International Journal of
Molecular Microbiology

Research Article 2021 | Volume 4 | Issue 2 | 14-24

Open Access
Article Information

Received: July 25, 2021

Accepted: August 20, 2021

Published: August 30, 2021

Keywords
Tea garden soil,
Microbial diversity,
Acidity–tolerant bacteria,
Functions.

How to cite
Mohammad, S., Qian, P., Jin, L., Jin, L., Ou, L., Iqbal, M.N., Zeng, G., Hu, X-F., 2021. Isolation and Identification of Acid-tolerant Bacteria from Tea (*Camellia sinensis*) Plant Soil. Int. J. Mol. Microbiol., 4(2): 14-24.

***Correspondence**
Hu Xiufang
Email:
huxiuf@sina.com

Isolation and Identification of Acid-tolerant Bacteria from Tea (*Camellia sinensis*) Plant Soil

Shakeela Mohammad[1], Pu Qian[1], Li Jin[1], Lu Jin[1], Li Ou[1], Muhammad Naeem Iqbal[2], Guohong Zeng[1], Xiu-Fang Hu[1*]

[1]Zhejiang Province Key Laboratory of Plant Secondary Metabolism and Regulation, College of Life Science, Zhejiang Sci-Tech University, 310018, Hangzhou, P.R. China.
[2]Pakistan Science Mission (PSM), Narowal (Noor Kot 51770), Pakistan.

Abstract:
This study aimed to explore the acid-tolerant bacteria isolated from tea plant soils that elaborate low pH range of (4.5–6.0). However, acidic soils require low water holding capacity and poor fertility rate; therefore continuously increasing acid in soils inhibits the growth and reduces the quality of *Camellia sinensis*. Microorganisms provide essential support to plants in several environmental conditions. According to the final results, 25 isolates were obtained from the soils; most of them belong to the genus *Pseudomonas sp.* and *Bacillus sp*. Some isolates, such as *Paraburkholderia sp*. LJCY 02, *Paenarthrobacter sp.* LJCY 09, *Pseudomonas azotoformans* LJCY 11, *Bacillus pumilus* LJCY 17 and *Lelliottia nimipressuralis* LJCY 18 grew very well in acidic medium, indicating good resistance against acidity. Some isolates like *Pseudomonas sp.* LJCY 04, *Pseudomonas sp.* LJCY 12, *Arthrobacter oryzae* LJCY 16, and *Saccharomyces cerevisiae* LJCY 25 made the pH value increase to 6.0-6.5. Inoculated in acid soil, *Saccharomyces cerevisiae* LJCY 25 increased their pH, which provides the potential to regulate the pH value of the acidic soil.

INTRODUCTION

Tea is a native plant in China, Tibet, northern India, and it is cultivated in some other countries around the world. Tea is the most popular, caffeine-containing beverage in the biosphere (Aliasgharzad *et al.*, 2011). The point is that almost all kinds of tea come from the same plant (*Camellia sinensis*) bushes; but the simple technique in which they are managed varies, yielding the main arrangements and varieties of tea. Different varieties such as White, Green, and Yellow tea are manufactured by steaming the leaves, accordingly eradicating the oxidation process (Gonçalves Bortolini *et al.*, 2021).

The production and cultivation of the tea plant frequently have great commercial importance. In 2019 around the world overall tea-growing land area was 1 million hectares; and the annual production of a total of 5.79 million tons of tea (FAOSTAT, 2021). Chemically tea plant contains approximately 4000 bioactive compounds of which one-third is contributed by polyphenols (Mahmood *et al.*, 2010).

Global land has received more than 50 kg accumulated N deposition during 2000–2010 (Penuelas *et al.*, 2013) which has been well documented as the main causation of soil acidification in terrestrial ecosystems (Yang *et al.*, 2012). The microbial populations associated with tea soils, some special groups of microbes such as bacteria, actinomycetes, and fungi either symbiotic or free-living are key for plant nutrition and health and, because of this, the rhizosphere is frequently compared with the human gut (Berendsen *et al.*, 2012). The rhizosphere is considered as a complete microbial profile that has higher microbial biomass and activity when compared to the surrounding bulk soil (Finzi *et al.*, 2015; Islam, 2018; Siyar *et al.*, 2019).

Microbes play main role in the remediation process to control soil pollution. Several species of microbes are used to control acidification through different mechanisms (Guan and Liu, 2020). Three main mechanisms are usually input forward to clarify how microbial activity can increase plant growth in several conditions: (1) controlling the hormonal signaling of plants in different ways; (2) outcompeting pathogenic microbial strains; and (3) increasing the bioavailability of soil-borne nutrients. The rhizospheric soil of *C. sinensis* is a typical micro-environment with high acidity (He *et al.*, 2021). This acidic soil enriches a group of bacteria resisting against acidity, and it also inhibits some beneficial bacteria, which might promote the growth of the tea plant. In this study, acid-tolerant bacteria were isolated and identified, which might be helpful to remediate the acidic soil and thus promote the growth of the tea plant.

