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# Screening and Molecular Identification of NON-Rhizobial Nodules Associated Bacteria in Some Leuminous Crops in Sohag Governorate

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## ABSTRACT

This study was conducted at the Department of Genetics, Faculty of Agriculture, Sohag University to isolate and identify the non-rhizobial nodule-associated bacteria at molecular level from different leguminous crops (Egyptian clover, Peanut and Lupine) in Sohag Governorate. A total of 25 nodule-associated bacteria were isolated during this study. All 25 isolates were characterized using symbiotic (*nodA*) gene loci which found normal in *Rhizobium* sp. It was found that all isolated bacteria produced clear PCR fragments of approximately 666 base pairs (bp) except the seven isolates (1, 9, 10, and 17) of Egyptian clover and (19, 21 and 22) of Lupine. Among the seven non-rhizobial nodule-associated bacterial isolates, three isolates (no., 1 and 9 from Egyptian clover and no. 21 from Lupine) were identified by 16S rRNA gene sequencing analysis. These three isolates were symbolized as EMC, EME and EM7, respectively. The obtained data indicated that all of the bacterial isolates were belonging to the genus *Pseudomonas*. It was found that the two isolates, EME and EM7 gave 100% of similarity with *Pseudomonas lini* strain DLE411J, NR_029042, and *Pseudomonas viciae* strain 11K1, NR_180948, respectively. While, the third isolate (EMC) gave 97.72% of similarity with *Pseudomonas lini* strain DLE411J, NR_029042. The phylogenetic tree based on the 16S rRNA gene sequence of the three isolates supported the obtained results. *Pseudomonas* genus is commonly found coexisting with rhizobia in leguminous crop nodules. It could be used as a single bio-inoculant or in combination with rhizobia to improve nodulation and plant growth of leguminous crops.

**KEYWORDS:** Pseudomonas, nodA gene, 16S rRNA gene, non-nodules associated bacteria

## 1. INTRODUCTION

Worldwide, leguminous crops are crucial to the provision of food for human and animal consumption due to their high protein, vitamins, minerals, and fiber contents as well as, it is very cheap for people in poor countries (Ferreira *et al.*,

2021). It is also used as a cover crop that increases soil vitality and fertility which in turn leads to improve yield and productivity of the successive crops, preserve the soil's organic matter and nutrients (Stagnari *et al.,* 2017 and Ferreira *et al.,* 2021). Plants belonging to legumes are associated with numerous soil bacteria genera called rhizobia, that able to fix atmospheric nitrogen (Poole *et al.,* 2018). Rhizobia forms root nodules inside the host plant where the nitrogen fixation process occurs after absorbing the necessary nutrients from the plant (Spaink 2000 and Poole *et al.,* 2018). It was found that legume nodules are not only occupied by rhizobia, but numerous bacterial varieties inhabit inside nodules which are called non-rhizobial nodule-associated bacteria (Martinez-Hidalgo and Hirsch, 2017**,** Rajendran *et al.,* 2012 and Noreen *et al.,* 2015ab)**.** These bacteria did not involve directly in nodule formation process, but it colonizes nodules with rhizobia to promote nodule development and encourage the growth of host plant (Noreen *et al.,* 2019)**.**

Non-rhizobial nodule associated bacteria, containing different bacterial genera such as *Pseudomonas, Bacillus, Agrobacterium, Burkholderia, Variovorax,* and *Acinetobacter* (Shiraishi *et al.,* 2010, Bessadok *et al.,* 2020, Tapia-García *et al.,* 2020 and Pang *et al.,* 2021).These bacterial genera improve significantly the yield of legumes, provide plant with additional nitrogen, produce different phyto-hormones and protect the host against different phytopathogens (Das *et al.,* 2017**)**. Nowadays, identification of bacteria by molecular techniques has become more desirable more than traditional biochemical and morphological methods which did not give a true evolutionary relationship **(**Giller, 2001). 16S rRNA gene sequencing technique is considered one of the faster and more accurate methods that are widely used for bacterial identification **(**Srinivasan *et al.,* 2015). The 16S rRNA gene sequence (1.5 kp) is conserved in almost all bacteria and large enough to be used in informatics studies (Schröder, 2014 and Sabat *et al.,* 2017). So, it is widely used in bacterial phylogenetic relationships analyses due to its stability and its vital functions in bacterial cells (Pulawska *et al.,* 2000).

