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RHODOCOCCUS ERYTHROPOLIS AN ENDOPHYTIC BACTERIUM ISOLATED FROM ROOT NODULES OF *LUPINUS LATIFOLIUS*

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ABSTRACT

Lupinus latifolius is a perennial herbaceous and one of the most nitrogen-fixing legumes. The bacterial strain that amplified part of the16S rRNA gene, DT02, was isolated from the root nodule of *L. latifolius* planted in Nezahat Gökyiğit Botanical Garden, Istanbul, Turkey. The isolated bacterium is Gram-positive, rod-shaped, acidic, facultatively anaerobic, and fast-growing. gDNA was isolated from the bacterium, and amplified gDNA of the expected size were sequenced and analyzed using bioinformatics tools. The identified partial 16S rRNA sequence was submitted to the NCBI database (accession number MN599098.1). Phylogenetic analysis based on the 16S rRNA gene sequence showed that strain DT02 belonged to the genus *Rhodococcus* within the family Nocardiaceae and was closely related to *Rhodococcus* sp. strain 137A-21with a similarity value of 99.93%. This bacterium can have plant-growth-promoting abilities. Additionally, the rhizome of *L. latifolius* was also harvested and analyzed for mineral element content by ICP-OES analysis. According to the measurement, the rhizome was rich in terms of Ca (2000 mg kg-1), Na (153 mg kg-1), Mg (2000 mg kg-1), and K (143 mg kg-1).

KEYWORDS:
MIS, Nezahat Gökyiğit Botanical Garden, 16S rRNA, *Lupinus latifolius*

INTRODUCTION

Lupinus latifolius Lindl. Ex J. Agardh (broadleaf lupin) belongs to the genus *Lupinus*, family Fabaceae. *L. latifolius*, a dicot, is perennial herbaceous and varies in morphology due to its adaptation in several types of habitat. The inflorescence bears many whorls (Figure 1A). The flowers of this species are purple to blue (Figure 1B). This species is one of the most nitrogen-fixing legumes since it has nitrogen-fixing nodules; hence it has great potential for studies concerning nitrogen fixation. Also, some legumes like *L. latifolius* establish symbiosis with rhizobia to develop specialized structures named root nodules, where rhizobia can fix nitrogen; this interaction depends on each part of the symbiosis. Plant growth and development was affected by rhizosphere and rhizoplane microorganisms. Various bacterial genera such as *Alcaligenes*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, and *Serratia* have plant growth-promoting characteristics [1, 2].

This study mainly focused on *L. latifolius*. The bacterial strain identified in this study as a member of the genus *Rhodococcus*, DT02. The genus *Rhodococcus*, described by Bell et al. (1998) [3], comprises Gram-positive, rod-shaped, non-motile, acidic, facultatively anaerobic, and fast-growing.

Most species of the genus *Rhodococcus* have been isolated from soil. However, there are also some legume endophytes in this genus, such as *Rhodococcus erythropolis*, isolated from the rhizome of *L. latifolius* growing at the Nezahat Gökyiğit Botanical Garden (NGBG) in Istanbul, Turkey.

MATERIALS AND METHODS

Nodule samples were removed from the rhizome of *L. latifolius* planted at NGBG (Figure 2) and were taken into sterile cups containing sterile distilled water. Samples were directly kept at +4 ℃ until bacterial isolation.

For mineral element analysis, the collected rhizome was dried at 70°C for 2 days, then weighed at room temperature, and crushed into powder in a mortar. Acid digestion method using a closed-vessel microwave system (MarsExpress; CEM Corp., Matthews, NC, USA) with 5 ml of 65 % HNO_3 and 2 ml of 30 % H_2O_2 for a 0.3-0.4 g sample was implemented for mineral analysis. Mineral element (Ca, K, Mg, Na) content was determined according to the method described by Olsen et al. (1954) [4] using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (Vista-Pro Axial; Varian Pty Ltd, Mulgrave, Vic., Australia). Certified standard reference materials obtained from the National Institute of Standards and Technology (NIST; Gaithersburg, MD, USA) were used for normalization of the measurements.

The collected nodule samples were used for bacterial isolation. Isolated nodules were surface sterilized in 2.5% (w/v) $HgCl_2$ for 3 min., rinsed three times with sterile distilled water, and then grinded in liquid yeast extract mannitol (YM) medium (l-1: 10 g mannitol, 1 g yeast extract, 0.2 g K2HPO4, 0.2 g MgSO4 .7H2O, 0.5 g NaCl) described by Vincent (1970) [5]. The diluted nodules-liquid YM medium mix was taken on YM agar medium and incubated at 28°C until bacterial colonies were obtained. For the pre-selection of plant growth-promoting rhizobacteria (PGPRs), YM agar medium containing either Bromothymol blue or Congo red was used. Gram staining of the isolates was also conducted. To obtain single colonies, suspensions of bacteria were diluted in liquid medium as 1 in 6 serial dilutions and reseed on solid medium.

