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Isolation and Molecular Characterization of Root-Associated Bacteria from *Butea monosperma* (Lam.) Taub. Narowal, Punjab, Pakistan

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Butea monosperma (Lam.) Taub. is a medium-sized leguminous arboreal species in the family Fabaceae, characterized by its commercial value, pharmaceutical properties, and ecological importance. The overexploitation of this plant has caused it to be listed in the Red List of endangered species. This research aimed to isolate and molecularly identify plant root-associated bacteria from *B. monosperma*. Root samples were carefully collected, and pure cultures were prepared. Biochemical and morphological characterization was performed through staining and microscopy, respectively. Genomic DNA was isolated using a phenol–chloroform extraction method. The isolated DNA was quantified using spectrophotometry (A260/280) and 1% agarose gel electrophoresis. Molecular identification was carried out by amplifying the bacterial 16S rRNA gene using polymerase chain reaction (PCR). The amplicons were sent for Next-Generation Sequencing (NGS). The obtained sequence was submitted to NCBI to obtain an accession number. The phylogenetic tree was constructed using MEGA 12. The isolated bacterium was Gram-positive and morphologically appeared rod-shaped. The spectrophotometric ratio A260/280 was 1.85, and the amplicon size on agarose gel electrophoresis was 1360 base pairs. Phylogenetic analysis showed a 98% genetic similarity to *Bacillus subtilis*, which was confirmed through BLAST results. The accession number obtained was PX480147. *Bacillus subtilis* is a plant growth-promoting bacterium, and it could be applied in the plantation of B. monosperma.. The development of plant–root associations could be the best strategy for the conservation of this endangered species.

Keywords: *B. monosperma*, Rhizobacteria, Plant Conservation, 16S rRNA Gene Sequencing, PGPR

Introduction:

Butea monosperma (Lam.) Taub., commonly known as Palash or Flame of the Forest, is a tree belonging to the Fabaceae family and is valued for its beneficial properties [1]. This tree species is indigenous to the region and fulfills a variety of important functions. It is common in the Indian subcontinent, particularly in Pakistan, Nepal, and India [2]. *B. monosperma* possesses numerous medicinal uses in Ayurveda, Unani, and homeopathy [3]. Natural chemicals, such as alkaloids, tannins, polyphenols, glycosides, and flavonoids, are found in its stems, bark, roots, fruits, leaves, flowers, gum, and seeds [4][5]. These phytochemicals are vital for both traditional and modern treatments [6]. The anti-inflammatory and analgesic characteristics of *B. monosperma* highlight its long history of application in traditional Asian medicine and the increasing interest in natural remedies as alternatives to pharmacological drugs [7].

B. monosperma plays an essential role in environmental restoration. The plant can be used to regenerate degraded sites, enhance ecological processes, and support other plants by fixing nitrogen, as it is a legume [8]. Furthermore, B. monosperma is economically valuable and has numerous uses [9]. Research indicates that it improves polluted soils by enhancing chemical, microbial, and enzymatic activities [10]. However, due to overuse and lack of conservation knowledge, the number of *B. monosperma* plants has significantly declined, and it is now endangered in most locations in Punjab, Pakistan [11]. Thus, there is a dire need to preserve it and ensure sustainability by understanding its interactions with soil microbes.

Root-associated bacteria, especially those inhabiting the rhizosphere, have a significant symbiotic relationship with host plants. This association significantly enhances plant growth and contributes to stronger resistance against biotic and abiotic stresses [12]. Increasing levels of chemical contaminants and heavy metals from anthropogenic activities negatively affect plants and animals, contributing to abiotic stress. Further, climatic conditions (e.g., drought and nutrient deficiency) may hinder plant growth and survival [13]. Plants are also exposed to biotic stressors, such as pathogenic pests and microbes. Fortunately, plant growth-promoting rhizobacteria in the root zone help plants overcome both forms of stress [14].

Bacillus subtilis is one of the most widely studied bacteria for stimulating plant growth in the rhizosphere [15]. This bacterium promotes plant growth and controls plant pathogens in multiple ways, such as enhancing nutrient availability, altering phytohormone balance, producing antimicrobial compounds, and developing systemic resistance. Although its usefulness in crop production has been widely researched under laboratory conditions, its performance in the field is highly variable [16].