Due to the substantial sequence similarity between certain species, it is qualified to identify bacteria at both the genus and species levels (Deurenberg *et al.,* 2017). Furthermore, the nucleotide sequences of this region are determined and compared with sequences available at databases to produce homology matches, which allows bacterial identification of the target samples. This makes the 16S rDNA gene sequencing is a viable technique for classifying bacteria (Clarridge, 2004, Salman *et al.,* 2012 and Sabir *et al.,* 2013). So, the present investigation aimed to isolate and identify the non-rhizobial nodule-associated bacteria from root of some leguminous crops cultivated in Sohag governorate at molecular level.

## 2. MATERIALS AND METHODS

This research was carried out at Microbial Genetics Lab., Department of Genetics, Faculty of Agriculture Sohag University, Egypt.

### 2.1. Nodules-associated bacteria isolation

Nodules-associated bacteria were isolated from three leguminous crops grown at various geographical regions at Sohag Governorate as following: Egyptian clover (*Trifolium alexandrinum* L.), Lupine (*Lupinus termis* L.) and Peanut (*Arachis hypogaea* L.) according to the procedure of (Vincent's, 1970). Healthy fresh nodules were washed many times with tap water; surface sterilization was done by soaking them in ethanol for 30 seconds, submerged in 10% commercial chloride for 10 minutes, and then washing five times with sterile water. To confirm the sterilization, the water from the previous rinse was utilized. Sterilized nodules were crushed and streaked onto Yeast Extract Mannitol media (YEM). After 3 incubation days at 28 °C, bacterial single colonies were purified on YEM media several times and purified single colonies were stored on slant agar of YEM at 4°C. A total of 25 isolates were isolated from root nodules of three chosen leguminous crops, 18 isolates from Egyptian clover, 4 from Lupine, and 3 from Peanut. These isolates were numbered from 1 to 25.

### 2.2. Molecular identification of nodules associated bacteria

#### 2.2.1. Genomic DNA isolation

A 10-minute DNA preparation method involved the vortex of a single colony of bacteria in 0.5 milliliters of distilled water and boiling it for ten minutes (Hoffman and Winston, 1987). After that, place on ice for ten to thirty minutes. After a final 10-minute centrifugation at 10,000 rpm, the extracted DNA was utilized as a template for the PCR reactions.

#### 2.2.2. Amplification of Nod A region

The PCR primers used for amplifying *nodA* region were: *nodA*1 5'- TGC RGT GGA ARN TRN NCT GGG AAA -3' and *nodA*2 5'- GGN CCG TCR TCR AAW GTC ARG TA -3' (Zhang *et al.,* 2000 and Moulin *et al.,* 2004). Reaction mixtures containing 25 µl were used for the PCR.. The composition of the reaction mixture was as follows:

| Particulars | Content |
|---|---|
| **10 X Dream TaqTM Green Buffer** | 12.5 µL |
| **Forward primer** | 1.5 µL |
| **Reverse primer** | 1.5 µL |
| **DNA template** | 2 µL |
| **Water Deionized** | 7.5µL |
| **Total volume** | 25 µL |

The amplification programme was as follows:

| St. No | Steps | Temperature (oC) | Time |
|---|---|---|---|
| **1** | **Denaturation (initial)** | 95 | 4 min |
| **2** | **Denaturation** | 95 | 45s |
| **3** | **Annealing** | 49 | 60s |
| **4** | **Extension** | 72 | 2 min |
| | **Final extension** | 72 | 10 min |

The total numbers of cycle (2-4) were 30 cycles.

#### 2.2.3. Gel electrophoresis of PCR products

Aliquots of 5 µl of PCR products were analyzed by horizontal electrophoresis in 2% agarose gels in TAE buffer (40 mM Tris-HCl, 4 mM sodium acetate, and 1 mM EDTA pH 7.9). With standard gels, electrophoresis was performed at 100 V for 1 hour. Ethidium bromide was used to stain the gel, which was then photographed under UV light.

#### 2.2.4. Amplification and analysis of 16S rRNA gene

The amplification of the 16S-rRNA gene sequence was done according to (Haukka *et al.,* 1998**)** by using the universal bacterial primers 16S-1F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S-1509R (5′-ACGGCTACCTTGTTACGACTT-3′). The PCR reaction mixture was done in (50µL) of the final volume by mixing (4µL) of the DNA template with (2µL) from each primer and (17µL) of sterilized double distilled water then completing the final volume by adding (25µL) of 10 X Dream TaqTM Green Buffer as a Master Mix. PCR reaction conditions were carried out as follows: Initial denaturation at 94°C for 3 minutes, denaturation at 94°C for 30 seconds, primer annealing at 57°C for 30 seconds, chain extension at 72°C for 2 minutes, and a final extension at 72°C for 10 minutes. Thirty cycles of denaturation, annealing and extension were performed.

