



## Isolation Endophytic Bacteria from Leaf Glands and Seeds of *Ardisia crenata* Plant and its Molecular Detection



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### Abstract

**I**SOLATION endophytic bacteria from leaf glands (nodules) and seeds of *Ardisia crenata* plant and cultured on Nutrient agar (NA) medium individually. The results of two types of bacterial isolated were grown in light yellow after incubation for 24 hours and took a circular shape slightly convex and Gram positive. The isolated bacteria from leaf nodules sensitive to all antibiotics used in this study, while the bacteria from seeds was resistance only to 10 µg/ml Cefotaxime and 10 µg/ml Ceftriaxone. The data of DNA sequencing analysis of the nitrogenous bases for 16S rRNA gene after PCR technique with the global database, National Center for Biotechnology Information (NCBI), showed 99.29% similarity between sequence of leaf isolated bacteria and *Bacillus megaterium* strain YSBM5 registered, therefore its recorded for the first times at *Bacillus* sp. JA Leaves, while 92.02% similarity between sequence of seeds isolated bacteria and *Bacillus subtilis* then its recorded at *Bacillus* sp. JA seed strain in NCBI.

**Keywords:** *Ardisia crenata*, *Bacillus* sp., 16S rRNA, PCR.

### Introduction

One of the most common bacterial genera in soil is the genus *Bacillus*, belong to the family *Bacillaceae*, order *Bacillales*, class *Bacilli*, and phylum *Firmicutes* [1, 2]. These endophytic bacteria are incredibly diverse and have been shown to support crop growth through a variety of mechanisms, such as fixing atmospheric nitrogen, producing phytohormones including auxin, cytokinin and gibberellin [3], increasing the bioavailability of minerals like zinc, phosphorus and sequestering iron through siderophores. In addition, production of lytic enzymes, antibiosis, detoxification, destruction of pathogen virulence factors, and the manufacture of ethylene catabolism-related 1-aminocyclopropane-1-carboxylate (ACC) deaminase [4, 5, 6].

Many species in this genus have been isolated from a wide range of environments, including plant-associated habitats. For example *B. licheniformis*, *B. polymyxa*, *B. cereus*, *B. megaterium*, *B. subtilis*, *B. amyloliquefaciens* isolated from tomato roots [7], *B. safensis*, *B. pumilus* and *B. altitudinis* were isolated from rhizosphere of crabgrass, maize and rice [8]. The rhizosphere of sugarcane revealed the presence of a novel diazotrophic bacterium called

*Bacillus rhizosphaerae*, which could raise plants' dry biomass [9].

*Ardisia crenata* is a species belonging to the genus: *Ardisia*, subfamily: *Myrsinoideae*, family: *Primulaceae* according to the APGIII classification (2009). Common names include Hen's Eyes, Christmas berry, Australian Holly, Coral Berry, Coral *Ardisia*, and Scratch Throat. [10].

The leaves of *Ardisia crenata* are characterized by having special structures or nodes along the edges of the leaves sometimes they are referred to as the leaf gland, which is specialized glandular tissue that contains endophytic bacteria within it [11], the presence of endophytic bacteria is not limited to leaves only, as recent molecular studies have proven their presence in leaf nodules, bud tips, seeds, and ovaries [12], and this endophytic have beneficial by synthesizing defensive secondary metabolites or growth factors [11].

This study aims to isolate bacteria from leaf glands (nodules) and seeds of the *Ardisia crenata* plant and diagnose them at the microbiological and molecular levels.

