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ISOLATION AND MOLECULAR IDENTIFICATION OF BACTERIA ASSOCIATED WITH SOIL SURROUNDING THE ROOT OF CITRUS (*Citrus sinensis* L.) TREE

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ABSTRACT

Microbiological processes occur in the soil around the roots of land plants. This dynamic area, where plants and microorganisms interact, is crucial for the health and productivity of these plants. This study was aimed at isolating and molecularly identifying bacteria associated with the soil surrounding the root of an orange tree growing in the University of Ilorin, Nigeria to be able to know their roles, whether beneficial or harmful. Securing the soil is a way of having a food-secured world. Soil samples were collected at four different points from an orange tree trunk into separately labeled Ziploc bags using a separate sterile hand trowel for each collection. The labeled ziploc bags were brought to the University of Ilorin's Biology Laboratory for physicochemical analysis of the soil sample and isolation of bacteria using serial dilution method. Results of the physicochemical assessment of the bulked sample indicated that it is a sandy-loam soil with pH value of 6.7 (slightly neutral). Four bacterial species, i.e. *Bacillus cereus*, *Lysinibacillusmacroides*, *Leucobacterkomogatae*, and *Alcaligenesfaecalis* were isolated and identified molecularly. According to existing literature, the four bacterial species identified in this study are known for their abilities to enhance plant development and solubilize phosphorus, which are crucial for improving supply of nutrient.

Keywords: Bacterial species, Microbiological activities, Physicochemical analysis**,** Phosphorous solubilization**,** Serial dilution

INTRODUCTION

Citrus (*Citrus sinensis* L.) belongs to the Rutaceae family and are extensively grown in tropical and subtropical zones worldwide (Khan *et al*., 2021). They are believed to have originated from the southern Himalayan area, covering northeastern India and neighboring China, which is the top producer of citrus globally (Rao *et al*., 2021). Citrus fruits are rich in nutrients, providing sugars, organic acids, amino acids, vitamin C, and minerals such as calcium and magnesium, making them important for human nutrition. They are consumed globally in both their fresh and processed forms (Olahan *et al*., 2023).

The soil area around the roots of terrestrial plants, enriched by rhizodeposits and their microbial communities, is highly sensitive to environmental changes, making it an excellent indicator of soil health and quality (Fierer *et al*., 2021). Rich diversity of soil microorganisms is crucial for maintaining the sustainability and productivity of terrestrial plants, citrus trees inclusive. Many of these microorganisms benefit terrestrial trees by balancing crop hormones, enhancing stress resilience and ensuring consistent yields (Dukare *et al*., 2022). Bacteria, integral members of soil microbial communities, are crucial for nutrient cycling, organic matter breakdown, enhancing soil fertility, and suppressing diseases (Dai *et al*., 2021).

The bacterial composition in soil is a strong indicator of soil ecological health and can significantly influence crop yield (Hermans *et al*., 2020). Factors influencing the diversity of soil bacteria in soils include the soil's parent material and pH levels (Jin *et al*., 2022), temperature (Gao *et al*., 2021), fertilization practices (Wan *et al*., 2021), irrigation water quality (Gao *et al*., 2021) and the application of copper-based fungicides (Huang *et al*., 2021). The specific configuration of soil bacterial organisms in citrus trees remains unclear. There is a lack of published research on the bacterial species found in the soil surrounding citrus trees in Nigeria. Understanding these communities is crucial, given that soil microbes play a crucial role in preserving the balance of agroecosystems. Consequently, this research aimed to isolate and molecularly identify the bacterial species present in the soil surrounding a citrus tree in the University of Ilorin, so as to be able to ascertain their roles in the life of the plant.

MATERIALS AND METHODS

Collection of soil samples

Soil samples were gathered from four points (5, 10, 15, and 20 cm) around a randomly selected citrus tree at the University of Ilorin using sterilized hand trowels and placed into labeled Ziploc bags (Olahan *et al*., 2017). The samples were swiftly moved to the Biological Research Facility at the University of Ilorin for bacterial isolation and determination of the soil types, as well as their pH values.

Mechanical analysis of the soil samples

Ten grams (10 g) of soil sample were collected from each labeled Ziploc bag and bulked together, then analyzed following the techniques described by Oyeyiola & Agbaje (2013).

Determination of pH values of the soil sample

Ten grams (10 g) of the bulked soil sample was separately placed into Petri dishes, and labeled Plates A and B. 10 ml of normal potassium chloride was added to plate A, while Plate B received an equal amount of distilled water. After thorough stirring, both plates were allowed to settle for 30 minutes. The pH meter (PHS-25 model) was calibrated using a blank at pH values of 4 and 7. The electrode was then placed into the suspension in plate B, followed by plate A. The pH values were measured and recorded.