The cultures purified from a single colony were used for further molecular analysis. A PCR 16S rRNA characterized the isolates. 16S rRNA genes were amplified using D1F (5' AGAGTTTGATCCTGGCTCAG -3') and D1R (5' AAGGAGGTGATCCAGCC -3') primers. PCR was implemented in a 25-µL reaction volume by mixing the final concentration of 5 ng DNA extract with the polymerase reaction buffer (1X), Taq DNA polymerase (0.125 U/µL), dNTPs (0.2 mM), and each primer (0.8 µM). PCR amplification was performed in a thermal cycler (Applied Biosystems, Singapore) adjusted to the following PCR conditions: initial denaturation at 95 °C for 7 min, 35 amplification cycles (denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 min), and final extension step at 72°C for 5 min. PCR products were checked by agarose (1%, w/v) gel electrophoresis.

Isolated and purified PCR products were directly sequenced. The sequencing service was commercially provided by BM Laboratory Systems, Ankara, Turkey (https://www.bmlabosis.com/). The consensus sequences of 16S rRNA were used to search for similar sequences in GenBank at the National Center for Biotechnology Information (NCBI), Bethesda, USA, using the BLAST search program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [6]. Phylogenetic analysis was performed by the Molecular Evolutionary Genetics Analysis (MEGA) program version 7.0 (https://www.megasoftware.net/). The phylogenetic tree was generated from the nucleotide sequences in the dataset using the Neighbour Joining method based on the Tamura 3-parameter method [7]. Aligned 16S rRNA sequence was evaluated with bootstrap analysis (1000 replicates) [8].

MIS analysis of the bacterial isolate was implemented using gas chromatography (6890N GC, Agilent Technologies INC., USA) and MIS Software (Sherlock 6.0 MIDI, Inc., Newark, DE, 2005). The resulting data were compared with the commercial database (RTSBA6). Fatty acid methyl ester (FAME) groups were identified by MIS software. The unit with the highest score between the diagnosis results was considered as the ideal result. The 16S rRNA sequence of the isolate DT02 was submitted to GenBank under accession numbers MN599098.1.

RESULTS

Nodules of leguminous crops present an excellent environment for the development of various endophytic bacteria, which have a role in plant growth in a positive way. The present study reports the morphological and molecular characterization of *R. erythropolis* collected from the rhizome of *L. latifolius* planted at NGBG in Turkey. According to pre-selection testing given in the Materials and Methods section in detail, twenty bacterial colonies, which were morphological similarities in color, size, and shape were developed on the YM medium; hence one single colony was selected for further analysis, including molecular characterization.

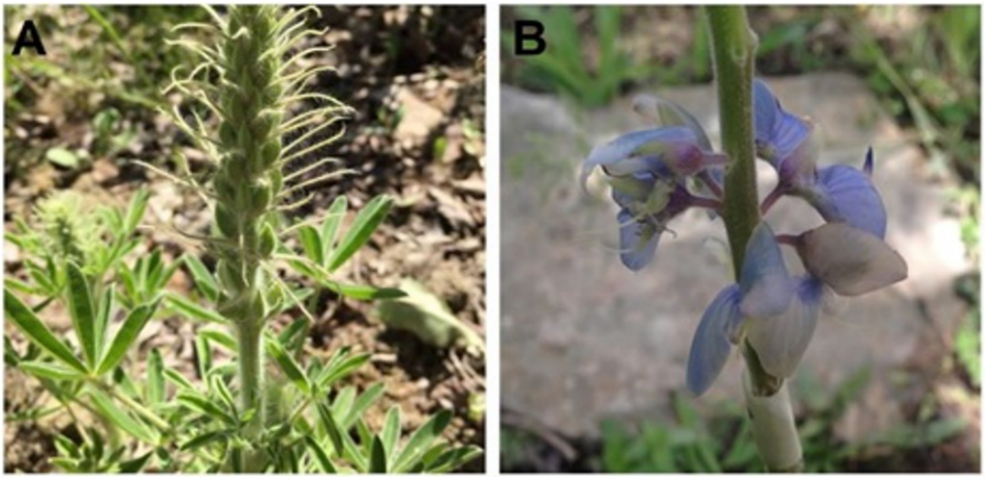

FIGURE 1
General view of *L. latifolius* planted in NGBG; (A) Buds of *L. latifolius* (B) Flowers of *L. latifolius*

