah, et al., 2021) by dual culture method using Potato Dextrose Agar (PDA) medium. The experiment was in a completely randomized design (CRD) in 10 (rhizospheric bacteria) by 4 (rot fungi) factorial, replicated three times. An agar disc cut with a diameter of 5 mm of 5-days old of each of the test pathogens was placed at the centre of Petri plate that contained 15 ml of PDA and rhizobacterial isolates were streaked 3 cm away from the fungi in the same plate. The control experiment contained only the test pathogen placed in the same way without rhizobacterial isolates on a fresh Petri dish. These were incubated for seven days at room 28±2 °C. The antifungal property of the bacteria was determined by measuring the radial growth of pathogenic fungi towards the direction of the rhizobacteria for seven days. The PIRG was calculated using the formula described by Fadhilah et al. (2021)

2.6 Data Analysis

Data on PIRG were transformed by Arcsine transformation and analyzed by ANOVA using the generalized linear model of the statistical analysis software (SAS, 2008). Means with significant differences were separated using Duncan's Multiple Range Test (DMRT) at P = .01.

3. RESULTS

3.1 Identification of Apple Postharvest Rot Fungi

The identity of the fungal isolates of apple fruit collected was verified to be *Lasiodiplodia theobromae*, *F. equiseti, Curvularia aeria* and *Penicillium sp*ecies using macroscopic and microscopic examinations (Fig. 1).

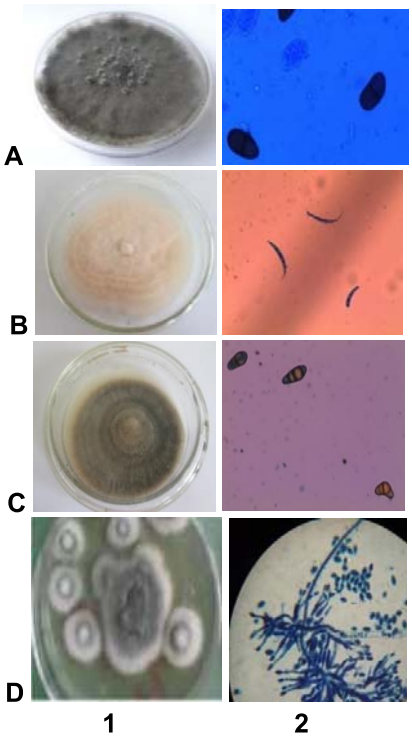


Fig. 1. Culture plates (1) and Photomicrograph (2) of postharvest rot fungi of apple fruits: A, *Lasiodiplodia theobromae*; B, *Fusarium equiseti;* C, *Curvularia aeria* and D, *Penicillium sp*ecies (mg = x 400)

Table 1. Morphological, microscopic and biochemical identification of bacteria isolated from rhizospheric soils

Isolate code	Colour	Shape	Elevation	Gram reaction	Cell shape	Catalase	Oxidase	Indole	Motility	Citrate	H2S	Lactose	Glucose	Gas
SPA 1	Cream	punctiform	Convex	-	Rods	+	+	+	-	+	-	-	+	-
SPA 2	Light yellow	Round	Raised	+	Rods	+	-	+	+	-	+	-	+	-
SPA 3	Cream	irregular	Flat	+	Rods	-	+	-	-	-	-	+	+	-
SPB 1	Yellow	Round	Convex	-	Rods	+	+	+	-	-	-	-	-	-
SPB 2	Whitish	Filamentous	Flat	+	Rods	+	+	-	-	-	-	-	-	-
SPB3	Yellow	Round	Convex	+	Rods	+	-	-	+	-	+	+	+	+
SPC 1	Cream	irregular	Flat	+	Rods	+	+	-	+	-	-	-	-	-
SPC 2	Whitish	Round	Convex	+	Rods	+	-	-	+	-	-	-	+	-
SPC 3	Yellow	Round	Convex	-	Rods	+	-	-	-	-	-	-	-	-
SPC 4	Cream	Round	Convex	+	Rods	+	-	-	-	-	-	-	-	-

3.2 Morphological, Microscopic and Biochemical Identification of Rhizobacteria

Results of colony morphology, microscopic and biochemical identification of the isolated bacterial from rhizosphere were presented in Table 1. The colonies of the 10 isolated rhizobacteria varied widely in their morphological characteristics. The colour of the isolates was either whitish, cream or yellow and their shapes ranged from roundish, irregular, and filamentous to punctiform. Colonies with raised, convex and flat elevation were also produced by the isolates. Microscopic observations of the isolates showed three gram negative bacteria (coded as SPA1, SPB1 and SPC3) indicated by pink colour gram staining reaction, whereas seven were gram positive (SPA2, SPA3, SPB2, SPB3, SPC1, SPC2 and SPC4) with purple coloration. Their cellular morphology showed that they were all rod-shaped bacteria while four of the isolates (SPA2, SPB3, SPC1 and SPC2) were motile.