MATERIALS AND METHODS

The soil of the tea plant is a micro-environment with low pH, which provides a chance for acid-tolerant bacteria to succeed to live inside. To test the capacity of bacteria to remediate the acid soil, acid-tolerant bacteria were isolated. The soils used for the isolation of bacteria were from Longjing tea Garden in Hangzhou. Each sample contained the soils from three tea plants. The samples were mixed completely and stored at low temperature. The medium used for isolation was GM. The component of the medium includes 10.0 g of glucose, 0.5 g of peptone, 0.2 g of yeast extract, 0.2g of Magnesium Sulphate ($MgSO_4$), and 25.0g of agar respectively. The reagents include primary dyeing of ammonium oxalate crystal violet solution, Iodine as mordant dyeing, 95% ethanol solution, restraining with fuchsin solution, and microscopic examination using cedar oil.

Isolation of bacteria (Culture)

The fresh wet soil samples were collected from the tea garden, dried, and then sifted with 2 mm nylon filter. 5.0 g of the screened soil sample was added to the conical bottle of 45 ml sterile water and cultured for 24 hours. The supernatant of 1 ml was then diluted with a gradient of 10 to 1 to 10 to 9, respectively. Then streaked on the solid GM medium and cultured at 28 ℃ for 3 to 5 days. A single colony was selected and purified on the same GM solid plate until a single colony was obtained. The

single colony was cultured at 28ºC for 36 hours under vigorous mixing at 220 r/min, and stored in 50% glycerol tubing, and placed in the refrigerator at -80ºC.

Identification of strains

Bacterial strains were morphologically identified according to their different characteristics. The single colony was cultured on GM medium at 28ºC for 3 to 5 days. Bacterial isolates were identified based on microscopic, morphological, and biochemical characters following Bergey's Manual of Systematic Bacteriology (Bergey *et al.*, 1994; Iqbal *et al.*, 2016; Iqbal *et al.*, 2015; Saleem *et al.*, 2018).

Molecular identification

The colonies cultured at 28ºC for 48 h on GM agar plate were collected and the 16S rDNA nucleotide sequence (1500-1600bp) of the strain was amplified by polymerase chain reaction (PCR). The PCR primers used were the universal primers of bacterial 16S rDNA, and the sequence of primers is shown in table 1.

The primers were made from Sangon Biotech (Shanghai) Co., Ltd. Synthesis. The samples were analyzed according to the PCR system (Table 2) and procedures (Table 3). PCR products were sent to Sangon Biotech (Shanghai) Co., Ltd for sequencing and further analysis. After sequencing, the 16S rDNA region sequences were compared with each other in the Gene Bank database to analyze the taxonomic status of the strains.

The nucleotide sequences of 16S rDNA were lined up with representatives from similar genera and were placed in the Gene Bank with the program MEGA version 4.1. The phylogenetic trees positioned on the 16S rDNA were assembled by using the following methods: the neighbor-joining (Mega version 3.1), determined parsimony, and maximum probability (Phylip version 3.65) methods. Bootstrap resampling analysis was performed to estimate the confidence of the tree topologies.

Table 1. Primer sequence used in the study.

Primer name	Primer sequence
Forward 27F	5′-AGAGTTTGATCATGGCTCAG-3′
Reverse 1492R	5′-ACGGTTACCTTGTTACGACTT-3′

Table 2. The PCR amplification system is as follows.

Reagent	Volume (μ L)
2×Vazyme LAmp Master Mix	25
Primer 1	2
Primer 2	2
Primer DNA	2
ddH_2O	19
Total	50

Table 3. The PCR amplification procedure is as follows.

Step	Time
94ºC	10min
94ºC	30s
55ºC	30s
72ºC	30s
(2-4)step	35 cycles
72ºC	30s
4ºC	∞

RESULTS

Isolation of acidity tolerant bacteria

Isolation of acid-tolerant bacteria was based on the differences in colony morphology and color. In the present study, twenty-five strains of acid-resistant bacteria were isolated from tea plant soils (Figure 1). Different colonies were screened from initial level of acidic soil supplemented on GM medium with adjusted pH 4.5. All the isolates were tested for morphological characterization and their tolerance in acidic conditions. Further 16S rDNA sequences were analyzed, and their growth in different pH. Twenty isolates were found capable to grow at pH range 4.5 (medium acidic condition), and all the isolates that showed the ability to tolerate pH range 4.5 were selected for more revisions. The Microscopic studies of bacterial isolates were observed. A significant difference in morphological activity was observed between twenty-five isolates of bacteria. As shown in figure 1, the name of bacterial isolates is indicated as LJCY.

Morphological identification

Colonies of the isolates on GM medium appeared thin, flat, faint yellow, opaque, round with smooth edge, and approximately 1.2~3mm in diameter. The ultra-structure of the isolated cell under microscopy showed that most isolates were short, rod-like organisms. Gram staining test showed that the isolates were Gram-negative or Gram-positive bacterium (Figure 2). All the isolates showed different colors, shapes, and sizes, which indicated the diversity of the acid-tolerant isolates in soils of the tea plant (Table 4). For the morphological study of isolates a significant difference was observed between the all isolates shown in figure 2 LJCY 01 to LJCY 23 showed different cell structures, sizes, and shapes according to their morphology.

