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Endophytic bacterial diversity of *Origanum rotundifolium*: isolation and molecular identification of strains

Origanum rotundifolium'un endofitik bakteri çeşitliliği: izolasyon ve moleküler tanımlama

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ABSTRACT

Endophytic bacteria in medicinal and aromatic plants provide valuable insights into the microbial diversity of these plants and form the basis for research on their ecological roles and biotechnological potential. In this study, the endophytic bacterial diversity of *Origanum rotundifolium* Boiss. was determined, and a total of eleven endophytic bacteria were isolated from the root, stem, and leaf tissues of *O. rotundifolium*. The isolated bacteria were characterized based on certain morphological and biochemical properties. Molecular identification was performed using 16S rRNA gene sequencing. The identified isolates were as follows: *Kocuria rosea* (OR1), *Schumannella* sp. (OR2), *Micrococcus luteus* (OR3, OR7), *Bacillus proteolyticus* (OR4), *Curtobacterium flaccumfaciens* (OR5), *Staphylococcus* sp. (OR6, OR10), *Microbacterium proteolyticum* (OR8), *Microbacterium* sp. (OR9), and *Actinomyces* sp. (OR11). This study reports, for the first time, the diversity of endophytic bacteria isolated from *Origanum rotundifolium*..

ÖZ

Tıbbi ve aromatik bitkilerdeki endofitik bakteriler, bu bitkilerin mikrobiyal çeşitliliği hakkında değerli bilgiler sunmakta ve ekolojik rollerinin yanı sıra biyoteknolojik potansiyellerine dair araştırmalar için temel oluşturmaktadır. Bu çalışmada, *Origanum rotundifolium* Boiss.'un endofitik bakteri çeşitliliği belirlenmiş ve toplamda on bir endofitik bakteri, *O. rotundifolium*'un kök, gövde ve yaprak dokularından izole edilmiştir. İzole edilen bakteriler, bazı morfolojik ve biyokimyasal özelliklerine göre karakterize edilmiştir. Moleküler tanımlama, 16S rRNA gen dizileme yöntemiyle yapılmıştır. Tanımlanan izolatlar sırasıyla: *Kocuria rosea* (OR1), *Schumannella sp.* (OR2), *Micrococcus luteus* (OR3, OR7), *Bacillus proteolyticus* (OR4), *Curtobacterium flaccumfaciens* (OR5), *Staphylococcus sp.* (OR6, OR10), *Microbacterium proteolyticum* (OR8), *Microbacterium sp.* (OR9) ve *Actinomyces sp.* (OR11) olarak belirlenmiştir. Bu çalışma, *Origanum rotundifolium*'dan izole edilen endofitik bakteri çeşitliliğini ilk kez rapor etmektedir.

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1. INTRODUCTION

The genus *Origanum*, a significant group within the Lamiaceae family, is of considerable medicinal value. Species within this genus are well-documented for their antimicrobial, antioxidant, and therapeutic properties. Phytochemical analyses of these species have led to the identification of numerous biologically active compounds (Baydar et al., 2004; Kulisic et al., 2004; Chisti et al., 2013). Historically, *Origanum* species have been extensively used both as culinary spices and in traditional medicine (Fleisher & Fleisher, 1988).

Origanum rotundifolium is known as "Mercan köşk" in Türkiye. It is naturally distributed in a limited area extending from northeastern Türkiye to the western part of Transcaucasia, which includes Georgia and Armenia (POWO, 2025). This species, which grows at altitudes ranging from 250 to 1300 meters on rocky slopes, is

shrub-like with soft, hairy leaves and can reach a height of approximately 30 cm. Its flowering period occurs between June and September (Davis, 1982). In Türkiye, it naturally grows, particularly in the Artvin province of the Black Sea Region.