All isolates did not utilize citrate and ammonia as the sole source of carbon and ammonia respectively, except SPA1. Nine of them produced catalase enzyme, while SPA2 was negative. Isolate SPA1, SPA3, SPB1, SPB2 and SPC1 produced cytochrome c oxidase enzyme while the remaining were negative. In addition, only two rhizobacterial, (SPA2 and SPB3) produced Hydrogen Sulfide (H_2S) gas. Two isolates SPA3 and SPB3 have ability to ferment both lactose and glucose sugars. Indole production was only observed on SPA1, SPA2, and SPB1isolate.

3.3 Molecular Identification of Isolated Antagonistic Rhizobacteria

Ten rhizobacteria were isolated from the rhizosphere of soil collected from three different locations on CATES farms, Bells University of Technology, Ota, Ogun state, Nigeria. Phylogenetic analysis using Molecular Evolutionary Genetics Analysis version 7.0 confirmed the morphological identification of the bacteria (Fig. 2). The sequence similarity search using BLASTn against the GenBank database revealed high homology (97.08 – 98.65%) with the known bacterial sequences (Table 2). The evolutionary history was inferred using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura, 1980). High bootstrap values supported the clustering of isolates with closely related sequences in the GenBank database. This suggests a strong correlation between the phylogenetic grouping and the morphological characteristics observed for the isolates. All isolates formed distinct clades when compared to their respective reference control from GenBank.

3.4 Antifungal Activity of Rhizobacteria against Rot Causing Fungi of Apple

The inhibitory effect of the rhizosphere bacteria paired with rot causing fungi of apple assessed at 5 days after inoculation varied (Table 3). The PIRG of the fungi by the rhizobacterial isolates varied significantly (P = .05) from 0 to 74.51%. *Calidifontibacillus erzurumensis* was the most effective among the 10 rhizobacteria. It significantly produced the highest inhibition on *F. equiseti* (74.5±16.2%), *Pennicillium* species (60.6± 5.2%), *L. theobromae* (60.0± 4.7%), and *C.* aeria (52.9± 5.9%). *Paenibacillus pabuli*, *Pseudomonas lactis* and *Bacillus aerius* also produced significantly high antimicrobial effects next to *C. erzurumensis* on two or three of the rot pathogens. Seven of the rhizobacteria did not show any antifungal effect on the radial growth of *L. theobromae*. The least PIRG of *F. equiseti* (10.8±1.7) was observed when paired with *C. firmus* while that of *Pennicillium* species (9.1±3.1%) and *C. aeria* (0.±0) were by *C. indologens* and *B. reusreri,* respectively.

Table 2. Results of molecular identification of isolated rhizobacteria based on 16S RNA gene sequence data

Isolate code	Closest match in GenBank database	% similarity to first hit on GenBank	Accession number
SPA 1	*Pseudomonas lactis* strain 001	97.78	OR361791
SPA 2	*Neobacillus kokaensis* strain 002	98.56	OR361792
SPA 3	*Paenibacillus pabuli* strain 003	98.65	OR361793
SPB 1	*Chryseobacterium indologenes* strain 004	97.49	OR361794
SPB 2	*Calidifontibacillus erzurumensis* strain 005	97.28	OR361795
SPB 3	*Rossellomorea marisflavi* strain 006	98.45	OR361796

Isolate code	Closest match in GenBank database	% similarity to first hit on GenBank	Accession number
SPC 1	*Bacillus aerius* strain 007	98.47	OR361797
SPC 2	*Brevibacillus reuszeri* strain 008	98.27	OR361798
SPC 3	*Stenotrophomonas pavanii* strain 009	97.08	OR361799
SPC 4	*Cytobacillus firmus* strain 010	97.12	OR361800