16S rDNA sequences analysis

The nucleotide sequences of 16S rDNA of the bacterial isolates were determined. The results of homology searched with the micro sequencing of bacterial full gene library using the BLAST system revealed that the 16S rDNA base sequence of many isolates had more than 99% homology with *Pseudomonas sp.* and *Bacillus sp.* (Table 5). Altogether, seven isolates (LJCY05, LJCY10, LJCY13, LJCY14, LJCY17, LJCY19, and LJCY24) had over 99% homology of 16S rDNA with *Bacillus sp.*, and thus identified as *Bacillus sp.*

At the same time, six isolates (LJCY04, LJCY06, LJCY11, LJCY12, LJCY15, and LJCY20) were identified as *Pseudomonas sp.* Two isolates were identified as *Cedecea neteri* (LJCY03, LJCY07) and *Arthrobacter sp.* (LJCY08, LJCY16), respectively. Among the other isolates, just one belonged to *Paenarthrobacter sp., Lelliottia sp*., *Arthrobacter sp., Staphylococcus sciuri, Salmonella enterica,* and *Saccharomyces cerevisiae* each. It indicates that the *Bacillus* and *Pseudomonas* might be the major species tolerant to acidity, which needs further evidence.

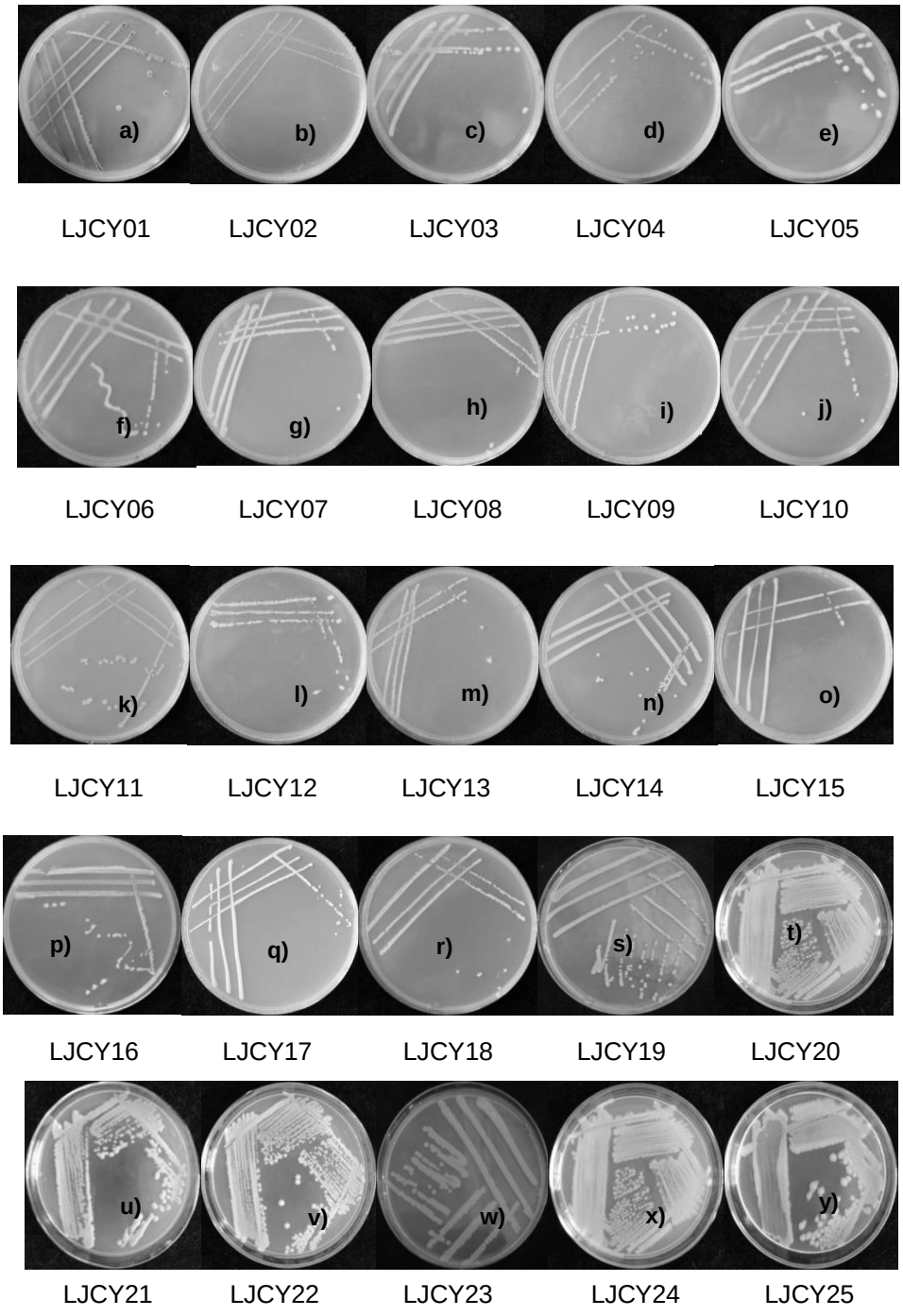

Fig. 1. Isolates of acidity tolerant bacteria on GM with pH 4.5.