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## **Material and Methods**

### *Isolation and purification of the bacteria from the leaf nodules and seeds of Ardisia crenata.*

Leaves that have mature glands (nodules) and fruits seeds of *Ardisia crenata* plant were separated and washed thoroughly with running water. Then leaves edges were carefully cutted using a sterilized scalpel with 0.5 cm. Subsequently, its and fruit seeds surface sterilized by immersed leaves in 96% ethanol for 1 minute followed by 2 minutes in 3% sodium hypochloride (NaOCl) and immersed fruit seeds in in 96% ethanol for two minutes, followed by 10 minutes in NaOCl respectively. Then were cleaned 3 times using sterile DW. and each one was separately crushed alone with 3.0 ml of liquid Nutrient Broth (NB) medium by sterile glass rod. After that, a single loopful of the suspension was scattered on to sterile plates of solid Nutrient Agar (NA) medium each one individually. At 28±2°C, the samples were incubated. A single well isolated colony was picked to solid NA medium in a petri plate and incubated after the purity and homogeneity of colony type was carefully examined through repeated re-streaked.

### *Preparation of isolated bacteria cell suspension*

Suspension of the isolated bacteria were prepared by taking a loopfull of bacteria isolated from leaf and seeds individually after 24 hours and placed in a vial contain 20 ml from liquid Nutrient Broth medium and incubated for 24 hours at 28 °C in a shaker incubator (New Brunswick Scientific Co., Inc. Edison. N.J. USA) with 100 r / min. rotational speed

### *Cultural characters*

Observed for Gram stain, color, and form of the cell colony for two types of bacteria isolates

### *Antibiotics tests*

Tested the sensitivity the two types of bacteria isolated in this study by Disk diffusion method Based on the method of [13], with the use of [10] types of prepared antibiotics 10 µg ml<sup>-1</sup> Tetracycline, 10 µg ml<sup>-1</sup> Cefotaxime, 30 µg ml<sup>-1</sup> Norfloxacin, 10 µg ml<sup>-1</sup> Ciprofloxacin, 26 µg ml<sup>-1</sup> Trimethoprim /Sulphamethoxazole, 10 µg ml<sup>-1</sup> Chloramphenicol, 100 µg ml<sup>-1</sup> Nitrofurantion, 10 µg ml<sup>-1</sup> Gentamicin, 30 µg ml<sup>-1</sup> Nalidixic acid, 10 µg ml<sup>-1</sup> Ceftriaxone Subsequently, the bacteria were spread evenly on the surface of the Mueller-Hinton agar medium by dipping a sterile cotton swab into each bacterial suspension and spreading it over the medium more than once and in all directions, using sterilized forceps the antibiotics discs were placed on the medium and softly pressed to ensure their adhesion. The plates were incubated at a temperature of 28°C for 24 hours. for determination the antibiotic test, the diameters of inhibition around the discs were measured using ruler and compare it with standard

tables based on the Clinical and Laboratory Standards Institute to determine if they are sensitive, moderately sensitive, or resistant to antibiotics [14,15].

### *16S rRNA amplified and sequenced using Polymerase Chain Reaction (PCR).*

Genomic DNA of two types of bacteria were extracted used a genomic DNA purification kit (Geneaid company). 16S rRNA is amplified and sequenced used Polymerase Chain Reaction (PCR), where the GoTaq G2 Green Master Mix from Promega (USA) was utilized in a 20 µL volume reaction. To amplified the full region of the 16S rRNA gene were used the universal primers 27F AGAGTTTGATCMTGGCTCAG and 1522R AAGGAGGTGATCCARCCGCA [16]. In accordance with the manufacturer's recommendations, (100 ng) of total template DNA and (1 µM each) of primer concentration were applied.

Mix the reaction components to prepare the reaction mixture in Premix tubes. The following was the PCR program set for the 16S rRNA gene: following a 3 minutes denaturation at 95 °C, there were 30 cycles total, each cycle consisted of (30 seconds of denaturation at 95 °C, 30 seconds of annealing at 55 °C, 1 minute of extension at 72 °C), and five minutes of final extension at 72 °C. The amplified products were visible on 1% agarose gel using Midori Green Advance DNA dye. A 100 bp DNA marker purchased from New England Biolabs, UK was used as a molecular weight marker. After being purified, the 16S rRNA gene PCR products were forwarded to the Psomagene sequencing company in the United States (USA) for sequencing. The sequence from the isolated bacterium was compared using the BLAST tool at the National Center for Biotechnology Information (NCBI) with the nucleotide database that is accessible at the Gene Bank.