Study Objectives:

The main objective of this study was to isolate and identify plant root-associated bacteria from Butea monosperma.

To achieve accurate identification and phylogenetic analysis of the isolated bacterial strains, the isolates were characterized using morphological, biochemical, and molecular techniques, including 16S rRNA gene sequencing.

Furthermore, this work aims to support the conservation and sustainable use of this multipurpose species, thereby increasing its value, creating demand, and promoting further awareness.

The uniqueness of this study lies in its first identification and isolation of *Bacillus subtilis* from the roots of the endangered *B. monosperma* in Narowal, Punjab. This discovery enhances our understanding of the species, contributing valuable insights for future research and conservation efforts.

Materials and Methods:

Sample collection:

Plant samples were collected to extract root-associated bacteria from Butea monosperma (Lam.) Taub., collected in Narowal, Punjab, Pakistan (32°20'13.2216"N, 74°54'12.0096"E). The root samples were placed in sterile polythene zippered bags for analysis in the Plant Molecular Biology Lab at the Department of Botany, Government College University, Lahore, following the research protocol [17].

Figure 1. Regional distribution of the sampling site, which was indicated by red, in the Narowal district of Punjab, Pakistan.

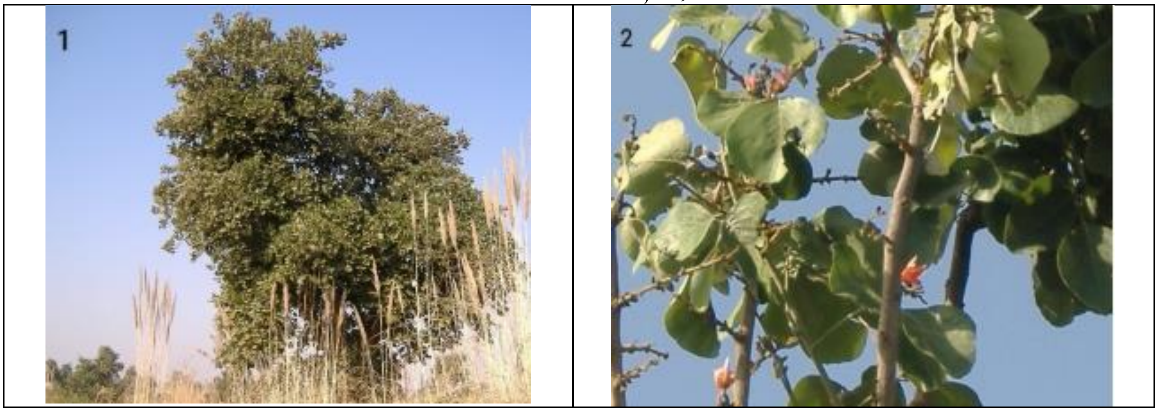

Figure 2. Samples were collected from the selected site

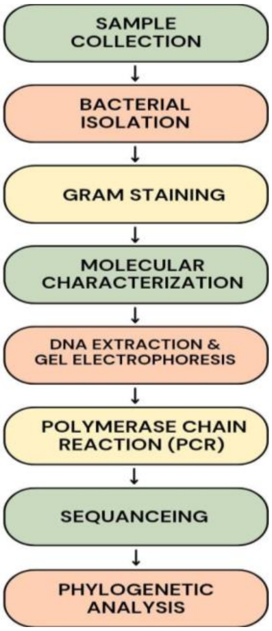

Figure 3. The workflow of the study is outlined above.

Bacterial Isolation:

The root samples were thoroughly washed with double-distilled water as well as a 70% ethanol solution for sterilization purposes. The bacteria were cultured in Luria Broth medium

[18]. The primary purpose of using Luria Broth is to support bacterial growth in liquid culture, allowing subsequent isolation of single colonies on plates [19].

Gram Staining:

Smears of each bacterial isolate were prepared on clean glass slides, allowed to dry, and then heat-fixed for Gram staining. The procedure involved several important reagents: Crystal Violet (primary stain), Iodine (mordant that fixes the dye), Acetone–Alcohol (decolorizer), and Safranin (counterstain). In the first step, Crystal Violet was applied for one minute, followed by rinsing and the addition of Gram Iodine for another minute. The slides were rinsed again and decolorized using acetone–alcohol until clear. Finally, the slides were immersed in Safranin for 30 seconds to 1 minute, washed, and air-dried. The slides were then observed under oil immersion. This procedure confirms the Gram reaction of bacterial isolates through the Gram reaction and provides essential morphological traits for further analysis [17].