O. rotundifolium is a plant characterized by its rich content of essential oils and a wide range of biological activities. Studies analyzing the chemical composition of its volatile oils and extracts have identified compounds such as thymol and carvacrol as major contributors to its antioxidant and antibacterial properties (Dikbaş et al., 2009; Goze et al., 2009; Nohutçu et al., 2024). Among these, carvacrol is particularly noteworthy due to its antimicrobial, antitumor, and analgesic effects (Başer, 2008). In addition, the essential oil of *O. rotundifolium* has been evaluated for its antibacterial activity against 20 different plant pathogenic bacteria, and its insecticidal potential has also been investigated (Görmez et al., 2016; Yıldırım et al., 2011).

Despite all these studies, to our best of knowledge, there is no information available in the literature regarding the endophytic bacterial diversity of *O. rotundifolium*. However, endophytic bacteria are microorganisms that promote plant growth, enhance the production of secondary metabolites, and contribute to reducing chemical inputs in sustainable agriculture (Arora et al., 2018). These bacteria function through various mechanisms, including hormone production, siderophore secretion, phosphate solubilization, nitrogen fixation, reduction of ethylene levels, and biological control of phytopathogens. In addition, they can enhance the plant's tolerance to biotic and abiotic stresses and increase its antioxidant capacity (Mohamad et al., 2018).

The aim of this study is to identify the endophytic bacterial diversity of *O. rotundifolium*, and to establish a foundation for future research into the biotechnological potential of these bacteria.

MATERIALS AND METHODS

2.1. Plant material

Various *Origanum rotundifolium* was collected in June 2022 in Artvin, Türkiye, at an elevation of 697 m (N41° 10' 26.4'', E041° 53' 38.6''). Plant parts (including stems, flowers, and leaves) were examined to ensure the selection of healthy specimens. The material was placed in plastic bags to prevent compression, with ventilation holes made on the top and sides to facilitate airflow. The samples were then transported to the laboratory and stored at –20°C for subsequent analysis.

2.2. Isolation of endophytic bacteria

The bacterial isolation process was carried out using root, stem, and leaf samples of *Origanum rotundifolium*. The surface sterilization protocol was adapted and optimized from the comprehensive review by Sahu et al. (2022) to suit different tissues of *O. rotundifolium*.

Each sample was washed with sterile distilled water to remove surface contaminants, followed by sterilization with ethanol and sodium hypochlorite solution in succession. Root samples were sterilized in 70% ethanol for 3 minutes, in a 2% sodium hypochlorite solution for 4 minutes, and again in 70% ethanol for 3 minutes. Stem samples were sterilized in the same solutions for 3 minutes each. Leaf samples were sterilized in 70% ethanol for 3 minutes, in 2% sodium hypochlorite for 2 minutes, and finally in 70% ethanol for 2 minutes. After sterilization, all samples were rinsed five times with sterile distilled water and dried using sterile filter paper. The samples were then divided into small pieces, transferred to sterile agar plates, and homogenized. Serial dilutions of the resulting homogenate were prepared and plated onto agar media. Incubation was performed at 30°C for 4-5 days, after which endophytic bacteria were isolated from the developed colonies, purified, and stored at -20°C.

2.3. Characterization of endophytic bacteria

Some morphological features (cell morphology, motility), biochemical characteristics (Gram reaction, catalase, oxidase) were determined (Harley & Prescott, 2002).

2.4. Molecular identification of endophytic bacteria

Endophytic bacterial colonies were cultured on agar plates at 30°C for approximately 18 hours. DNA was isolated from bacterial samples using a commercial DNA isolation kit (Norgen Biotek Corp., Canada), following the manufacturer's instructions. The 16S rRNA gene region was amplified by PCR using universal primers 27F (AGA GTT TGA TCM TGG CTC AG) and 1492R (GGY TAC CTT GTT ACG ACT T). A 20 µl PCR reaction mixture was prepared according to the PCR Master Mix protocol (A.B.T.™). For