## **Results and Discussion**

### *Cultural characters of endophytic bacterial isolated*

The colonies of novel strains of the endophytic bacteria isolated from leaves glands (nodules) and seeds of *Ardisia crenata* which grown on solid nutrient agar (NA) medium with light yellow after incubation for 24 hours, and took a circular shape slightly convex for each types (Fig. 1. A,B). The results gave positive reaction to Gram's staining. The findings presented above are consistent with the endophytic *Bacillus* bacteria that were isolated from several crops. Where the light yellow color of the colony belong to the *Bacillus* genus [17]. Previous studies have confirmed that bacillus bacteria are capable of producing a number of colors [18].

### Resistance and sensitivity to antibiotics

The results of the two isolated bacteria in this study were shown on a Mueller-Hinton agar medium by measuring the diameter of the inhibition zone using ruler and resulting from 10 different types of antibiotics, as described in Table (1).

### 16S rRNA sequencing

The concentration of DNA extracted from the endophytic bacteria reached to 443 µg/ml, for isolated leaf bacteria and reached to 541 µg/ml, for isolated seeds bacteria with purity was 1.8 for two types. The results of the electrophoresis in 1% agarose gel of the above chromosomal DNA amplified product by PCR using the specific primer of 16s rRNA gene showed the separation of a two band with a molecular weight of 1495 bp (Fig.2) similar to the molecular weight of the specific primer used.

The 16S rRNA gene sequence nucleotide analysis findings in the DNA Blast program showed there is a 99.29% similarity between sequence of leaves isolated bacteria and the sequence of *Bacillus megaterium* strain YSBM5 registered with the Gene bank in NCBI and there is a 92.02% similarity between sequence of seeds isolated bacteria and the sequences of *Bacillus subtilis* . and was given code PP215359.1 to seed isolated bacteria. After sending the results of nitrogenous bases sequences analysis of two isolates to the gene bank at the NCBI, the study was able to register it for the first time as a new strains and was given code PP213275.1 to leaves isolated bacteria and was known as *Bacillus* sp. JA Leaves (Fig.3). These findings are in line with numerous investigations into the possibilities of identifying genetic variation among the examined isolates through the technique of studying nitrogenous bases sequences conducted by the researcher [19] to isolate 13 endophytes bacteria

from the leaves of both indigenous medicinal plants that grew naturally in the dry South Sinai region(Egypt) (*Achillea fragrantissima* and *Fagonia mollis*), where the researchers used DNA sequencing technology for 16S rRNA region. The results showed that 9 bacterial isolated from *Fagonia mollis* ,8 of this isolates belong to *Bacillus* genus and 1 isolate belong to *Paenibacillus* genus while 4 bacterial isolated from *Achillea fragrantissima* showed 3 isolated belong to *Brevibacillus* genus and 1 isolated belong to *Paenibacillus* genus. The results of another study conducted by [20] which included the isolation Seven endophytic bacterial isolates from the roots, stems, petioles, and leaves of healthy melon plant tissues in Yogyakarta and Central Java, Indonesia, and their genetic diversity was determined by molecular diagnostics of the 16s rRNA region between them. According to a molecular identification based on 16S rRNA gene sequence analysis, the putative endophytic bacteria were closely linked to the genera *Burkholderia* and *Bacillus* and was known as *Bacillus* sp. JA seed (Fig.4).

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The authors declare that the present study hasno financial issues to disclose.

### Conflict of interest

The authors declares that there is no conflict interest.