Isolation of Bacteria

Nutrient Agar (NA) (500 ml) was prepared following the manufacturer's instructions. Serial dilution method as explained by Fawole & Oso (2007) was adopted for isolation

of bacterial species from the soil samples. Distilled water (9 ml) was pipetted into each of 16 sterilized test tubes arranged in a set of 4, representing the four points from which soil samples were collected from the orange tree. The first test tube in each set was labeled 10^{-1}, the second test tube 10^{-2}, the third test tube 10^{-3} and the fourth 10^{-4}. A gram of the soil sample taken at distance 5 cm from the citrus tree was weighed into the test tube labeled 10^{-1} in order to prepare the stock solution. This was shaken to give a good mixture.

One ml (1 ml) of the mixture in the test tube marked 10^{-1} was dispensed into the test tube marked 10^{-2} and shaken. This process continued until the dilution factor 10^{-4} was obtained. Thereafter, 1 ml each of the 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilution factors was inoculated on separate NA plates using a sterilized 10 ml sized pipette and then allowed to set. After setting, the plates were kept in an incubator at a temperature of 37°C for a duration of 2 to 3 days. The procedures were repeated for each of the remaining soil samples collected at distances 10, 15 and 30 cm respectively. After incubation, the pure cultures discovered on each plate were appropriately labeled as Isolate 1, Isolate 2, Isolate 3 and Isolate 4. They were subsequently subjected to molecular identification.

Molecular Identification of the bacterial isolates

DNA Extraction

Genomic DNA from bacterial isolates was extracted using the Quick-DNA Bacterial Miniprep Kit. The procedure began by adding 50-100 mg of bacterial cells to a specialized tube containing 750 µl of lysis buffer. The mixture was then subjected to mechanical disruption using beads for 20 minutes, followed by centrifugation. The resulting supernatant underwent filtration to eliminate impurities, and the obtained filtrate was mixed with a genomic lysis buffer. The solution was then transferred to a spin column and centrifuged multiple times with wash buffers to purify the DNA. Finally, the pure DNA was extracted in a 50 µl DNA elution buffer.

PCR Amplification

The target region was subjected to amplification using the OneTaq® Quick-Load® 2X Master Mix (NEB, Catalog No. M0486) in accordance with a standardized procedure. The reaction mixture contained 1 µL of template DNA, 0.25 µL each of 10 µM forward and reverse primers (16S-27F: AGAGTTTGATCMTGGCTCAG and 16S-1492R: CGGTTACCTTGTTACGACTT), 6.25 µL of OneTaq Quick-Load 2X Master Mix with Standard Buffer, and 4.75 µL of nuclease-free water. Thermal cycling was carried out using the Eppendorf Mastercycler nexus gradient 230, following a program consisting of an initial denaturation at 94°C for 5 minutes, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 1 minute, and extension at 68°C for 1.5 minutes. This was followed by a final extension at 68°C for 10 minutes, and the reaction was then held at 4°C.

Sequencing

The PCR products were subjected to sequencing utilizing both forward and reverse primers at Inqaba Biotechnol. Ltd in Ibadan, Oyo State. Sequencing was performed using the PRISM™ Ready Reaction Dye Terminator Cycle Sequencing Kit, employing the dideoxy chain termination. The resulting products were subsequently analyzed with an ABI PRISM 3500XL DNA Sequencer (Applied Biosystems, USA), adhering to the manufacturer's instructions.

Phylogenetic analysis

The chromatograms obtained from the sequencing reaction were analyzed using the BioEdit Sequence Alignment Editor. The resulting 16S rRNA consensus sequences were compared to reference sequences in the National Center for Biotechnological Information database using BLASTn. To ensure accessibility, the sequences were deposited into GenBank in accordance with established guidelines (Yakubu & Ajayi, 2024). Subsequent analysis included phylogenetic and molecular evolutionary assessments using MEGA software (version 11).

RESULTS AND DISCUSSION

Mechanical analysis of the soil sample

The soil sample is made up of 20% silt, 70% sand and 10% clay particles. It is therefore classified as a sandy-loam soil (Table 1), while the pH value is 6.7 (weakly acidic).

