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Isolation, Identification and Molecular Characterization of Distinctive Isolates in Rhizosperic Soil of Coffee (*Coffea arabica* L.)

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A B S T R A C T

The current investigation was to identify and characterise the rhizospheric isolates in coffee leaves (*Coffea arabica* L.) that have many functions for stimulating plant growth. Coffee leaves were harvested from the plants growing at the Lower Pulney Hills Regional Coffee Research Station in Thandigudi. Morphological and biochemical characterization of seven different rhizospheric bacterial isolates was conducted. Recent studies have found varying degrees of resistance in rhizospheric bacterial isolates. Nitrogen fixation, phosphate solubilization, and potassium mobilization are all characteristics that help plants thrive. Additionally, the rhizospheric bacteria were prospective plant symbionts for granting biotic and abiotic stress tolerance. The three effective isolates were subjected to 16S rRNA and 18S rRNA sequencing, and the findings revealed that CPR-2 was *Bacillus subtilis*, CPR-4 was *Pseudomonas fluorescens*, and CPR-7 was *Trichoderma harzianum* and CPR-8 was *Rhizoctonia solani*.

Introduction

The most consuming beverage in the world and the second most traded commodity after oil is coffee (*Coffea arabica* L.), which is a Rubiaceae plant. Around the world, tropical and subtropical climates are where people drink coffee the most.

One of the major crops, coffee has a considerable economic effect. The genus *Coffea* belongs to the Rubiaceae family. The existence of hundreds of additional physiologically active phytochemicals in coffee, such as polyphenols like chlorogenic acid and lignans, the alkaloid trigonelline, melanoidins produced during roasting, and modest quantities of magnesium, potassium, and vitamin B_3 in the caffeine research (Dam, 2020). Three to five cups of coffee per day have consistently been linked to a lower risk of numerous chronic illnesses.

India's most significant beverage crop is tea, while its second-most important beverage crop is coffee. It

was brought to Arabia from Ethiopia's Abyssinia Plateau in the eleventh century. Coffee is a brewed beverage derived from coffee beans that give you immediate energy and relaxation. The coffee genus includes 100 species of tropical trees and shrubs.

This genus was initially discovered in the 18th century by Swedish botanist Carolus Linnaeus. The ancestor of the species was Ethiopian coffee (Davis *et al.*, 2012). Climate change poses a serious threat to this crop, which is necessary and crucial to the expansion of the world economy.

This is the world's most important agricultural product. It provides over 120 million jobs and is a significant source of income for more than 40 tropical countries. This research covers a variety of topics, including how climate change affects coffee, the importance of coffee for society and health, ideal conditions for coffee growth, and how to prepare seeds for sowing and nurseries.

The impact of nanotechnology and molecular markers on the development of coffee crops, as well as the difficulties, were also covered, along with planting and irrigation techniques, harvesting and roasting procedures, significant diseases, and chemical components in coffee plants. The amount of bushes required to provide shade and protection from the sun during coffee manufacturing is an economic activity that stimulates biodiversity.

Additionally, from an economic perspective, coffee farming gives families and other value chain participants work and income. Both its flavour and its health advantages make coffee a popular beverage. Conservation assessments are based on the International Union for the Conservation of Nature (IUCN) Red List Categories. 72 (or roughly 70%) of the 103 Coffea species are in danger of going extinct due to deteriorating habitat quantity and quality. *Hemileia vastatrix Berk et Br*. is the most common coffee disease, causing production losses of 35-40% on average, and up to 60% in extreme cases (Gouveia *et al.*, 2005). Many cultivated plants, such as tomato, lettuce, potato, and corn (Hallmann *et al.*, 1997), are associated with growth promotion by boosting certain growth related features including root number, dry matter weight, leaf area and seed germination (Frommel *et al.*, 1991).

Coffee leaf rust, known as *Hemileia vastatrix*, is a dangerous illness that affects both the quality and yield of coffee berries. Rhizospheric bacterial and fungal isolates from coffee leaves function as plant growth regulators and biocontrol agents. Examples of activities that encourage plant development include nitrogen fixation, increasing nitrogen availability through phosphorous solubilization, and mineralization of organic compounds (Melnick *et al.*, 2008).

The strains generate in vitro phosphatase and indole acetic acid. The variety of PGPR found in coffee farms in different geographical regions ranges from PGPR was initially described as a form of bacteria and fungi with properties that promoted plant development while being resistant to plant diseases.