Molecular Characterization:

DNA Extraction and Gel Electrophoresis:

Genomic DNA was isolated from bacteria using the phenol–chloroform extraction method as described by [20]. DNA samples were analyzed using 1% agarose gel electrophoresis to assess integrity [21]. The purity of the isolated DNA was determined using the spectrophotometric ratio A260/280.

Polymerase Chain Reaction (PCR):

Molecular identification was performed using 16S rRNA universal primers PA-F (5'-AGAGTTGATCCTGGCTCAG-3') and PH-R (5'-AAGGAGGTGATCCAGCCGCA-3') as described by [22].

Phylogenetic Analysis:

The evolutionary relationship among the bacterial isolates was determined based on their 16S rRNA gene sequences. Sequences were aligned using ClustalW within MEGA 12 software [23]. Phylogenetic trees were generated using the Maximum Likelihood (ML) method with the Tamura–Nei substitution model [24].

Results:

Morphological Identification of Isolated Bacteria:

Gram staining was used to confirm pure bacterial cultures. The presence of a purple stain indicated that the cultured bacteria were Gram-positive (Figure 4), exhibiting a rod-shaped morphology and primarily arranged in pairs, suggesting that the specimen was a pure culture of Bacillus subtilis. Gram-positive bacteria are characterized by a thick peptidoglycan layer, allowing them to retain the purple stain even after treatment with ethanol, as they are not decolorized during this process.

Table 1. A summary table of the characteristics of bacterial isolates ID, including colony morphology, Gram reaction, DNA quality metrics, and BLAST results.

Isolated ID	**Morphology of the colony**	**Gram stain**	**Quality of DNA (ul)** spectrophotometric ratio A260/280	**PCR product (bp)**	**Blast identity (%)**	**Accession No.**
B1	Purple colour, Rod shape, Arrange in pairs,	Gram +	50ul, 1.85	1360	98%	PX480147

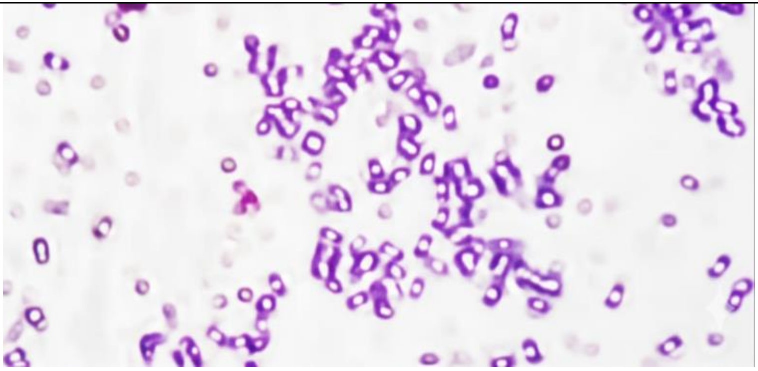

Figure 4. Gram-positive bacteria are observed under a microscope after Gram staining

Isolation and Quantification of DNA:

Extraction and Agarose Gel Electrophoresis:

The quality of genomic DNA was carefully assessed using a spectrophotometer. All samples exhibited high-quality DNA, as shown in Figure 5 below.

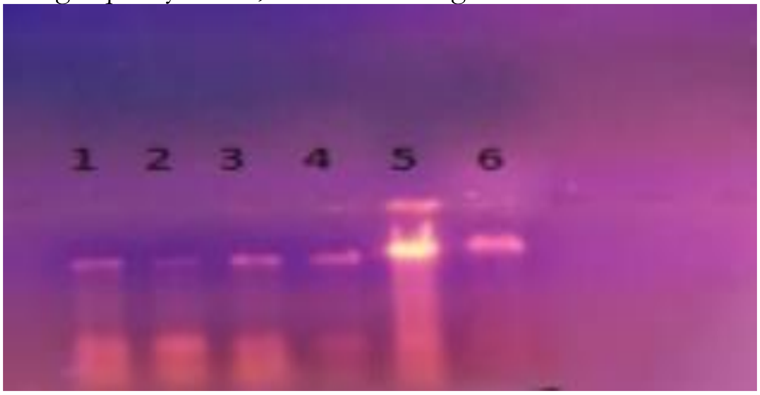

Figure 5. Genomic DNA band on 1% agarose gel

16S rRNA Primers Amplification through PCR:

The 16S rRNA gene of the bacterial isolate was amplified using universal primers via PCR, resulting in a distinct band on the agarose gel (Figure 6), which confirmed successful amplification. Sequencing and BLAST analysis identified the isolate as Bacillus subtilis, and phylogenetic analysis confirmed its placement within the Bacillus clade.