#### 2.2.5. 16S rRNA gene sequencing and phylogenetic analysis.

Sequencing of the purified DNA samples was done at GATC Company (GATC Biotech Ltd. - The London BioScience Innovation Centre - London, United Kingdom) by using ABI 3730xl DNA sequences. The obtained sequences were compared to sequences in the public database using the Basic Local Alignment Search Tool (BLASTn) on the website "http://www.ncbi.nih.gov" to ascertain similarity to sequences in the GenBank database. The sequences of the 16S rRNA gene found during this work are available in the National Center for Biotechnology Information (NCBI) database under the accession numbers OR855460 to OR855462. By the MEGA X software package, the neighbor-joining (NJ) method in conjunction with the unweighted pair group approach was used to create the phylogenetic tree (Kumar *et al.,* 2018).

## 3. RESULTS AND DISCUSSION

All the 25 nodules associated bacterial isolates were morphologically and biochemically characterized in this work, data published by (Ismail *et al.*, 2022 and Ismail and Soltan, 2023). During their growth on YEM, it was observed that growth of seven isolates did not look like *Rhizobium*. These isolates were separated and purified on agar plates for molecular identification.

### *Nod A* region amplification

To emphasize that all isolated bacteria are belonging to the genus *Rhizobium*, All the 25 isolates were characterized using symbiotic (*nodA*) genes loci which are found normally in *Rhizobium* sp. It was found that all bacterial isolates produced clear PCR fragments with approximately 666 base pairs (bp) except the seven isolates (1, 9, 10 and 17) of Egyptian clover and (19, 21 and 22) of Lupine (Figure 1). These results were similar to those of (Youseif *et al.*, 2014 and Sijilmassi *et al.*, 2021**)**. *NodA* is a gene which is coding for a protein that is required for generating factors of the rhizobial nodulation involved in legume infection signaling (Dhaoui *et al.*, 2016).

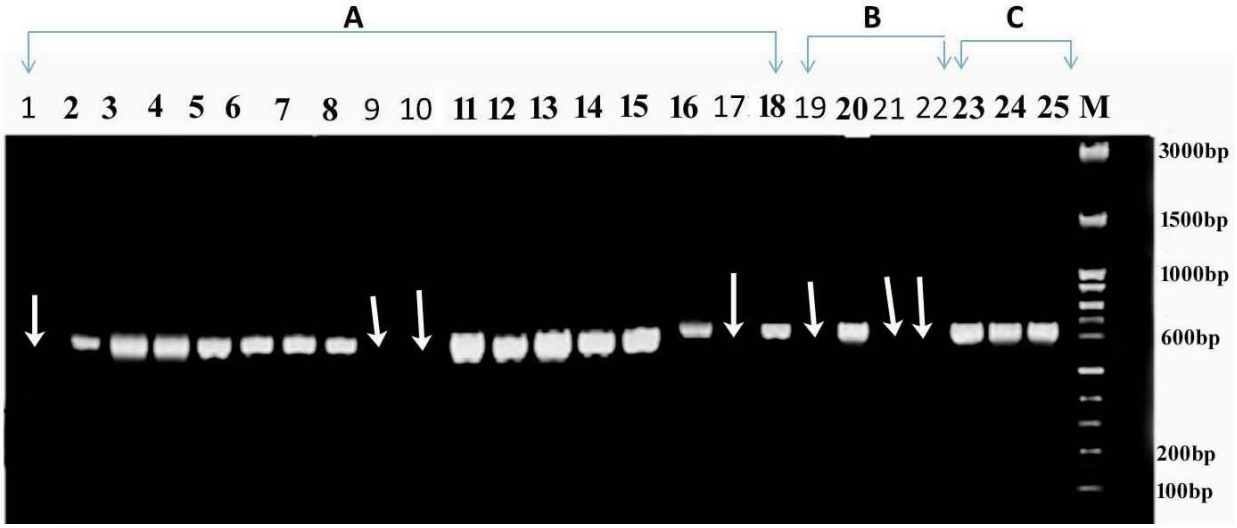


**Figure 1. The amplification product of *Nod A* region of 25 nodules associated bacterial isolates (A) Egyptian clover, (B) Lupine and (C) Peanut. M, 100bp DNA Ladder. Arrows refer to unamplified *nodA* bands.**

### 3.1. Identification of non-rhizobial nodules associated bacteria by 16S rRNA gene sequencing

Among of the seven non-rhizobial nodules-associated bacterial isolates, three isolates (no., 1 and 9 from Egyptian clover and no. 21 from Lupine) were identified by 16S rRNA gene sequencing techniques. These isolates were symbolized as EMC, EME, and EM7, respectively. The 16S rRNA gene sequences are very useful for studying the taxonomy and phylogeny of bacteria. The 16S rRNA gene's function has not changed over time, even though it is present in almost all bacteria and is usually found in operons or multigene families. This implies that the 16S rRNA gene (1500 bp) is large enough for informatics applications and that random sequence changes are a more accurate approach to measuring time (Patel, 2001). The PCR product fragments of the 16S rRNA gene of the 3 bacterial isolates gave a one band of 1500 base pairs (Figure 2).