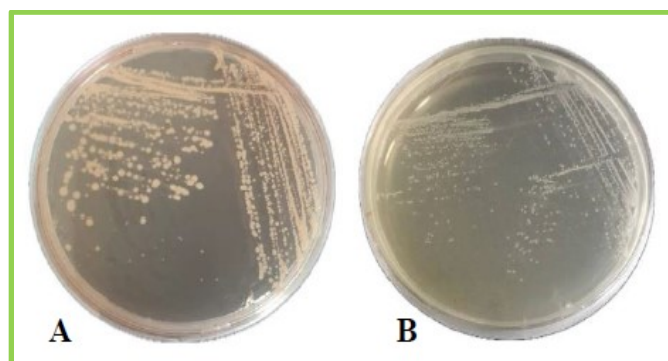


Fig. 1. Endophytic bacteria isolated form leaves glands (nodules) (A) and seeds (B) of *Ardisia crenata* grown on NA medium after 24 hour of inocubation.

TABLE 1. Resistance and sensitivity to antibiotic test

Antibiotics ( $\mu\text{g/ml}$ )	Bacteria isolated from	
	Leaves glands (nodules)	Seeds
Tetracycline (10)	S	S
Cefotaxime (10)	S	R
Norfloxacin (30)	S	S
Ciprofloxacin (10)	S	S
Trimethoprim/ Sulphamethoxazole (26)	S	S
Chloramphenicol (10)	S	S
Nitrofurantion (100)	S	S
Gentamicin (10)	S	S
Nalidixic acid (30)	S	S
Ceftriaxone (10)	S	R

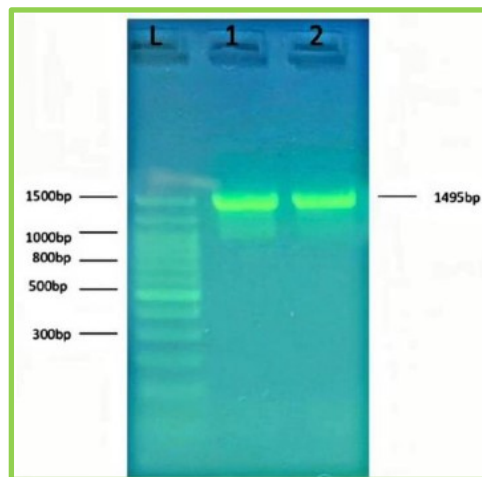


Fig. 2. Electrophoresis of the DNA amplified to the 16s rRNA region by PCR and isolated from the studied bacteria in 1.0% agarose

Fig. 3. Register the novel *Bacillus* sp. strain JA Leaves with code PP213275.1 in NCBI



**Bacillus sp. (in: firmicutes) strain JA seed 16S ribosomal RNA gene, partial sequence**

GenBank: PP215359.1  
[FASTA](#) [Graphics](#)

Go to:

LOCUS PP215359 717 bp DNA linear BCT 31-JAN-2024  
 DEFINITION Bacillus sp. (in: firmicutes) strain JA seed 16S ribosomal RNA gene, partial sequence.  
 ACCESSION PP215359  
 VERSION PP215359.1  
 KEYWORDS  
 SOURCE Bacillus sp. (in: firmicutes)  
 ORGANISM Bacillus sp. (in: firmicutes)  
 Bacteria; Bacillota; Bacilli; Bacillales; Bacillaceae; Bacillus.  
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 301 gaggcagcag tagggaatct tccgcaatgg angaaagtct gacggatcaa cgccccgtga  
 361 gtgatgaagg ttttcggatc gtaaaactct gttgtttggg aagaacaagt acaaaagtaa  
 421 ctgctcgtac cttgaccgtc cctaaaccga angccacggc taactacntg ccagcanccc  
 481 cgtaataacn tangtngcna gcgtgtgccg gaattattgg gcgtatagcg cgcgcacgca  
 541 gtcctttaa tctgatgtgg aaggccacgg gctcaccccc tgtacgggtcn tntaaactg  
 601 nnggacttga gtgcccaana naaaantgta attccncgtg ttgcggtgat atgcggagag  
 661 atgtgnanga acaccntnn tcaacgnnac cctctgggac gtcaactgaca cttaatc  
 //