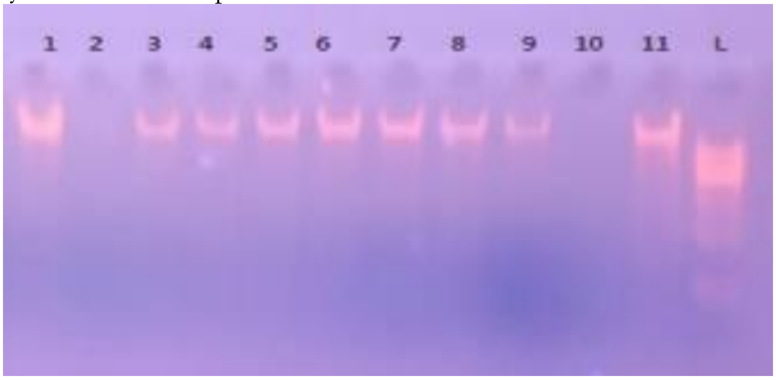

Figure 6. PCR primer result amplified band of 1.3 kb associated with *B. subtilis.*

Phylogenetic Analysis:

Bacillus subtilis isolates were obtained from root-associated samples of B. monosperma. Clustal W was used to align the sequences with reference bacterial species in the phylogenetic tree Figure 7.

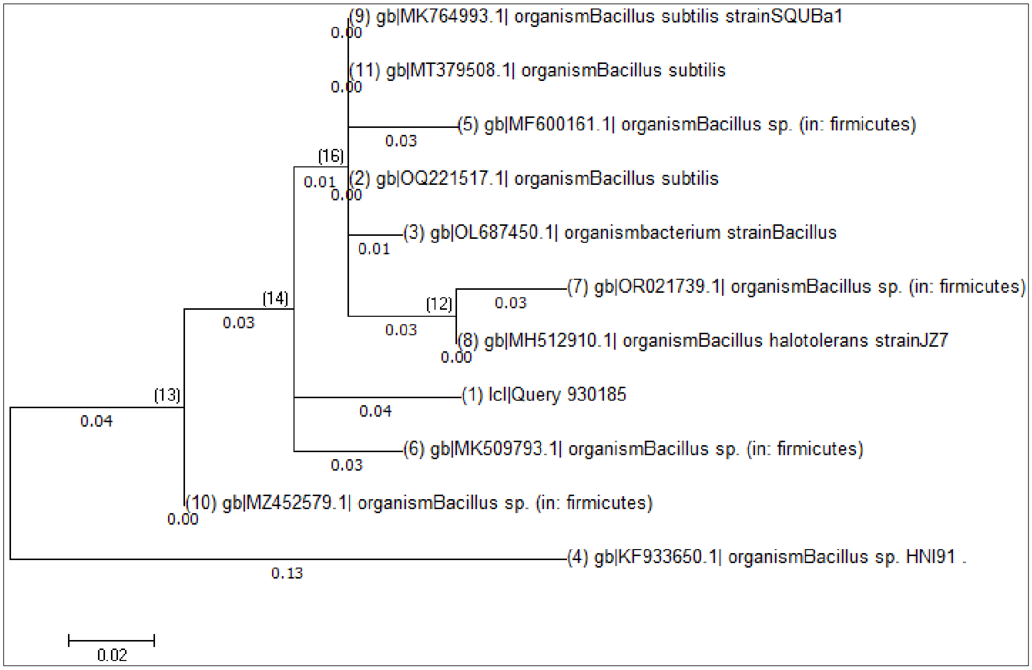

Figure 7. Eleven reference sequences used in phylogenetic analysis were obtained from GenBank, and a bootstrap analysis was done on a thousand replicas.