Table 1: Textural classes and pH value of the soil

Soil sample	% of Silt in the soil sample	% of Clay in the soil sample	% of Sand in the soil sample	Soil Type
****	20	10	70	Sandy-loam

Distribution of the Bacterial Isolates

Four bacterial isolates were obtained from the soil sample and they were denoted as Isolates 1, 2, 3, and 4. Soil samples collected at various distances from the tree trunk (5 cm, 10 cm, 15 cm, and 30 cm) revealed the presence of Isolates 1 and 4 across all points, while Isolate 2 was only found in samples from points 2 and 3. Isolate 3, on the other hand, was detected in samples from points 1, 3, and 4 (Table 2).

Table 2: Distribution of bacterial isolates in the various distances away from the trunk of the citrus tree

	POINTS			
Isolates	**1**	**2**	**3**	**4**
1	+	+	+	+
2	-	+	+	-
3	+	-	+	+
4	+	+	+	+

Identification of the Bacterial Isolates

The 16S rRNA amplification revealed that the recovered isolates belong to four different genera. The 16S rRNA sequence BLAST search showed that isolate 1 is 99.13% similar to *Bacillus cereus* (MK202350) (Table 3), and also the resultant phylogenetic tree indicated that isolate 1 and the same *Bacillus cereus* (MK202350) are closely related and these two lie on the same branch (Fig. 1). The 16S rRNA sequence BLAST search characteristic of the Isolate 2 has 98.76% similarity with *Lysinibacillus macroides* (MN198100) (Table 3 and Fig.2). Also, the 16S rRNA sequence analysis revealed that isolate 3 is 97.13% similar to

Leucobacter komagatae (NR_114966) (Table 3 and Figure 3). The 16SrRNA sequence comparison showed that isolate 4 has high similarity of 99.80% to *Alcaligenes faecalis* (MK312671) (Table 3). The 16S rRNA sequence analysis indicated that isolate 4 in the phylogenetic tree lies on the same branch close to *Alcaligenes faecalis* (MK312671) (Fig 4). Therefore the four isolate were named and recorded in the genbank as follows: *Bacillus cereus* strain F, *Lynsibacillus macroides* strain 18474T, *Leucobacter komagatae* strain RA, and *Alcaligenes faecalis* strain JBW4 under accession numbers PP732648, PP732649, PP732650, and PP732651, respectively.

Table 3: Identification of bacterial species based on sequences similarities

S/no	Organism	Sequence length (bp)	% identity	Accession no of BLAST hit	Highest query coverage (%)	NCBI No
1	*Bacillus cereus*	1523	99.15%	MK202350	99%	PP732648
2	*Lysinibacillus macrolides*	1454	98.76%	MN198100	99%	PP732649
3	*Leucobacter komagatae*	689	97.13%	NR_114966	100%	PP732650
4	*Alcaligenes faecalis*	1499	99.80%	MK312671	100%	PP732651

Discussion

Results of this study revealed that the sample analyzed is a slightly acidic sandy-loam soil. Soil that drains well while holding onto sufficient moisture and nutrients is ideal for citrus trees. They also require a specific soil pH, ranging from slightly acidic to neutral (6.0-7.0), which enables them to absorb the necessary nutrients for healthy growth (Lazaneo *et al.*, 2014). The bacterial isolates from the analysed soil were identified at the molecular level as *Bacillus cereus* (Isolate 1), *Lynsibacillus macroides* (Isolate 2), *Leucobacter komogatae* (Isolate 3) and *Alcaligenes faecalis* (Isolate 4). This seems to be a novel report on the bacterial composition of soil surrounding the roots of an Orange tree in Nigeria. The identified bacterial species showed varying distribution across the points from where the soil samples were collected for bacterial isolation, with *Bacillus cereus* and *Alcaligenes faecalis* being present in the soil samples collected from points 1 to 4 in this study. The consistency of these species could be attributed to their ubiquitous nature (Okanlawon *et al*., 2010; Fernadez *et al*., 2021).

Trivedi *et al.* (2011) identified species from the genera *Burkholderia*, *Pantoea*, *Pseudomonas*, *Bacillus*, *Painibacillus*, and *Serratia* in association with citrus roots in Florida. Fang *et al*. (2014) identified *Alcaligenes faecalis* N1-4 in rhizosphere soil from tea plants located in Cheyun Mountain, Xinyang City, Henan, China. Similarly, Saimmai *et al*. (2012) found *Leucobacterkomagatae* 183 (accession number AB542942) in mangrove sediment from Trang Province in southern Thailand. This finding was part of a broader search for bacteria that produce biosurfactants, which are surface-active compounds with potential industrial applications. Jyolsna *et al*. (2021) isolated *Lynsibacillus macroides* from rhizospheric soil samples surrounding *Pisuksativum* in Indian Institute of Horticultural Sciences. In their exploratory and bioprospecting study, Burkett-Cadena et al. (2019) found beneficial microorganisms in the rhizospheric soil of a pepper plant.