The most investigated genera among them include *Alcaligenes, Pseudomonas, Azospirillum, Bacillus, Klebsiella, Azotobacter, Enterobacter, Gluconacetobacter, Burkholderia, Arthrobacter, Rhizobium, Bradyrhizobium, Trichoderma* spp. and *Rhizoctonia solani* respectively.

Materials and Methods

Rhizospheric bacterial isolates of Coffee

For the current research, the coffee rhizospheric soil were collected from Coffee plantations of RCRS.

Coffee Rhizospheric soil isolation method

10g of soil sample rhizospheric soil was taken for serial dilution series (10-1 until 10-6) by using saline water (0.85%). The cultures were isolated by pour plate technique and the incubation period was at 27±2°C for 24 hours. Purification of isolates was done after the incubation period was over and

maintained in LB and PDA slants at 4^{o}C for further studies.

Morphological characterisation

Bacterial rhizospheric were classified based on phenotypic characteristics such as colony colour, margin, shape, texture, gram reaction, elevation and spore-forming abilities (Steinbach and Shetty, 2001). The rhizospheric fungal genus was identified using Lactophenol cotton blue staining (Barnett and Hunter, 1972).

Gram staining (Hucker and conn, 1923)

Hucker's modified technique was used to stain the inoculants using Gram staining.

Endospore staining (Hussey and Zayaitz, 2007)

The common method used to perform the endospore staining technique is the Schaeffer-Fulton method (or a modification).

Motility test (Jain, 2020)

Fresh culture of bacteria was taken. Then bacterial motility was determined using Microscopy. The microscopic technique commonly used is the 'hanging drop method' of Jain.

Biochemical characterisation

Identification of bacteria was done by biochemical tests for tentative characterization.

Catalase test (Aneja, 2006)

A loop containing a 24-hour-old culture of a rhizospheric bacterial isolate grown on nutritional agar slants was dipped into a glass tube containing 0.5 ml distilled water and mixed thoroughly, take one drop of culture and place it in the glass slide. Add, a few drops of 3% hydrogen peroxide. To detect the presence of effervescence.

Oxidative fermentation test (Aneja, 2006)

Fermentation media was prepared. The media was autoclaved at 15lb pressure for 15 minutes. Each specified fermentation tube of media was inoculated with the cultures and labelled. A layer of oil was added on top of the media present in a test tube. Incubated at 35^{O}C for 24-48 hours and observed for colour change.

IAA test (Aneja, 2006)

The test cultures were inoculated into test tubes containing 1% tryptone broth and cultured at 35°C for 48 hours. Followed by the incubation time, the tubes were filled with 1 ml of Kovac's reagent and the observations were recorded.

Methyl Red test (Aneja, 2006)

The rhizospheric bacterial isolates were inoculated with Methyl Red test broth made in a set of test tubes and the incubation temperature was at 30°C for 48 hours duration.

Few drops of methyl red alcoholic solution were added to the set of test tubes. The appearance of a distinct red hue was the prediction of positive MR test results.

Voges-proskauer test (Aneja, 2006)

The rhizospheric bacterial isolates were inoculated in the set of tubes. A naphthol solution (5% solution in 70% ethyl alcohol) was added. Then shook for 15 minutes. The emergence of a red hue suggested a favourable response of acetyl methyl carbinol synthesis. This showed a positive VP test result.

Citrate utilization test (Aneja, 2006)

Inoculations of rhizospheric bacterial isolates into Simmons citrate agar medium were done. Culturing is made for 48 hours at 37°C. A change in pH causes the medium to discolour from green to blue, indicating a favourable response.

Starch hydrolysis test (Aneja, 2006)

Make a single line streak across the plate with the unknown microorganism. Incubate at either 25 or 37^{o}C. Flood the plate with iodine after incubation and development.

Urease test (Aneja, 2006)

The rhizospheric bacterial isolates were inoculated to urea agar plates and the incubation period was up to 24 to 48 hours. The medium's hue shifts from blue to red.

Hydrogen sulphide production (Aneja, 2006)

The production of hydrogen sulphide was studied using a sulphide indole motility (SIM) medium. SIM agar stabs were made, and rhizospheric bacterial isolates were inoculated into them. Stabs were inoculated and the incubation period was at 30°C for 48 hours duration. The results were observed and recorded.

TSI test (Aneja, 2006)

Test cultures were streaked above the agar slants and incubated for 24 hours at 37 °C with a loose top to allow for air ambience. The colour indication from orange to deep reddish was observed.