Fig. 4. Register the novel *Bacillus* sp. strain JA seed with code PP215359.1 in NCBI

## References

- Harirchi, S., Sar, T., Ramezani, M., Aliyu, H., Etemadifar, Z., Nojumi, S. A., Yazdian, F., Awasthi, M. K. and Taherzadeh, M. J. *Bacillales*: From Taxonomy to Biotechnological and Industrial Perspectives. *Microorganisms*, **10**(12),2355 (2022).
- Dobrzyński, J., Wróbel, B. and Górka, E. B. Taxonomy, Ecology, and Cellulolytic Properties of the Genus *Bacillus* and Related Genera. *Agriculture*, **13**(10), 1979 (2023).
- Mukhtar, T., Ali, F., Rafique, M., Ali, J., Afridi, M. S., Smith, D., Mehmood, S., Amna Souleimanov, A., Jellani, G., Sultan, T., Munis, F. H. and Chaudhary, H. J. Biochemical Characterization and Potential of *Bacillus safensis* Strain SCAL1 to Mitigate Heat Stress in *Solanum lycopersicum* L. *Journal of Plant Growth Regulation*, **42**(1), 523-538 (2023).
- Barea, J. M. and Richardson, A. E. Phosphate mobilisation by soil microorganisms. Principles of Plant-Microbe Interactions: *Microbes for Sustainable Agriculture*, 225-234 (2015).
- Din, B. U., Sarfraz, S., Xia, Y., Kamran, M. A., Javed, M. T., Sultan, T., Munis, M. F. H. and Chaudhary, H. J. Mechanistic elucidation of germination potential and growth of wheat inoculated with exopolysaccharide and ACC-deaminase producing *Bacillus* strains under induced salinity stress. *Ecotoxicology and Environmental Safety*, **183**, 109466 (2019).
- Modi, K. and Jha, S. *Bacillus* consortia as a Sustainable Approach for Plant Growth Promotion in Rice (*Oryza Sativa* L.). *Curr. Agric.*, **39**, 28-49 (2022).
- Zhou, D., Huang, X. F., Chaparro, J. M., Badri, D. V., Manter, D. K., Vivanco, J. M. and Guo, J. Root and bacterial secretions regulate the interaction between plants and PGPR leading to distinct plant growth promotion effects. *Plant and Soil*, **401**, 259-272 (2016).
- Habibi, S., Djedidi, S., Prongjunthuek, K., Mortuza, M. F., Ohkama-Ohtsu, N., Sekimoto, H. and Yokoyama, T. Physiological and genetic characterization of rice nitrogen fixer PGPR isolated from rhizosphere soils of different crops. *Plant and Soil*, **379**, 51-66 (2014).
- Madhaiyan, M., Poonguzhali, S., Lee, J. S., Lee, K. C. and Hari, K. *Bacillus rhizosphaerae* sp. nov., an novel diazotrophic bacterium isolated from sugarcane rhizosphere soil. *Antonie van Leeuwenhoek*, **100**, 437-444 (2011)
- Jackes, B. *Ardisia*. (2021). Flora of Australia (2012).
- Yang, C. J. and Hu, J. M. Bacterial leaf nodule symbiosis in flowering plants. *Symbiosis*, **2**, 1-24 (2018).
- Ku, C. and Hu, J. M. Phylogenetic and cophylogenetic analyses of the leaf-nodule symbiosis in *Ardisia* subgenus *Crispardisia* (Myrsinaceae): evidence from