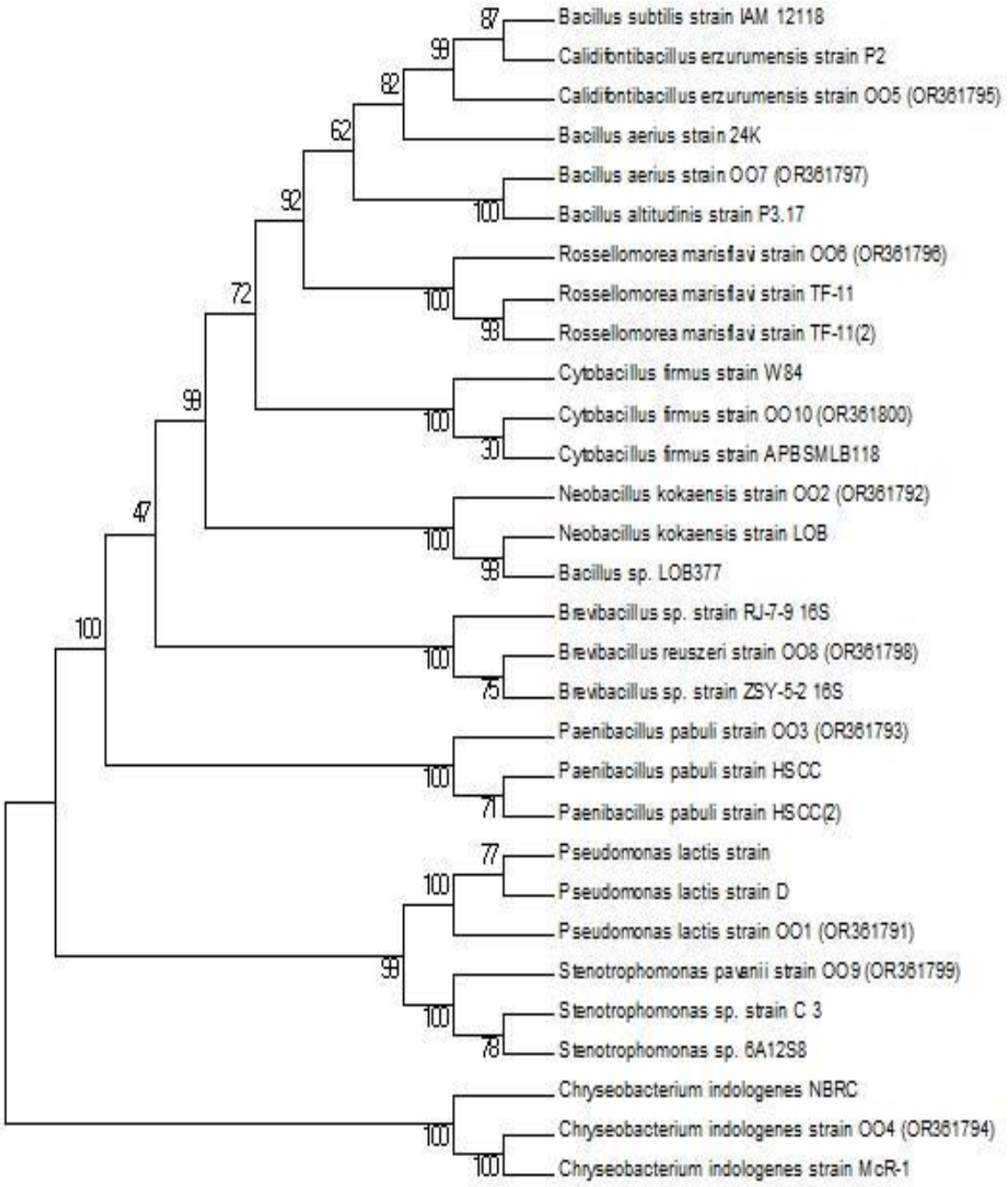


Fig. 2. Phylogenetic tree showing the relatedness of the 16S rRNA partial gene sequences of the isolated rhizospheric bacteria (those with accession numbers in parentheses) to others from the GenBank database

Table 3. Percentage inhibition of radial growth of rhizobacteria against rot causing fungi of apple at five days