The Maximum Likelihood technique, based on the Tamura–Nei model, was used to determine the evolutionary history. The tree with the highest log probability (-242.8619) is presented. After estimating pairwise distances using the Maximum Composite Likelihood (MCL) approach, the resulting matrix underwent the Neighbor-Joining procedures. The initial tree(s) for the heuristic search were selected based on the topology having the highest log probability value. The tree is drawn to scale, and the number of substitutions per site is used to represent the length of each branch. A total of eleven nucleotide sequences were analyzed. The included sites comprised noncoding regions and first, second, and third codon positions. Positions with missing or incomplete data were excluded. The final dataset consisted of a total of 80 sites. MEGA 12 was utilized for the implementation of evolutionary analysis.

Discussion:

This study aimed at molecular characterization and phylogenetic analysis, focusing on bacteria that were isolated from the roots of *B. monosperma* (Lam.) Taub. from Narowal, Pakistan. To achieve these objectives, root-associated bacteria were isolated using a series of steps to obtain growth-promoting bacterial strains. Molecular identification was performed using 16S rRNA genetic markers, followed by further analysis. Sequencing of isolated species using 16S rRNA allowed the characterization of a strain of B. subtilis. In a similar study [25], the recommended method for identifying a bacterial species is typically rRNA sequence-based analysis. The bacterial species' 16S rRNA gene comprises highly conserved regions. 16S rRNA analysis is widely used to characterize novel strains. In a similar study conducted by [26], isolation was performed on collected samples. The researchers carried out MIS analysis, 16S rRNA sequencing, and region-specific sequencing. Based on nucleotide homology, the results indicated that *B. megaterium* is a highly prevalent bacterial species in the rhizomes of *V. turcica*. These findings support the isolation of bacteria belonging to the genus *Bacillus*.

Morphological attributes of the isolated bacteria were observed using a compound microscope and Gram staining methods. *B. subtilis*, a growth-promoting bacterium, was identified (Fig. 4), supported by [27]. The isolates' morphological characteristics were documented in accordance with Bergey's Manual of Systematic Bacteriology, and biochemical assays were conducted. Using standard methods, the potential symbiotic organism was molecularly characterized through PCR of 16S rRNA genes, and the resulting PCR product was sequenced. The plant growth-promoting bacterial properties were also assessed. In a similar study [28], identification of a PGPR at the molecular level was performed using 16S rRNA sequencing. DNA was isolated from a homogeneous culture, and its integrity was assessed using 1% agarose gel to confirm the presence of a single high-molecular-weight band (Fig. 6). A single PCR amplicon band was detected on a resolving agarose gel, indicating that the gene fragment had been amplified using PCR. The PCR amplicon underwent a column purification step to remove any contaminants. The PCR amplicon was then subjected to DNA sequencing using primer 27F. The next step involved BLAST analysis to compare the gene sequence with the NCBI GenBank database. The top ten sequences were selected based on the highest identity scores (Fig. 7) and then aligned using multiple alignment software.

The purpose of the current study was to isolate and identify plant growth-promoting rhizobacteria (PGPR) using forest soil samples. Characterization of *Bacillus cereus* was performed after 16S rRNA gene sequencing of isolate T1.4, which showed high sequence homology with well-known *B. cereus* strains [29]. A similar procedure was used in another study, whereby *Bacillus subtilis* isolates with plant growth-promoting effects were also retrieved. However, further studies are justified to identify other growth-promoting bacterial taxa that might protect the threatened species *B. monosperma* and enhance its robustness and growth under unfavorable environmental conditions.

Conclusion:

This study aimed to isolate and genetically profile plant root-associated bacteria of *B. monosperma*. The process involved collecting root samples and isolating bacteria using Luria–Bertani (LB) medium and extracting DNA. The 16S rRNA gene was amplified using PCR, and sequence chromatograms were examined. Using BLAST, multiple sequence alignments, MS Viewer, and NCBI taxonomy tools, the bacteria were identified as *Bacillus subtilis*. A phylogenetic tree was constructed to show the evolutionary relationships of the discovered *B. subtilis* strains. The present work provides valuable information on the microbial associates of the plant and contributes to knowledge about its microbiome. Further studies should focus on a broader range of microbial associates by utilizing advanced sequencing technologies. Laboratory and field trials are necessary to evaluate their functional characteristics and to investigate their roles in plant health, survival, conservation of endangered species, and the optimization of agricultural solutions.

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