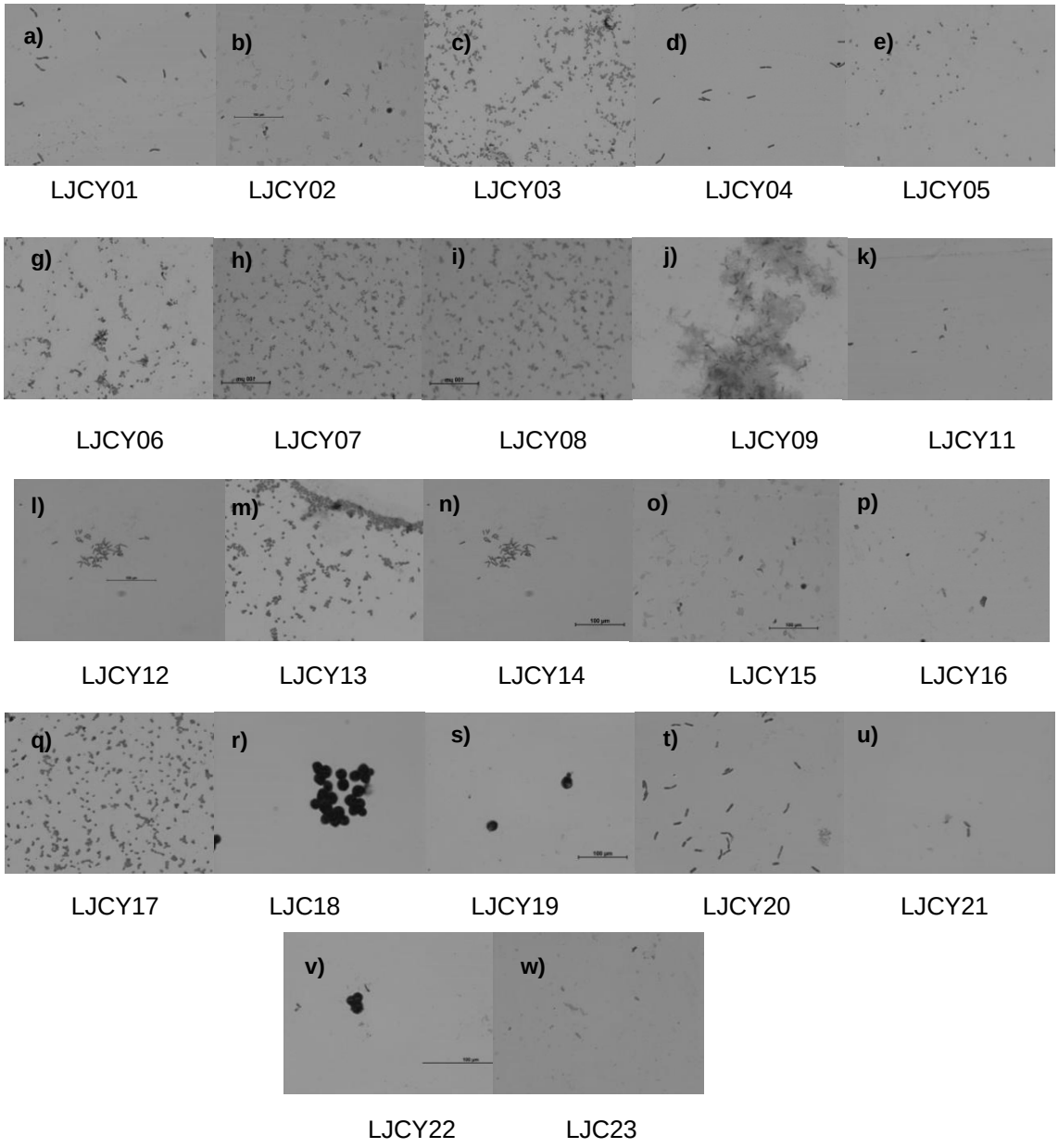

Fig. 2. Microscopic cells of bacterial isolates.

Table 4. Colony morphological characterizations of the isolates.