Rhizobacteria	*Fusarium equiseti*	*Lasiodiplodia theobromae*	*Pennicillium* species	*Curvularia aeria*
Pseudomonas lactis (strain 001)	55.9±38.5ab	0±0c	30.3±5.2b	3.9±2.4de
Neobacillus kokaensis (strain 002)	20.6±7.8bc	0±0c	36.4±9.1b	5.9±4.8de
Paenibacillus pabuli (strain 003)	46.1±30.6abc	0±0c	27.3±18.2bcd	33.3±6.8b
Chryseobacterium indologenes (strain 004)	53.9±27.9ab	0.8±0.7c	9.1±3.1d	2.0±1.9e
Calidifontibacillus erzurumensis (strain 005)	74.5±16.2a	60.0±4.7a	60.6±5.2a	52.9±5.8a
Rossellomorea marisflavi (strain 006)	34.3±18.9abc	0±0c	24.2±5.2bcd	11.8±5.9cd
Bacillus aerius (strain 007)	22.6±6.8bc	5.5±5.3b	21.2±5.2bcd	15.7±3.4c
Brevibacillus reuszeri (strain 008)	14.7±5.1bc	0±0c	12.1±8.3cd	0±0e
Stenotrophomonas pavanii (strain 009)	24.5±27.3bc	0±0c	12.1±8.5cd	15.7±3.4c
Cytobacillus firmus (strain 010)	10.8±1.7c	0±0c	18.2±9.1bcd	7.8±3.4cd

Means with similar letter along each row are not significantly different according to DMRT (p = .01).

4. DISCUSSION

Although, apple is an important fruit globally with great benefits to human's diet because of its nutritional content, postharvest fruit rot diseases greatly reduce its quality, shelf-life and profits to marketers in West Africa (Tripathi, et al., 2008). Unlike synthetic pesticide, the use of biological control of the rot-causing pathogens proffers a safe eco-friendly management option (Ogunsola, et al., 2024). This study evaluated the antifungal property of some rhizospheric bacteria against *L. theobromae*, *F. equiseti, C. aeria* and *Pennicillium species* which have been reported to cause rot diseases on apple fruits (Olumuyiwa, et al., 2024, Spadaro, 2024).

The ten rhizobacteria isolated were initially subjected to microscopic and biochemical analyses and identified through sequencing of 16S rRNA gene with BLASTn analysis of the final consensus sequences in the GenBank database (Alam, et al., 2011). These isolates, found to be closely related to the various available gram-negative and gram-positive bacterial strains, further confirmed the fact that diverse microbial species usually colonize a niche in a symbiotic relationship (Hentschel, et al., 2000). The *C. erzurumensis strain* used has high relatedness to *B. subtilis* strain which has earlier demonstrated high biocontrol capacity against several postharvest fruits and vegetables pathogens including *Alternaria alternata, A citri, Botryosphaeria berengeriana, B. cinerea, Colletotrichum gloeosporioides, C. musae, G. candidum, L. theobromae* and *Phomopsis citri* (Spadaro, 2024).The phylogenetic analysis also revealed that all the bacterial strain fitted in to an evolutionary cluster comprising members of *Pseudomonas, Bacillus, Stenotrophomonas, Chryseobacterium* and *Rossellomorea,* grouped into separate cluster.

These rhizobacteria produced varied *in vitro* antifungal effects against the apple fruit rot pathogens. Some bacterial isolates produced significant inhibitory effects on the growth of the test pathogens whereas others showed mild or no effect, indicating variation in the mode of action and/or the type of antifungal metabolite produced by the rhizobacteria (Kumar, et al., 2002). Among the ten rhizobacteria, *C. erzurumensis* was most effective which significantly inhibited the mycelial growth of *Fusarium equiseti, L. theobromae, Penicillium* species *and Curvularia aeria.* This was followed by *P. pabuli*, *P. lactis* and *B. aerius.*

The antimicrobial capacity of some of these rhizobacteria and their related species against the diseases of other crop species have been reported. For instance, some *Bacillus* species (such as *B. subtilis, B. velezensis, B. flexus* and *B. amyloliquefacien*s) produced antimicrobial effects against plant pathogenic bacteria (Dowarah, et al., 2021) and fungi (Ogunsola, et al., 2024). *Calidifontibacillus erzurumensis* that produced the highest antifungal potential against apple fruit rot pathogens, is a newly classified strain from the Bacillaceae family with great similarity to *Bacillus azotoformans* and *Bacillus oryziterrae* (Adiguzel, et al., 2020). Although, similar finding on high antimicrobial capacity of C. *erzurumensis* was recently reported by Chee (2023), there has not been much study on this bacterium. It was first isolated from a hot spring in Turkey and categorized as a new gram positive bacterium (Adiguzel, et al., 2020). The high antimicrobial potential of this novel bacterium can be exploited for postharvest crop protection of apple fruits.