each sample, the reaction mixture contained 10 µl Hs-Taq master mix, 1 µl forward primer, 1 µl reverse primer, 4 µl sample DNA, and 4 µl dH2O. The prepared mixtures were placed in Thermal Cycler (Bio-Rad, Hercules, California, USA) and programmed under the following conditions: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, primer annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 5 minutes. PCR products were examined by electrophoresis on a 1% agarose gel in 1x TAE buffer to confirm the presence of the desired amplicon. The amplified product was sequenced by a commercial sequencing service (Macrogen, Netherlands), and bidirectional sequences were obtained. The sequencing results were used for identification through BLAST at NCBI GenBank. The obtained sequences were processed using BioEdit (version 7.09) and aligned using ClustalW (Hall, 1999). The phylogenetic analysis was performed based on the obtained results using MEGA 11 software (Tamura et al., 2021).

3. RESULTS AND DISCUSSIONS

In this study, the endophytic bacterial diversity of *Origanum rotundifolium* was determined for the first time, and a total of eleven endophytic bacterial isolates were obtained. The isolation process was carried out using root, stem, and leaf tissues of *O. rotundifolium*. The isolated bacteria were characterized by examining their morphological features and biochemical characteristics. All isolates were identified as Gram-positive. Isolates OR1, OR2, OR3, OR6, OR7, and OR10 exhibited coccus morphology, while isolate OR4 displayed rod shapes. The OR11 isolate demonstrated irregular rod shapes, and the remaining isolates exhibited short rod morphology. All isolates were non-motile and catalase-positive. The oxidase test results were positive for isolates OR1 (weakly positive), OR3, and OR4 (Table 1).

Table 1. Morphological and biochemical properties of endophytic isolates

Isolate Code	Gram Stain	Cell Morphology	Catalase	Oxidase	Motility
OR1	+	Coccus	+	wp	-
OR2	+	Coccus	+	-	-
OR3	+	Coccus	+	+	-
OR4	+	Rod	+	+	-
OR5	+	Short rod	+	-	nd
OR6	+	Coccus	+	-	-
OR7	+	Coccus	+	+	-
OR8	+	Short rod	+	-	-
OR9	+	Short rod	+	-	-
OR10	+	Coccus	+	-	-
OR11	+	Irregular rod	+	nd	-

+: positive result, wp: weakly positive, –: negative result, nd: not determined

Molecular identification was performed using the 16S rRNA gene sequencing method, and the isolates were identified as follows: *Kocuria rosea* (OR1), *Schumannella sp.* (OR2), *Micrococcus luteus* (OR3, OR7), *Bacillus proteolyticus* (OR4), *Curtobacterium flaccumfaciens* (OR5), *Staphylococcus sp.* (OR6, OR10), *Microbacterium proteolyticum* (OR8), *Microbacterium sp.* (OR9), and *Actinomyces sp.* (OR11). The sequencing data for each isolate were deposited in the GenBank database, and accession numbers were obtained for each isolate: PV652936 (OR1), PV652937 (OR2), PV652938 (OR3), PV652939 (OR4), PV652940 (OR5), PV652941 (OR6), PV652942 (OR7), PV652943 (OR8), PV652944 (OR9), PV652945 (OR10), and PV652946 (OR11). Phylogenetic analysis was conducted using MEGA 11 software, developed in the USA (Tamura et al., 2021), based on the obtained data, as illustrated in Figure 1.