FIGURE 2
The image of the root nodule. The left-to-right image is focused and indicated by an arrow

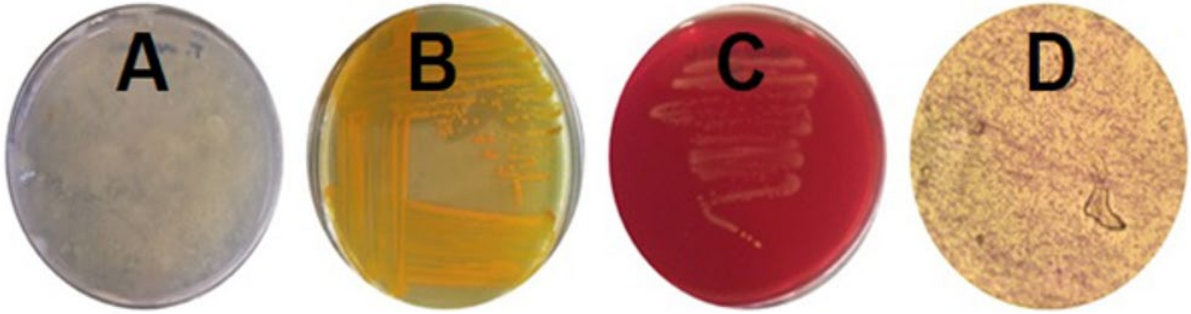

FIGURE 3
Morphological and physiological characteristics of isolated bacterial species from rhizomes of *L. latifolius*

Single colonies were purified to obtain bacterial cultures. Single colonies were detected at dilutions until 10^{-6} from the nodules of collected rhizome samples. Isolated bacteria were cultured on the media, including Congo red and Bromothymol blue, to identify their reactions against these chemicals for pre-selection. All isolated bacteria showed acidic and pinkish color. According to the Gram staining of the isolates, all of them were Gram-positive. Since the twenty isolates gave the same response in pre-selection tests, further analysis was conducted on one bacterial isolate to save time and labor. The reaction of the isolate against Congo red, Bromothymol blue, and gram-staining was shown in Figure 3. The characteristics such as colony colors, the absorption of Congo red and Bromothymol blue, pigmentation, Gram staining of the isolated bacterium are similar to the *Rhodococcus* species described in Alvarez and Steinbuchel (2010) [9].

After the pre-selection of the bacterium, 16S rRNA gene sequence analysis and Microbial Identification System (MIS) analysis were conducted on the isolate. The bacterial identity of the isolate was tried to be identified by fatty acid methyl ester (FAME) analysis using the MIS. As a result of MIS analysis, *R. erythropolis* (0.607) was identified for the strain DT02. The 16S rRNA was successfully amplified from DNA using the primer pairs D1F/D1R. The PCR amplified product of the isolate was subjected to sequence analysis, and the obtained readings were compared using the BLAST search program. The BLAST analysis of the 16S rRNA sequence data supported the closest match (99% similarity) in the NCBI GenBank database was found to be with *R. erythropolis* (Table 1). Thus, other phylogenetic marker genes such as virulence genes and genes encoding for outer membrane porin proteins were not be needed to apply for confirmation due to high similarities of 16S rRNA sequence with other rhodococcal sequences by the BLAST analysis. 16S rRNA gene sequence-based phylogenetic tree was

generated, which shows the relationship between other rhodococcal species (Figure 4).

The sequence readings obtained for the isolate were also used for the construction of the phylogenetic tree to analyze the relationships among the *Rhodococcus* species based on their 16S rRNA sequence similarities (Figure 4). The phylogenetic tree proposed two major clades. The 26 isolates obtained from available data based at NCBI as a result of the comparison of 16S rRNA sequence similarities were clustered into two branches by comparison of the sequences identified from the 16S rRNA PCR profiles (Figure 4). 23 isolates were pooled together into the same branch, but three isolates were grouped into the different branches (Figure 4) ICP-OES is used often to quantify mineral element content in any material, and in this study, this instrument was to identify macronutrients (Ca, Na, Mg, and K) of collected rhizome. According to ICP-OES measurement of the rhizome, it was rich in terms of Ca (2000 mg kg-1), Na (153 mg kg-1), Mg (2000 mg kg-1), and K (143 mg kg-1) according to the critical level of mineral elements reported by Andersen (2007) [10].