Plant Growth Promoting Rhizobacteria (PGPR) include *Alcaligenes*, *Bacillus*, *Leucobacter*, and *Lynsibacillus*. These beneficial microorganisms enhance plant growth via various processes, such as producing iron-scavenging molecules called siderophores, converting insoluble phosphates into accessible nutrients, and producing antibiotics that support plant health (Timofeva *et al*., 2022; Joshi *et al.*, 2023). Plant growth-promoting bacteria within the rhizosphere can effectively solubilize phosphate, thereby enhancing phosphorus availability in the soil (Oteino *et al*., 2015; Elhaissoufi *et al*., 2022). Specifically, genera such as *Bacillus*, *Leucobacter*, and *Alcaligenes* are notable for their potent phosphate solubilization capabilities, having been isolated from natural sources and extensively studied for their beneficial properties in some previous studies (Haouas *et al*., 2021; Qingwei *et al*., 2023).

CONCLUSION

Bacillus cereus, *Lysinibacillus macroides*, *Leucobacter komogatae*, and *Alcaligenes faecalis* were reported in this study. These bacteria, known for their growth-promoting and phosphorus solubilization capabilities, highlight the potential for utilizing microbial inoculants to enhance citrus cultivation. The findings suggest that leveraging these beneficial bacteria can contribute to improved plant health and productivity, emphasizing the importance of microbial interactions in sustainable agricultural practices. Future research should focus on field trials to assess the practical benefits of these bacteria on citrus growth and explore their potential in other crops.

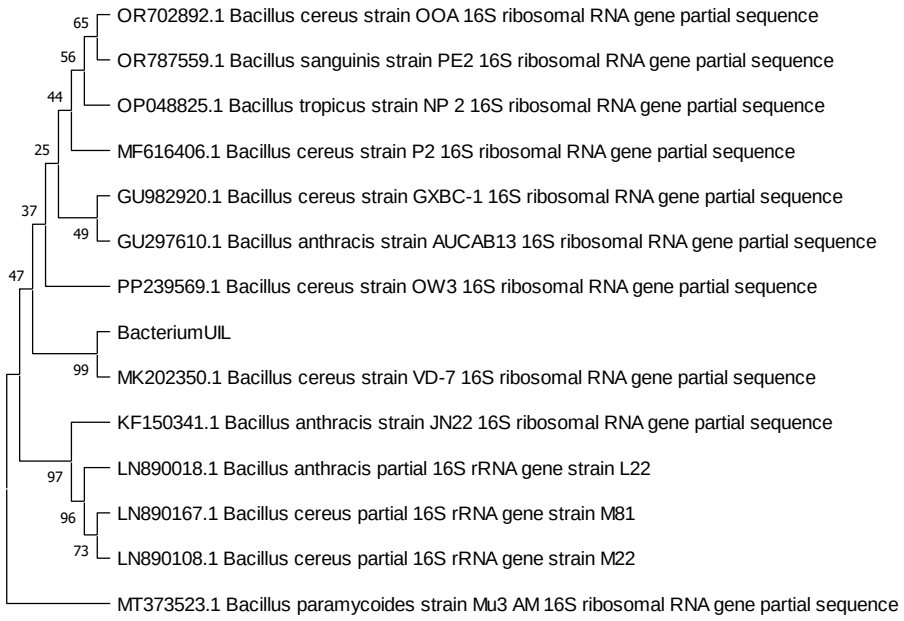

Figure 1: Molecular phylogenetic analysis of isolate 1 (*Bacillus cereus*) using Maximum Likelihood