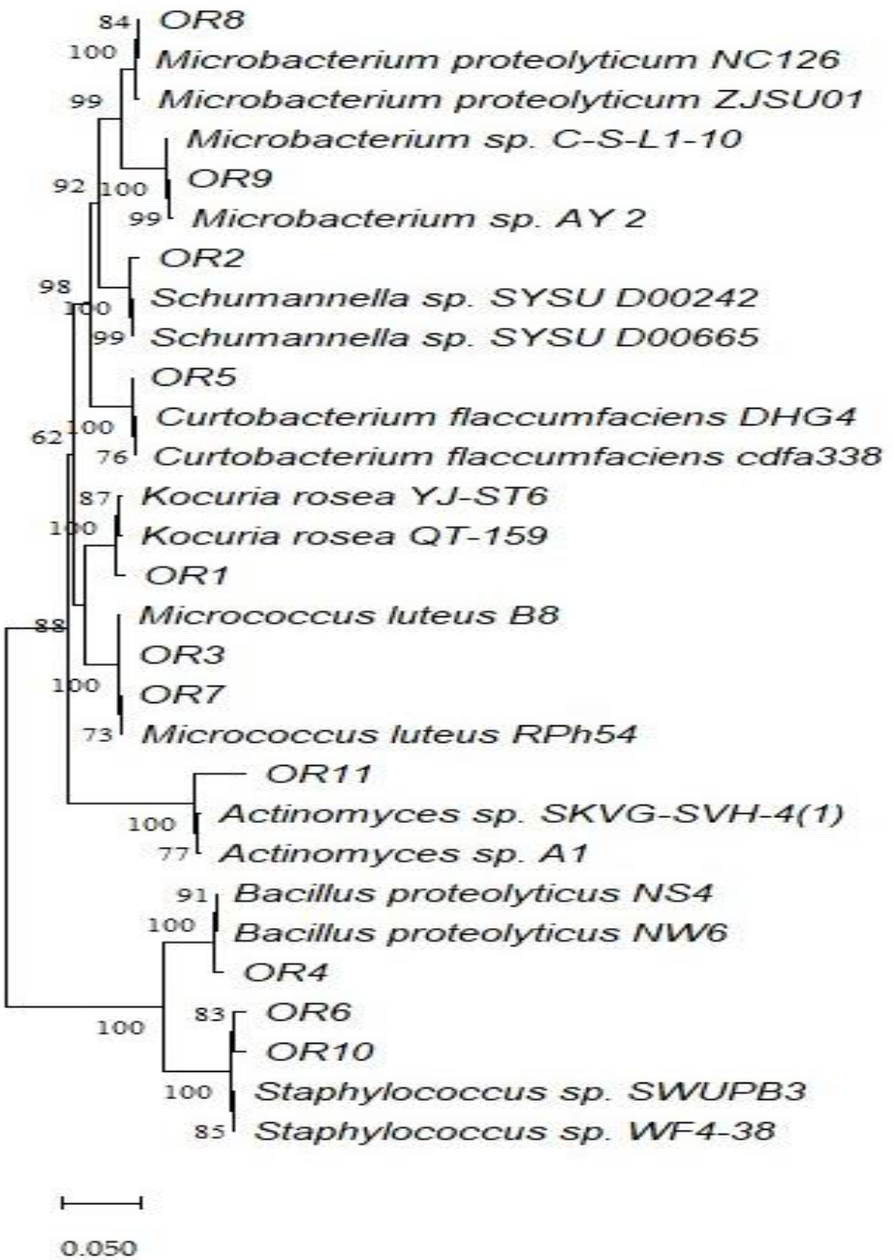

Figure 1. Phylogenetic relationships of endophytic bacteria (MEGA Software 11).

It was observed that some bacterial species and genera isolated from different tissues of *O. rotundifolium* were present in multiple tissue types. *Micrococcus luteus* (OR3, root; OR7, stem) and *Staphylococcus sp.* (OR6, stem; OR10, leaf) were detected in both tissue types. This suggests that these bacteria may exhibit a non-tissue-specific distribution within the plant and may possess a broad ecological tolerance. In contrast, the isolates such as *Kocuria rosea* (root), *Schumannella sp.* (root), *Bacillus proteolyticus* (stem), *Curtobacterium flaccumfaciens* (stem), *Microbacterium proteolyticum* (stem), *Microbacterium sp.* (stem), and *Actinomyces sp.* (leaf), isolated from a single tissue type, may show a tendency for tissue-specific colonization (Table 2). Furthermore, it is believed that this study represents the first report of the isolation of *Schumannella sp.* as an endophyte from *O. rotundifolium*.