Casein hydrolysis (Aneja, 2006)

Rhizospheric bacterial cultures were inoculated in plates consisting of milk agar medium. The incubation period was at 37^{o}C for 24-48 h. The results were observed.

Lactophenol cotton blue staining

Rhizospheric fungal cultures were identified by the method of Barnett and Hunter, 1972.

Molecular analysis

For characterization of selected rhizospheric cultures was based on the sequencing software v1 for 16S rRNA and 18S rRNA gene and phylogenesis investigation. The sequence alignment and modification were done by MEGA 7.0. For the sequence amplification and validation, NCBI's BLAST program's similarity index was used. The species used in this research were assigned to the following species based on their higher percentage resemblance to the species of the same genera.

Results and Discussion

Isolation and molecular characterization of rhizospheric bacterial isolates

The distinct rhizospheric isolates were obtained from the coffee leaves located at Regional Research Station, Thandigudi. The isolates were designated, as CPR-1, CPR-2, CPR-3, CPR-4, CPR-5, CPR-6, CPR-7 and CPR-8 isolated from coffee leaf extracts were arranged sequentially.

Seven different Plant growth promoting isolates were studied. The morphological and biochemical testing was conducted to identify the species of the genus. The morphological characteristics of the bacteria were obtained using Bergey's handbook of systematic bacteriology. Rhizospheric bacterial isolates from coffee leaves were tentatively identified as *Azospirillum*, *Bacillus*, and *Pseudomonas*, by biochemical analysis, and the details are included in (Table 2).

Molecular Analysis of effective rhizospheric isolates

The selected effective isolates of rhizospheric bacteria were closely related to *Bacillus* (CPR-2), *Pseudomonas* (CPR-4), *Trichoderma* (CPR-7) and *Rhizoctonia* (CPR-8) genera. The MEGA 7.0 phylogenetic analysis was carried out and the closely related sequences were aligned and a neighbour joining tree was generated (Figs 1 and 2).

Their 16S rRNA and 18S rRNA sequence reports were submitted to the NCBI gene bank under the accession codes OP326575, OP326574, OP326578 and OP268471.

Table.1 Morphological characterization of isolated Rhizospheric strains from coffee (*Coffea arabica* L.)

Isolate code	Margin	Colony colour	Shape	Texture	Gram reaction	Elevation	Mobility	Spore forming	Tentative Identification
CPR-1	Entire	White dense	Rod	Slightly curved	Negative	Convex	Nonmotile	Negative	*Azospirillum* sp.,
CPR-2	Irregular	Grey white	Rod	Rough	Positive	Flat	Motile	Central spore	*Bacillus subtilis*
CPR-3	Unbonate	White	Rod	Dull	Positive	Convex	Motile	Positive	*Bacillus* spp.
CPR-4	Wavy	Diffusible green	Oval and medium	Mucoid	Negative	Unbonate	Nonmotile	Negative	*Pseudomonas fluorescens*
CPR-5	Irregular	Grey white	Rod	Dull	Positive	Flat	Motile	Positive	*Bacillus* spp.
CPR-6	Smooth	Fluorescent	Rod	Mucoid	Negative	Unbonate	Nonmotile	Central spore	*Pseudomonas putida*
CPR-7	Regular	White	Variable	Loose	-	-	-	-	*Trichoderma harzianum*
CPR-8	Irregular	Brown	Irregular	-	-	-	-	-	*Rhizoctonia solani*

Table.2 Biochemical characterization of isolated bacterial strain from Coffee (*Coffea arabica* L.)

Isolate code	Catalase test	OF test	Urease test	IAA	MR test	IMvic test: VP test	Utilization of Citrate	Hydrolysis of Starch	Hydrogen sulphide Production test	TSI test	Hydrolysis of Casein	Tentative identification
CPR-1	+++	+	+++	-	-	+	+	+++	-	-	-	*Azospirillum* sp.,
CPR-2	+++	+	-	-	-	+	+	-	+	-	+	*Bacillus subtilis*
CPR-3	+++	+	-	-	-	-	+	+	+	-	-	*Bacillus* spp.
CPR-4	+++	+	+	-	-	+	-	-	-	-	+	*Pseudomonas fluorescens*
CPR-5	+++	+	-	-	-	+	+	-	+	-	+	*Bacillus* spp.
CPR-6	+++	+	-		-	-	-	-	-	-	-	*Pseudomonas putida*

Fig.1 Phylogram derived from sequences of 16S rRNA sequencing of PGP Isolates (OP326574, OP326575)