Name	Size	Shape	Colour	Wet/dry	Colony edge	Upper surface	Transparency
LJCY01	bigger	circular	purple	wet	rough	smooth	Not transparent
LJCY02	bigger	circular	white	wet	smooth	smooth	Translucent
LJCY03	bigger	circular	white	wet	smooth	smooth	Translucent
LJCY04	bigger	circular	white	wet	rough	smooth	Translucent
LJCY05	Medium	irregular	white	wet	smooth	smooth	Translucent
LJCY06	small	circular	yellow	wet	smooth	smooth	Transparent

LJCY07	medium	circular	white	wet	rough	smooth	Not transparent
LJCY08	medium	circular	white	wet	smooth	smooth	Not transparent
LJCY09	bigger	circular	Cyan	wet	smooth	smooth	Not transparent
LJCY10	small	irregular	white	wet	rough	rough	Not transparent
LJCY11	small	circular	white	Semi-humid	rough	smooth	Not transparent
LJCY12	medium	circular	yellow	wet	rough	smooth	Translucent
LJCY13	medium	circular	white	wet	smooth	smooth	Not transparent
LJCY14	medium	circular	white	wet	smooth	smooth	Not transparent
LJCY15	small	irregular	white	wet	rough	smooth	Translucent
LJCY16	medium	circular	white	wet	smooth	smooth	Not transparent
LJCY17	small	circular	white	dry	smooth	smooth	Not transparent
LJCY18	small	irregular	white	dry	rough	smooth	Not transparent
LJCY19	small	circular	yellow	dry	smooth	smooth	Not transparent
LJCY20	Medium	irregular	white	wet	rough	smooth	Translucent

Table 5. Molecular characterizations of bacterial isolates.

Name of the isolates	Generic name	Similarities
LJCY01	*Lelliottia sp*.	99.82%
LJCY02	*Paraburkholderia sp*.	99.23%
LJCY03	*Cedecea neteri*	99.79%
LJCY04	*Pseudomonas sp.*	99.93%
LJCY05	*Bacillus subtilis*	99.79%
LJCY06	*Pseudomonas brenneri*	99.70%
LJCY07	*Cedecea neteri*	99.79%
LJCY08	*Arthrobacter sp.*	99.15%
LJCY09	*Paenarthrobacter sp.*	99.15%
LJCY10	*Bacillus sp.*	99.57%
LJCY11	*Pseudomonas azotoformans*	99.58%
LJCY12	*Pseudomonas sp.*	99.79%
LJCY13	*Bacillus aryabhattai*	99.15%
LJCY14	*Bacillus simplex*	99.79%
LJCY15	*Pseudomonas sp.*	99.57%
LJCY16	*Arthrobacter oryzae*	99.65%
LJCY17	*Bacillus pumilus*	99.86%
LJCY18	*Lelliottia nimipressuralis*	99.86%
LJCY19	*Bacillus velezensis*	99.58%
LJCY20	*Pseudomonas fluorescens*	99.58%
LJCY21	*Staphylococcus sciuri*	99.70%
LJCY22	*Arthrobacter nicotianae*	100.00%
LJCY23	*Salmonella enterica*	99.44%
LJCY24	*Bacillus cucumis*	99.78%
LJCY25	*Saccharomyces cerevisiae*	98.92%

Phylogenetic analysis

Phylogenetic analysis revealed that isolates LJCY05, LJCY10, LJCY13, LJCY14, LJCY17, LJCY19, and LJCY24 clustered closely with *Bacillus* (Figure 3). Isolates LJCY04, LJCY06, LJCY11, LJCY12, LJCY15, and LJCY20) were clustered with *Pseudomonas* (Figures 4). Two isolates were clustered with *Cedecea neteri* (LJCY03, LJCY07) and *Arthrobacter sp.* (LJCY08, LJCY16), respectively. These results confirmed the above identification of the isolates.