Table 2. Endophytic bacteria isolated from *Origanum rotundifolium* and their isolation parts

Isolate Code	Plant Part	Bacterial Isolate
OR1	Root	*Kocuria rosea*
OR2	Root	*Schumannella sp.*
OR3	Root	*Micrococcus luteus*
OR4	Stem	*Bacillus proteolyticus*
OR5	Stem	*Curtobacterium flaccumfaciens*
OR6	Stem	*Staphylococcus sp.*
OR7	Stem	*Micrococcus luteus*
OR8	Stem	*Microbacterium proteolyticum*
OR9	Stem	*Microbacterium sp.*
OR10	Leaf	*Staphylococcus sp.*
OR11	Leaf	*Actinomyces sp.*

On the other hand, there are a limited number of studies on the endophytic bacteria of *Origanum* species. In a prior study, cultivable bacteria were isolated from different plant parts of *Origanum vulgare*, such as flowers, leaves, and stems. It was reported that a higher proportion of the isolated bacteria were Gram-positive. Additionally, the researchers detected genera such as *Pseudomonas*, *Sphingomonas*, *Curtobacterium*, *Arthrobacter*, *Bacillus*, *Staphylococcus*, and *Rhodococcus* in multiple tissue types, while bacteria like *Pantoea*, *Rhizobium*, *Paenibacillus*, *Micrococcus*, and *Rathayibacter* were only detected in the flower tissue. The leaf and stem were reported to be the plant parts with the highest species diversity (Castronovo et al., 2020). In contrast, it was found that all endophytic bacteria isolated from *O. rotundifolium* were Gram-positive in the current study. *Staphylococcus sp.* was similarly detected among bacteria isolated from different tissue types. *Micrococcus luteus* was also isolated from various parts of the plant, including the root and stem. In our study, the highest species diversity was found in the root and stem tissues.

Compared to the literature, the findings obtained in this study share certain similarities with the endophytic bacteria reported in other species of the *Origanum* genus. For example, endophytic bacteria isolated from the seeds of *O. heracleoticum* were identified as belonging to the genera *Pseudomonas*, *Pantoea*, *Erwinia*, *Paenibacillus*, *Peribacillus*, *Bacillus*, *Staphylococcus*, and *Kocuria*. Furthermore, the study highlighted the antibiotic resistance profiles and antibacterial activities of seed endophytes, revealing the pharmaceutical potential of these endophytic bacteria (Semenzato et al., 2022). The detection of *Bacillus*, *Staphylococcus*, and *Kocuria* genera in *O. rotundifolium* suggests that these genera are widespread among *Origanum* species and are likely endophytic genera adapted to the host tissue.

In other studies, conducted by Polito et al. (2022) and Vitali et al. (2023), endophytic bacteria of *O. vulgare* were isolated, including bacteria from the genera *Paenibacillus*, *Bacillus*, and *Arthrobacter*, as well as strains such as *Paenibacillus* sp., *Priestia* sp., and *Metabacillus* sp., which were found to possess antibacterial potential. Moreover, it has been reported that endophytic bacteria not only support plant health but also possess the ability to enhance the therapeutic properties of the plant, performing significant biological functions. In this context, it is believed that *O. rotundifolium* endophytes may also play similar functional roles.

4. CONCLUSION

This study is the first to identify the endophytic bacterial diversity of *O. rotundifolium*. Furthermore, the isolation of *Schumannella sp.* as an endophyte from an *Origanum* species for the first time makes a unique contribution to the literature and highlights the need for further investigation of its potential symbiotic relationships with different plant species. The isolated bacteria represent promising candidates for various biotechnological applications, such as the production of novel antimicrobial compounds and the development of bioformulations that promote plant growth, as they are associated with medicinal aromatic plants.

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