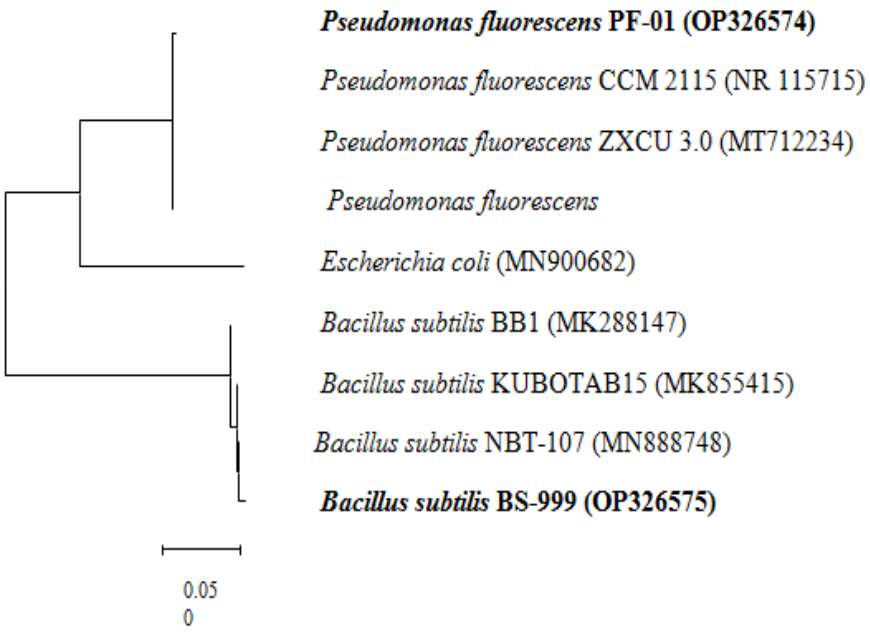


Fig.2 Phylogram derived from sequences of 18S rRNA sequencing of PGP Isolates (OP326578)