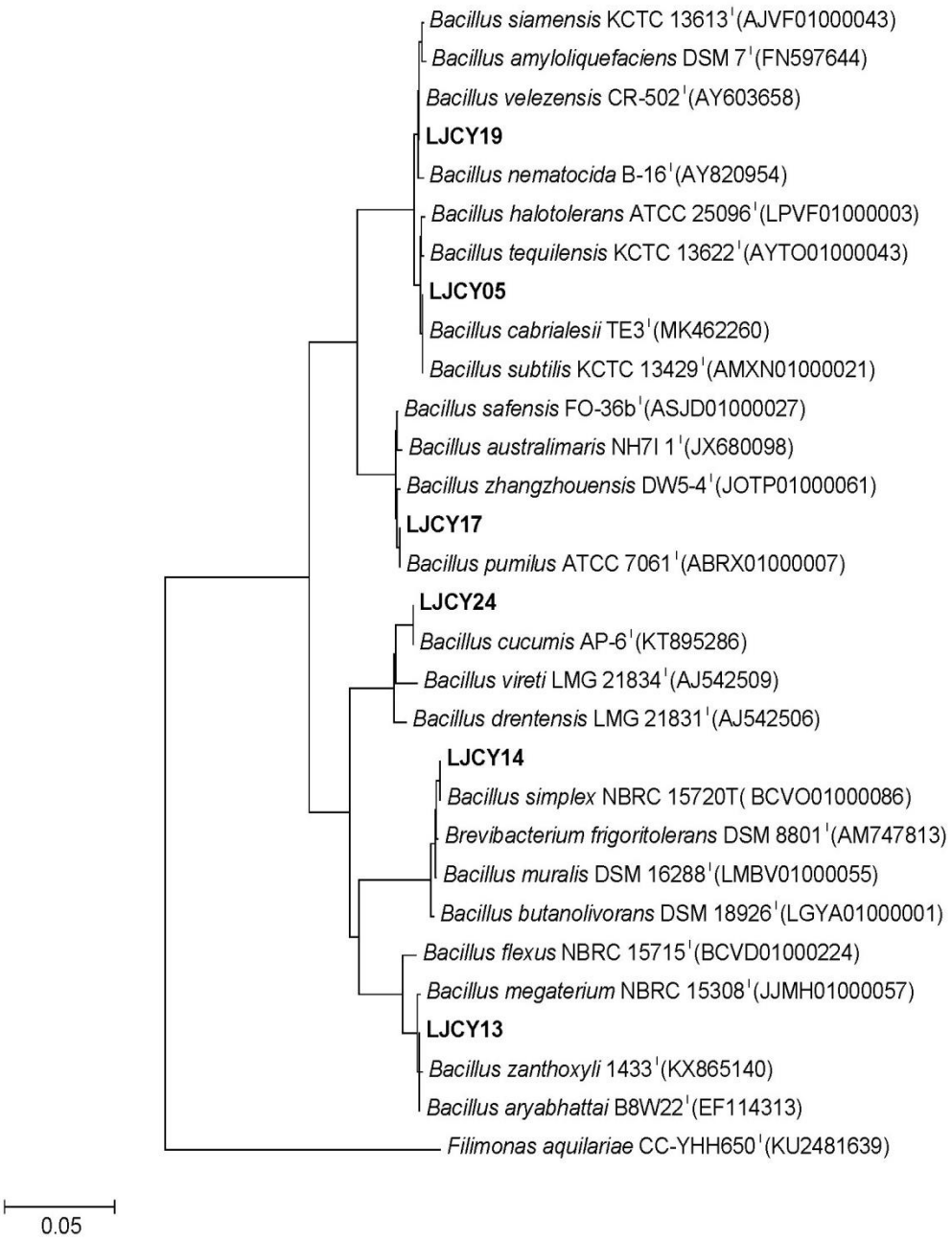

Fig. 3. Phylogenetic tree showing the relationship between the *Bacillus* isolates and the closely related strains. The tree is based on the alignment of 16S rRNA gene sequences and was constructed using the Neighbor Joining method (Mega version 4.1). The stability of the tree was assessed by 1000 bootstrap replications with Felsenstein confidence limits.