Plants are significant for the community and diversity of bacteria [11]. Due to the abundance of *R. erythropolis*, it is suggested that these species are well adapted or more competitive in *L. latifolius* rhizoplane and endorhizosphere. *R. erythropolis* might have great potential in fixing Nitrogen (N) in *L. latifolius*. Nitrogen fixation is due to the nitrogenase enzyme synthesized by rhizobia, not by the plant; in the absence of rhizobia, legumes are not able to fix

FIGURE 4

Neighbor-joining tree based on partial sequences of the 16S rDNA region of the isolate with each other for phylogenetic inference. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches.

TABLE 1
Sequences similarities according to the BLAST search against NCBI database based on identified 16S rRNA sequences

The BLAST search based on identified 16S rRNA ITS sequences		
Species	Accession No	Similarity (%)
Rhodococcus sp. strain 137A-21	MK143178.1	99.93
R. erythropolis isolate 19	LT891935.1	99.93
R. erythropolis strain S13006	KF956668.1	99.93
R. erythropolis strain BZ4	HQ588862.1	99.93
R. erythropolis strain SBUG 2060 L1	KU663058.1	99.93
R. erythropolis strain HS5	AY168583.1	99.85
R. erythropolis strain HO-KS22	MN622878.1	99.85
Rhodococcus sp. KUA-4	AB376625.1	99.85
Rhodococcus sp. 15-3	EU103620.1	99.85
Rhodococcus sp. 1B	EF143410.1	99.85
R. erythropolis strain HS17	AY168595.1	99.85
R. erythropolis strain HS4	AY168582.1	99.85
Rhodococcus sp. IA1XBOX	AY512640.1	99.85
Rhodococcus sp. Amico51	AY512639.1	99.85
R. erythropolis	AF532870.1	99.85
Rhodococcus sp. SA1	AY864650.1	99.85
Rhodococcus sp. CH-H63-1	KC881306.1	99.78
Rhodococcus sp. BEN	GU565219.1	99.78
R. erythropolis strain HS2	AY168580.1	99.78
Rhodococcus sp. An6	AJ551145.1	99.78
Rhodococcus sp. wp20	AJ551100.1	99.78
Uncultured *Rhodococcus* sp. isolate DGGE gel band zm4	KX911474.1	99.78
R. erythropolis strain K85	KF976880.1	99.78
Rhodococcus sp. LINCER1	JN896345.1	99.78
R. erythropolis strain DLC-terla	EU043316.1	99.78

nitrogen. *Rhodococci* are best known for their high potential in degrading organic and xenobiotic compounds. However, they can also be essential to produce industrial metabolites, including bio-surfactants and carotenoids. Quorum sensing disruption is well for nodulation and regulates several interactions in the rhizosphere. It has great potential in disrupting quorum sensing-based communication of Gram-negative bacteria by degrading N-acyl-homoserine lactone signaling molecules; hence it prevents plant disease [9]. Besides, according to mineral element analysis, levels of Ca (2000 mg kg-1), Na (153 mg kg-1), Mg (2000 mg kg-1), and K (143 mg kg-1) minerals were high in rhizomes of *L. latifolius*. It could be possible due to the plant growth-promoting abilities of *R. erythropolis* that was found in the endosphere of *L. latifolius*. *R. erythropolis* is known for its usefulness in removing industrial waste and oil degradation that this bacterium can enhance the oxidizing of oil and degrading of waste from the soil and increase the mineral element accumulation in plants [12]. *R. erythropolis* is a good producer of biosurfactants (microbial surfactants), which are used for environmentally safe technologies for bioremediation. It is known from the previous study that *R. erythropolis* can synthesize carotenoids and has plant growth-promoting activity [13, 14]. Besides, *R. erythropolis* can help root proliferation and increase mineral uptake by the plant.

CONCLUSIONS

R. erythropolis with plant growth-promoting ability was isolated from the rhizosphere of *L. latifolius*, where a significant nodule production of the analyzed plant. The present study contributed to the knowledge of the useful bacterial species associated with nodule production in *Lupinus* sp. Further analysis involving morpho-physiological characters of the isolate would be sufficient in order to characterize the species associated with root nodulation properly. Besides, the role of different species on the nodulation of *Lupinus* sp. can be studied in expression in different environmental conditions. The possible specific interactions between *R. erythropolis* and *L. latifolius* has not been studied. Further investigations on the *Rhodococcus* PGPR effect on *Lupinus* sp. should be carried out.

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