- nuclear and chloroplast markers and bacterial *rrn* operons. *International Journal of Plant Sciences*, **175**(1), 92-109 (2014).
13. Bauer, A. W; Kirby, W. M. M; Sherris, J. C. and Turck, E.M. Antibiotic susceptibility testing by a standardized single disk method. *American Journal of Clinical Pathology*, **45**(4), 493-496 (1966).
  14. Clinical and Laboratory Standards Institute (CLSI). M45-ED3rd, Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria. *3rd ed. Clinical and Laboratory Standards Institute*, Wayne, PA (2016)
  15. Osuntokun, O. T., Bakare, O. S., Bankole, O. M. and Ayodele, O. A. Comparative study of *Ocimum gratissimum* mediated nanoparticles and conventional antibiotics against endophytic leguminous bacteria and selected clinical isolates. *Asian Journal of Advanced Research and Reports*, **13**(1), 42-59 (2020).
  16. Abdulrazzaq, R. and Faisal, R. Efficiency of hichrome *Enterococcus faecium* agar in the isolation of *Enterococcus* spp. and other associated bacterial genera from water. *Journal of Life and Bio Sciences Research*, **3**(01), 01-06 (2022).
  17. Saha, M. L., Sadia, A., Sony, S. K. and Islam, M. N. Pigment producing soil bacteria of Sundarban Mangrove Forest. *Bangladesh Journal of Botany*, **46**(2), 717-724 (2017).
  18. Kharkhota, M., Hrabova, H., Kharchuk, M., Ivanytsia, T., Mozhaieva, L., Poliakova, A. and Avdieieva, L. Chromogenicity of aerobic spore-forming bacteria of the *Bacillaceae* family isolated from different ecological niches and physiographic zones. *Brazilian Journal of Microbiology*, **53**(3), 1395-1408 (2022).
  19. ALKahtani, M. D., Fouda, A., Attia, K. A., Al-Otaibi, F., Eid, A. M., Ewais, E. E. D., Hijri, M., Arnaud, M. S., Hassan, S. E. D., Khan, N., Hafez, Y. M. and Abdelaal, K. A. Isolation and characterization of plant growth promoting endophytic bacteria from desert plants and their application as bioinoculants for sustainable agriculture. *Agronomy*, **10**(9), 1325 (2020).
  20. Jayanti, R. M. and Joko, T. Research article plant growth promoting and antagonistic potential of endophytic bacteria isolated from melon in Indonesia. *Plant Pathol. J.*, **19**(3), 200-210 (2020).

## عزل البكتريا الداخلية من الغدد الورقية ومن بذور نبات *Ardisia crenata* والكشف عنها جزيئيا

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### الخلاصة

عزلت البكتريا من غدد أوراق (العقيدات) وبذور نبات *Ardisia crenata* واطهرت النتائج نمو العزلتين باللون الأصفر الفاتح بعد حضنها لمدة 24 ساعة واخذت الشكل الدائري المحدب قليلا وكانت كلتا العزلتين موجبة لصبغة الكرام. كانت البكتريا المعزولة من غدد الاوراق حساسة لجميع المضادات الحيوية المستخدمة في هذه الدراسة بينما البكتريا المعزولة من البذور كانت مقاومة فقط لـ 10 ميكروجرام/مل Cefotaxime و 10 ميكروجرام/مل Ceftriaxone. أظهرت بيانات تحليل تسلسل الحمض النووي للقواعد النيروجينية لجين 16S rRNA بعد استخدام تقنية PCR ومقارنتها مع قاعدة البيانات العالمية في المركز الوطني لمعلومات التكنولوجيا الحيوية (NCBI)، تشابهها بنسبة 99.29% بين تسلسل البكتريا المعزولة من الأوراق وبكتريا *Bacillus megaterium* سلالة YSBM5، وبالتالي تم تسجيل العزلة لأول مرة باسم *Bacillus* sp. strain JA Leaves في حين أن التشابه وصل بنسبة 92.02% بين تسلسل البكتريا المعزولة من البذور وبكتريا *Bacillus subtilis* وتم تسجيلها لأول مرة باسم في *Bacillus* sp. strain JA seed في NCBI.

الكلمات الدالة: *Ardisia crenata*، *Bacillus* sp.، 16S rRNA، PCR