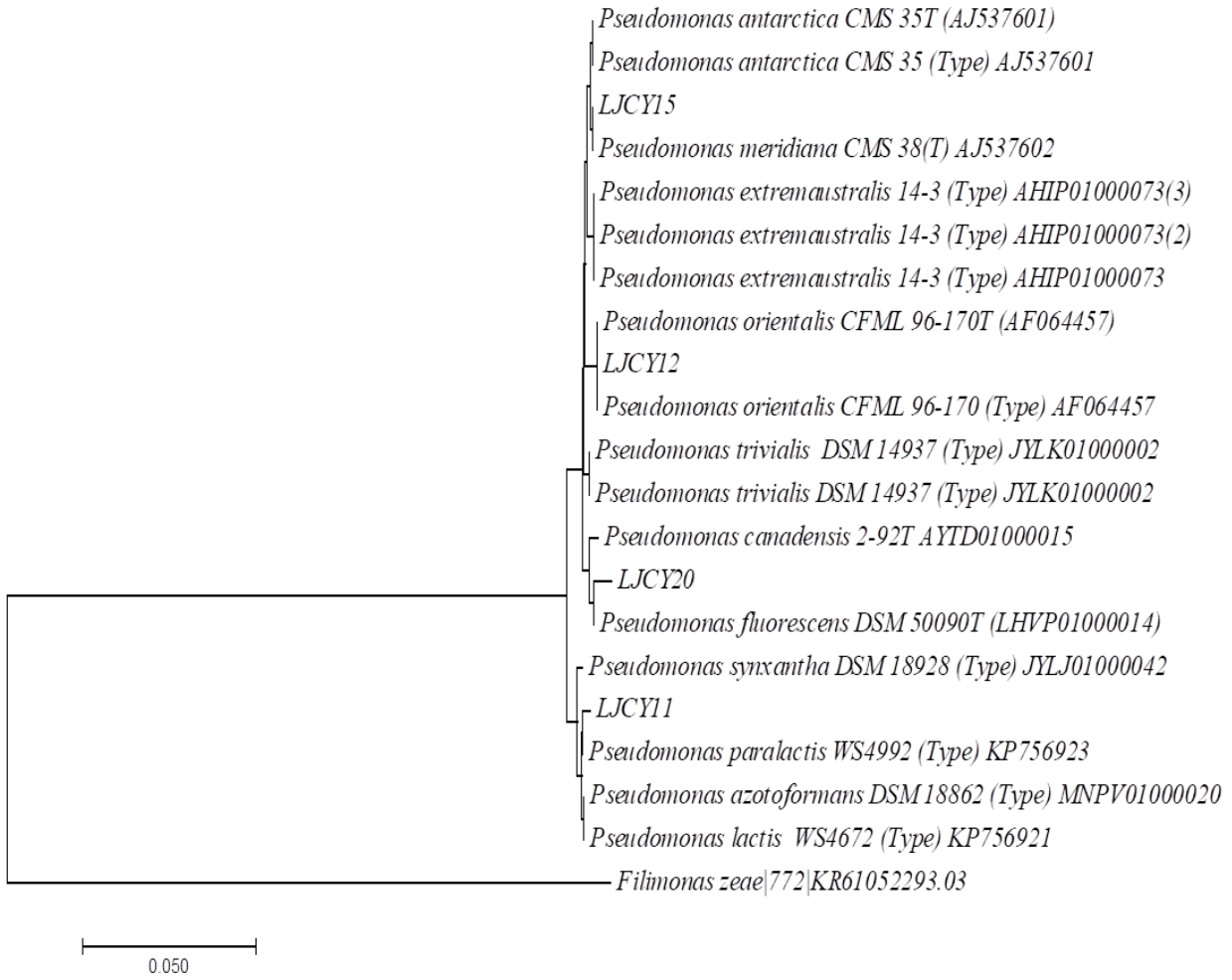

Fig. 4. Phylogenetic tree showing the relationship between the *Pseudomonas* isolates and the closely related strains. The tree is based on the alignment of 16S rRNA gene sequences and was constructed using the Neighbor-Joining method (Mega version 4.1). The stability of the tree was assessed by 1000 bootstrap replications with Felsenstein confidence limits.

DISCUSSION

In the present study, several species of bacterial isolates such as genus *Bacillus, Pseudomonas,* and some other isolated species were found in the acidic tea garden. Acidic soil, one of the important abiotic stresses, not only affects the growth, development, and yield of plants but also limits the geographical distribution of many wild plants (Gentili *et al.*, 2018). The tea crop favors acidic soil with pH range of 4.5-6.0. However, continuously increasing acid in soils inhibits the growth and quality of *C. sinensis.* Acidic soil is the main factor restricting the low water holding capacity and fertility level (Brodt *et al.*, 2011). Microorganisms usually support nutrient availability, resisting against diseases, and tolerate abiotic stresses (Johns, 2017).

In this study, acid-tolerant bacteria were isolated from the rhizosphere soils of *C. sinensis.* According to the results, 25 isolates were obtained from the soils; most of them belong to Genus *Pseudomonas sp.* and *Bacillus sp*. Some isolates, such as isolates *Paraburkholderia sp*. LJCY 02, *Paenarthrobacter sp.* LJCY 09, *Pseudomonas azotoformans* LJCY 11, *Bacillus pumilus* LJCY 17 and *Lelliottia nimipressuralis* LJCY 18 showed high tolerance against acidity. As cold-tolerant isolates

Pseudomonas sp. LJCY 04, *Pseudomonas sp.* LJCY 12, *Arthrobacter oryzae LJCY* 16 showed significant tolerance in acidic soil. Our results about the isolation of acid-tolerant bacteria from tea plant soil are supported by various studies (Goswami *et al.*, 2017; Kawai *et al.*, 2000).

The plant and microbiome showed higher interaction with each other through different metabolic mechanisms and formed other stress tolerance strategies (Dastogeer *et al.*, 2020; Siyar *et al.,*, 2019). Microorganisms produce different metabolites that act as signals during stress conditions. Furthermore, the isolation and morphological identification of bacterial isolates indicate that they have different shapes, sizes, and colors and also evaluate their functions in environmental stress conditions. A recent study showed that soil acidity exerts an adverse effect on microbial diversity (Msimbira and Smith, 2020).

The growth rate of recent results showed that isolates in the presence of low pH consistently have the ability to grow in GM liquid medium. Our results are supported by a previous study in which *Burkholderia* species, named *Burkholderia acidipaludis*, revealed strong tolerance to acidity (Aizawa *et al.*, 2010). The present results showed that the *Pseudomonas* sp. and *Bacillus sp*. species were highly resistant to low pH. Similar results were reported in the study conducted using tea plantation soil of Assam (Goswami *et al.,*, 2017).

CONCLUSION

In conclusion *Pseudomonas sp. Bacillus sp*. and seven other species had over 99% homology exhibits better acid tolerance than other isolates with better survival characteristics, and most isolated bacteria showed positive impacts in acidic conditions. After that, the isolates were further selected for morphological study and 16S r DNA sequence analysis. The soils used for the isolation of bacteria were from Longjing tea Garden in Hangzhou having a certain degree of acidic soil.

ACKNOWLEDGMENT

The author is grateful to the Zhejiang Province Key Laboratory of Plant Secondary Metabolism and Regulation, College of Life Science, Zhejiang Sci-Tech University, 310018, Hangzhou, P.R. China, for their cooperation and providing necessary facilities.

CONFLICT OF INTEREST

The author declares that this article's content has no conflict of interest.