The antimicrobial activity of *P. pabuli* has also been reported by Lorentz (2006) but yet to be well exploited. *Pseudomonas lactis* strain SW, a foliage endophyte, has been observed to be strongly antagonistic to the boxwood blight pathogen (*C. pseudonaviculata*) and provided a sustainable and effective control (Kong, 2019). Pretreating the plants with this strain reduced disease incidence by more than 70%. The *P. lactis* has not been found to have any adverse interactions or issues with human and environments although one of its close relatives, *P. azotoformans*, has been reported as a pathogen of cereal grains (Iizuka, et al., 2006). Additionally, the bacterium may be produced at minimal cost due to its origin from milk. However, it is referred to as a psychotropic bacterium with ability to cause spoilage in milk by the production of the heat-stable protease AprX which can withstand heat sterilization procedures and negatively impact the shelf life of milk products (Tanaka, et al., 2018). Despite the antimicrobial property of many Bacillus species against several plant pathogens, there are limited reports on that of *B. aerius.* It was however observed to have exhibited a broad-spectrum antibacterial activity inhibiting both gram-positive and gram-negative bacteria especially the fish pathogens, by producing a bacteriocin-like substance (Meidong, et al., 2018).

The disease suppressive property of the *C. erzurumensis* and *Pseudomonas lactis* could be

due to their ability to produce antifungal secondary metabolites and lytic enzymes (Gowtham, et al., 2016). The high inhibition of fungal growth in the dual culture experiment with wide zone of inhibition without physical contact with the pathogens, suggests that the bacterium might have produced the antifungal metabolites. Similar result was reported by Swain and Ray (2009) on antagonistic activity of *B. subtilis* against *F. oxysporium*. Some of these antifungal metabolites previously reported includes siderophores, hydrogen ions and lytic enzymes (chitinase and cellulase) (Gowtham, et al., 2016) and gaseous products (ethylene, hydrogen cyanide and ammonia) (Kumar, et al., 2002). The antifungal capacity of the metabolites seemed to vary among the isolates tested. The mycelia of *Fusarium equiseti* paired with *C. indologens* did not spread on the agar but showed raised growth. This suggests that the fungal mycelia might have been inhibited not only by antibiosis but also by other antifungal metabolites such as siderophores, hydrogen ions and gaseous products (Kumar et al., 2002).

In addition, synthesis of antibiotics is one of the most effective mechanisms that microorganism employ to prevent proliferation of phytopathogens. Raio and Puopolo (2021) reported that *Bacillus*, *Pseudomonas*, and *Serratia* genera secrete various secondary metabolites with potent inhibitory effects on plant pathogens. Some of the rhizobacteria isolated in this study belong to these three genera. *Pseudomonas* species produces antibiotics such as phenazine, pyrroronitrin, 2-hexyl-5-propylresorcinol, and hydrogen cyanide, as well as siderophores such as pyoverdine and achromobactin which might be responsible for the antifungal property (Raio & Puopolo, 2021).

The three rhizobacteria (*Calidifontibacillus erzurumensis, Paenibacillus pabuli* and *Pseudomonas lactis*) that showed antimicrobial property against apple fruit rot pathogens can be explored as a biocontrol agent towards an eco-friendly sustainable postharvest pest management.

The emergence of fungicide-resistant pathogens and the presence of harmful residues on fruits have driven the increase adoption of eco-friendly technologies for postharvest disease control. Recent decades have witnessed significant advancements in the application of biological and integrated approaches to manage postharvest diseases. The challenges of pesticide residues in fresh produce remains a major concern. Hence, research efforts continue to be on developing alternative management strategies to reduce the pre- and postharvest utilization of synthetic fungicides (Fenta, et al., 2023).

5. CONCLUSION

This study indicated the potential of *C. erzurumensis* followed by P. pabuli and P. lactis as active biocontrol agents for managing postharvest rot of apple. Out of the ten bacteria genera identified from rhizospheric soils, *C. erzurumensis* produced the highest growth inhibitory effect against postharvest apple rot fungi: *Lasiodiplodia theobromae, F. equiseti, Curvularia aeria* and *Penicillium* species *in vitro*, followed by the other two rhizobacteria. This result suggests that the three rhizobacteria could be used as biocontrol agents to serve as alternatives to synthetic agrochemicals for improving protection and profitability of apple fruits. These potential biocontrol agents can be standardized and formulated for used in greenhouse and field applications towards an eco-friendly sustainable agriculture. Extensive field trials to further investigate the effectiveness of these rhizobacteria strains and the mechanism of their antifungal property are required.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of this manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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