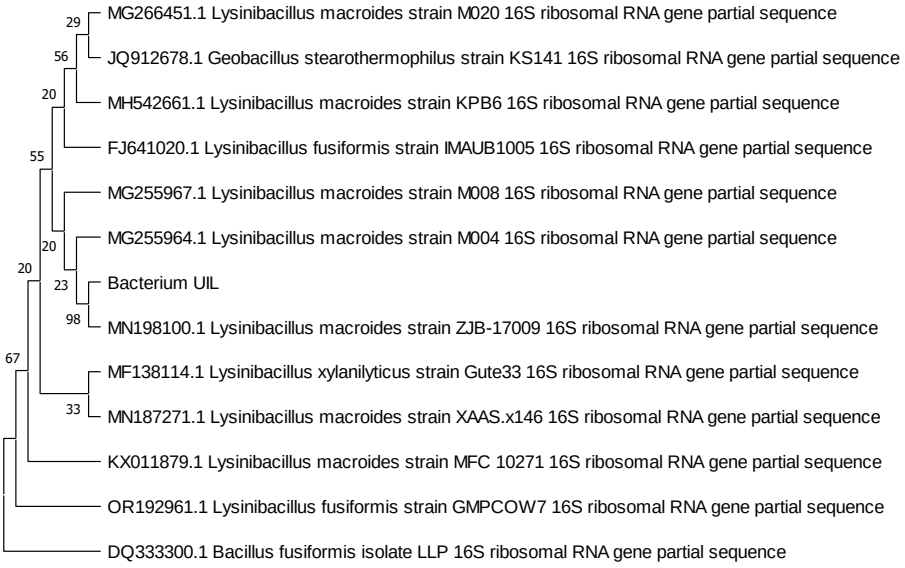

Figure 2: Molecular phylogenetic analysis of isolate 2 (*Lysinbacillusmacroides*) using Maximum Likelihood

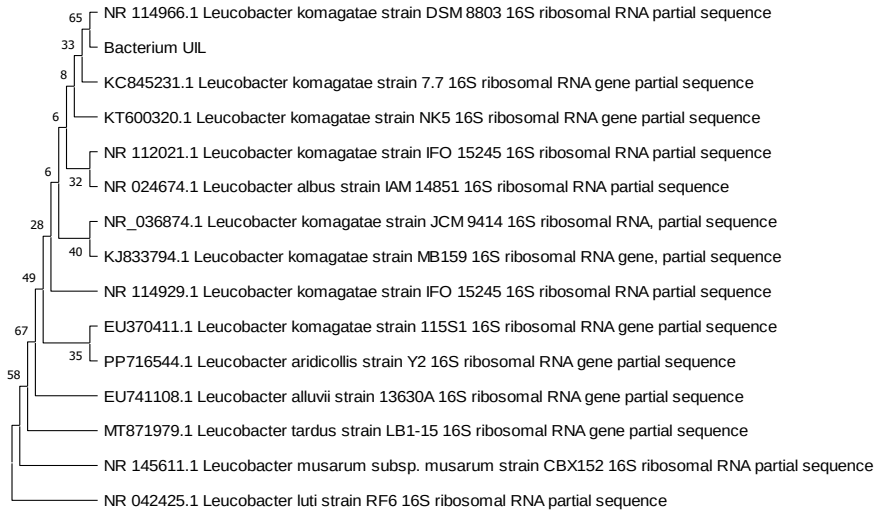

Figure 3: Molecular phylogenetic analysis of isolate 3 (*Leucobacterkomogatae)* using Maximum Likelihood

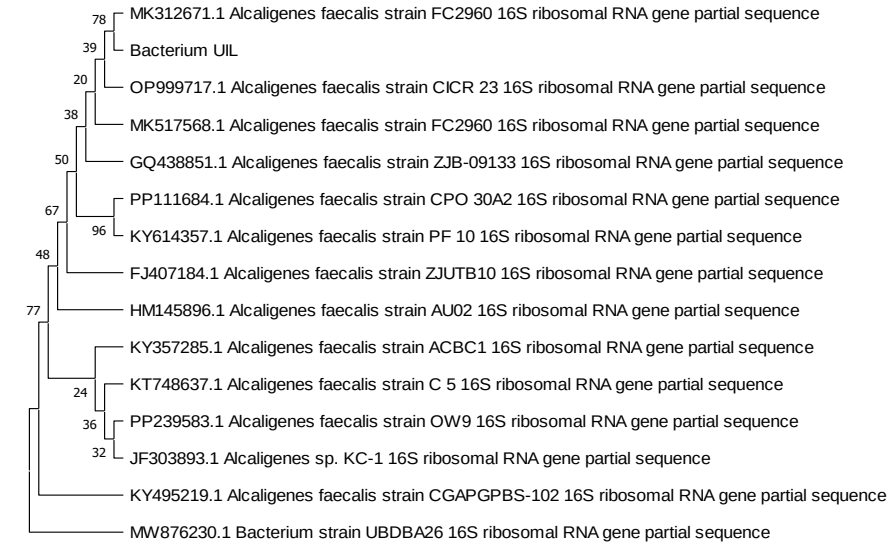

Figure 4: Molecular phylogenetic analysis of isolate 4 (*Alcaligenes faecalis)* using Maximum Likelihood

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