Funding: None.

REFERENCES

Aizawa, T., Bao Ve, N., Vijarnsorn, P., Nakajima, M., Sunairi, M., 2010. Burkholderia acidipaludis sp. nov., aluminum-tolerant bacteria isolated from Chinese water chestnut (Eleocharis dulcis) growing in highly acidic swamps in South-East Asia. Int. J. Syst. Evol. Microbiol., 60(Pt 9): 2036-2041.

Aliasgharzad, N., Hajiboland, R., Olsson, P.A., 2011. Lack of arbuscular mycorrhizal colonisation in tea (Camellia sinensis L.) plants cultivated in Northern Iran. Symb., 55(2): 91-95.

Berendsen, R.L., Pieterse, C.M., Bakker, P.A., 2012. The rhizosphere microbiome and plant health. Trends Plant Sci., 17(8): 478-486.

Bergey, D.H., Holt, J.G., Krieg, P., 1994. Bergey's manual of determinative bacteriology. 1994. Williams and Wilkins, Baltimore, MD, USA.

Brodt, S., Six, J., Feenstra, G., Ingels, C., Campbell, D., 2011. Sustainable agriculture. Nat. Educ. Knowl., 3(1).

Dastogeer, K.M.G., Tumpa, F.H., Sultana, A., Akter, M.A., Chakraborty, A., 2020. Plant microbiome–an account of the factors that shape community

composition and diversity. Curr. Plant Biol., 23: 100161.

FAOSTAT, 2021. Crops and livestock products: Tea. Retrieved August 4, 2021, from FAO Departments and Offices website:.

Finzi, A.C. et al., 2015. Rhizosphere processes are quantitatively important components of terrestrial carbon and nutrient cycles. Global Change Biol., 21(5): 2082-2094.

Gentili, R., Ambrosini, R., Montagnani, C., Caronni, S., Citterio, S., 2018. Effect of Soil pH on the Growth, Reproductive Investment and Pollen Allergenicity of Ambrosia artemisiifolia L. Front. Plant Sci., 9(1335).

Gonçalves Bortolini, D., Windson Isidoro Haminiuk, C., Cristina Pedro, A., de Andrade Arruda Fernandes, I., Maria Maciel, G., 2021. Processing, chemical signature and food industry applications of Camellia sinensis teas: An overview. Food Chem., X, 12: 100160.

Goswami, G. et al., 2017. Diversity and functional properties of acid-tolerant bacteria isolated from tea plantation soil of Assam. 3 Biotech., 7(3): 229-229.

Guan, N., Liu, L., 2020. Microbial response to acid stress: mechanisms and applications. Applied Microbiol. Biotechnol., 104(1): 51-65.

He, L. et al., 2021. Deciphering the Endophytic and Rhizospheric Microbial Communities of a Metallophyte Commelina communis in Different Cu-Polluted Soils. Microorgan., 9(8): 1689.

Iqbal, M.N. et al., 2016. Microbiological risk assessment of packed fruit juices and antibacterial activity of preservatives against bacterial isolates. Pak. J. Zool., 48(6): 1695-1703.

Iqbal, M.N. et al., 2015. Assessment of microbial load of un-pasteurized fruit juices and in vitro antibacterial potential of Honey against bacterial isolates. The Open Microbiol. J., 9: 26.

Islam, S., 2018. Microorganisms in the Rhizosphere and their Utilization in Agriculture: A Mini Review. PSM Microbiol., 3(3): 105-110.

Johns, C., 2017. Living soils: the role of microorganisms in soil health. Fut. Direct. Intl., 1-7.

Kawai, F., Zhang, D., Sugimoto, M., 2000. Isolation and characterization of acid- and Al-tolerant microorganisms. FEMS Microbiol. Lett., 189(2): 143-147.

Mahmood, T., Akhtar, N., Khan, B.A., 2010. The morphology, characteristics, and medicinal properties of Camellia sinensis tea. J. Medic. Plants Res., 4(19): 2028-2033.

Msimbira, L.A., Smith, D.L., 2020. The Roles of Plant Growth Promoting Microbes in Enhancing Plant Tolerance to Acidity and Alkalinity Stresses. Front. Sustain. Food Syst., 4(106).

Penuelas, J. et al., 2013. Human-induced nitrogen–phosphorus imbalances alter natural and managed ecosystems across the globe. Nat. Communic., 4(1): 1-10.

Saleem, M., Latif, A., Ashraf, A., Iqbal, M.N., 2018. Characterization of Carbapenem Resistance in Bacteria Isolated from Clinical Samples in Lahore, Pakistan. Int. J. Nanotechnol. Allied Sci., 2(2): 22-27.

Siyar, S., Inayat, N., Hussain, F., 2019. Plant growth promoting rhizobacteria and plants' improvement-a mini-review. PSM Biol. Res., 4(1): 1-5.

Yang, Y. et al., 2012. Significant soil acidification across northern China's grasslands during 1980s–2000s. Glob. Change Biol., 18(7): 2292-2300.