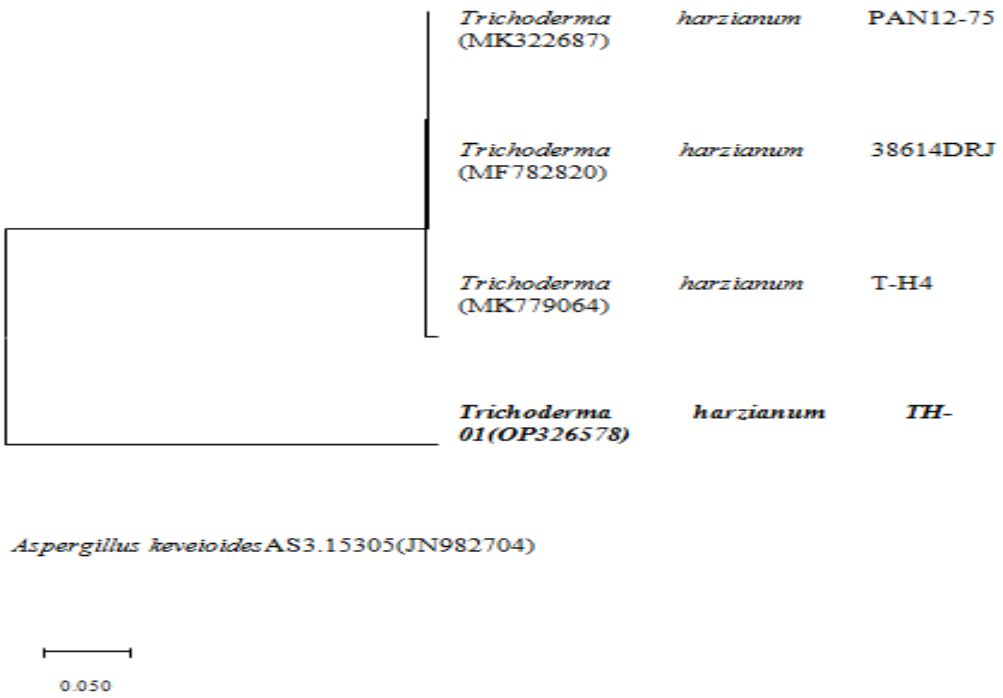
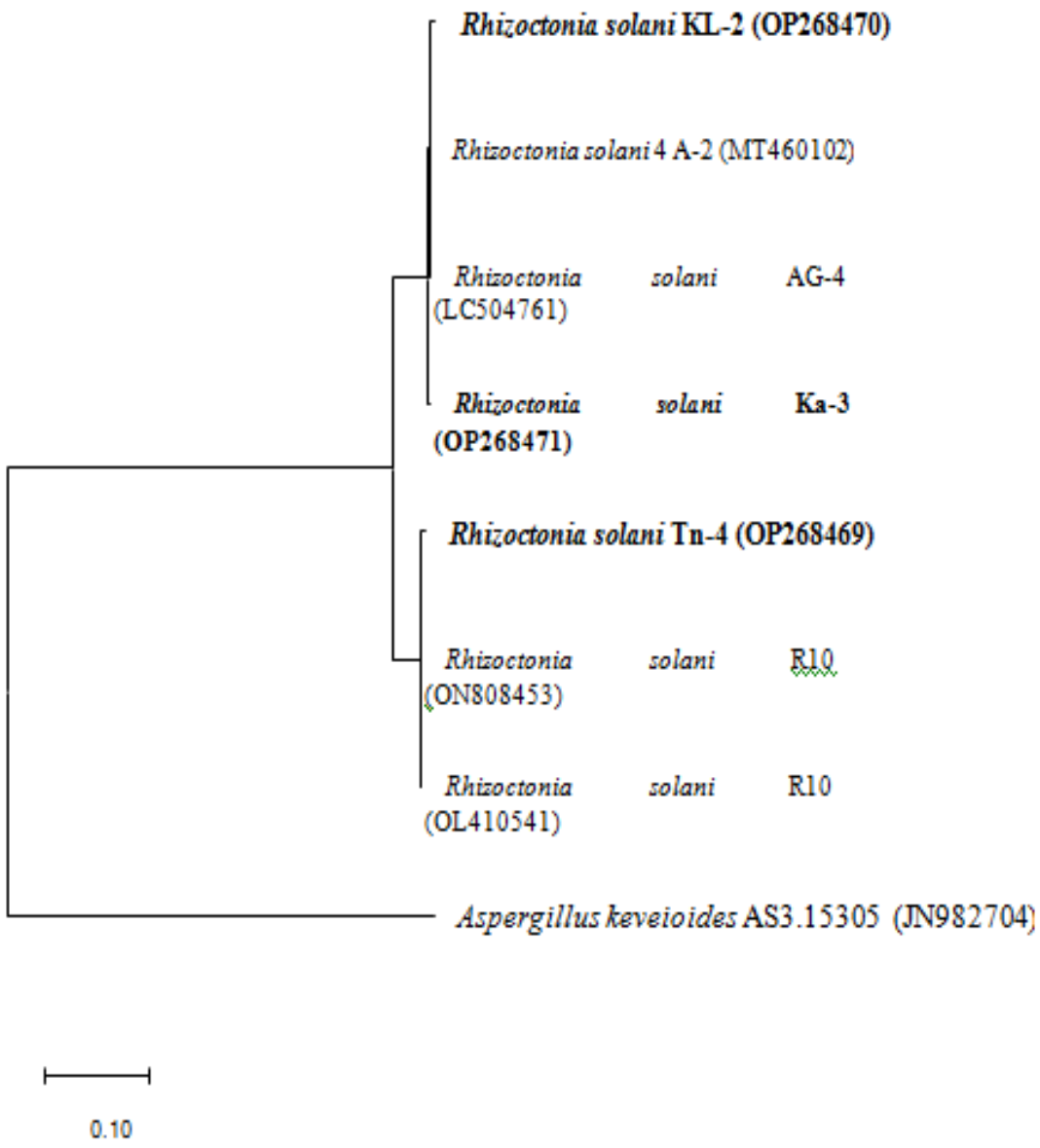


Fig.3 Phylogram derived from sequences of 18S rRNA sequencing of PGP Isolates (OP268471)



The potential of plants to promote the growth of rhizospheric bacterial isolates was scrutinized from Coffee leaves by Leaf extraction. The following isolates were tentatively identified by morphological and biochemical characterization.

Among all the following PGPR bacterial isolates, CPR-2 (*Bacillus subtilis*), CPR-4 (*Pseudomonas fluorescens*), CPR-7 (*Trichoderma harzianum*) and CPR-8 (*Rhizoctonia solani*) have been molecularly characterized. It could be possible to have a wider spectrum of activity, enhanced level and consistency in disease management against multiple pathogens/races of soil and airborne nature. In addition to disease control, better uptake of nutrients, improving plant growth and enhancing crop yield can also be achieved.

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