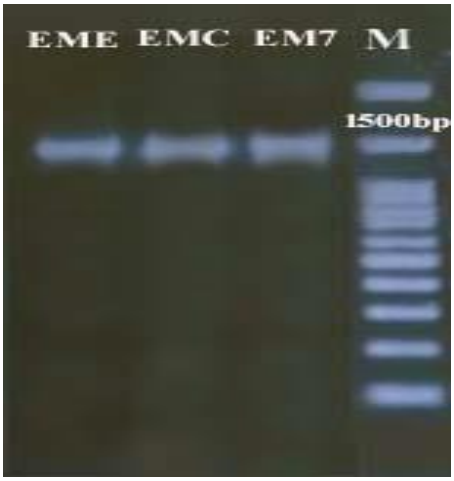


**Figure 2. 16S-rRNA gene banding pattern of 3 non-rhizobial nodules associated bacterial isolates (EME, EMC and EM7). M: 100 bp ladder marker.**

The percentages of similarity between the sequences of the three non-rhizobial nodules associated bacteria and the closest related NCBI strain(s) in the Genbank database are displayed in Table (2).

**Table 2. Identification of the three non-rhizobial nodules associated bacteria based on 16S rRNA gene sequence.**

| Isolates | accession No. | % Identity | Closest NCBI strain and accession No. | Class |
|---|---|---|---|---|
| **EMC** | OR855462 | 97.72 | *Pseudomonas lini* strain DLE411J 16S ribosomal RNA, partial sequence NR_029042. | Gamma-proteobacteria |
| **EME** | OR855461 | 100 | *Pseudomonas lini* strain DLE411J 16S ribosomal RNA, partial sequence NR_029042. | Gamma-proteobacteria |
| **EM7** | OR855460 | 100 | *Pseudomonas viciae* strain 11K1 16S ribosomal RNA, partial sequence. NR_180948. | Gamma-proteobacteria |

The obtained data in Table (2) shows that all identified bacteria were belonging to genus *Pseudomonas* which considered as one of the most common microorganisms which colonizing root nodules of different leguminous crops (Tapia-García *et al.,* 2020 and Pang *et al.,* 2021). It was found that the two isolates EME and EM7 gave 100% of similarity with *Pseudomonas lini* strain DLE411J, NR_029042, and *Pseudomonas viciae* strain 11K1, NR_180948, respectively. While the third isolate (EMC) gave 97.72% of similarity with *Pseudomonas lini* strain DLE411J, NR_029042. Mowafy *et al.* (2022) isolated and characterized molecularly the plant growth-promoting bacteria from *Phaseolus vulgaris* nodules. Their isolated bacteria were identified as *Bacillus* MAP3, *Brevibacillus* MAP4, *Pseudomonas* MAP5, and *Pseudomonas* MAP8. Numerous non-rhizobial bacteria are often associated with root nodules of several legume crops. *Pseudomonas* and *Bacillus* are two of the most prevalent of them (Dudeja *et al.,* 2012 and De Meyer *et al.,* 2015).

The phylogenetic tree of the 16S rRNA gene sequences from the three bacterial isolates (EMC *OR855461*, EME *OR855462* and EM7 *OR855460*) and their relatives in the GeneBank database was constructed using the neighbor-

joining method. The branch lengths were computed using the greatest composite likelihood method. The scale bar shows base substitutions per location. The percentage of duplicate trees in which the relevant taxa clustered together in the bootstrap test is shown next to each branch (1000 replicates). The sequence of the three chosen bacteria is marked by a red circle. The phylogenetic tree (Figure 3) showed that the two bacterial isolates EMC and EME of (Egyptian clover) had the closest relationship with *Pseudomonas lini* strain DLE411J 16S ribosomal RNA, partial sequence. (GeneBank accession No. NR_029042.1), and sharing the same clade. While the third isolate EM7 of (Lupine) was closely related to *Pseudomonas viciae* strain 11K1 (GeneBank accession No. NR_180948.1) and engaged the same clade.

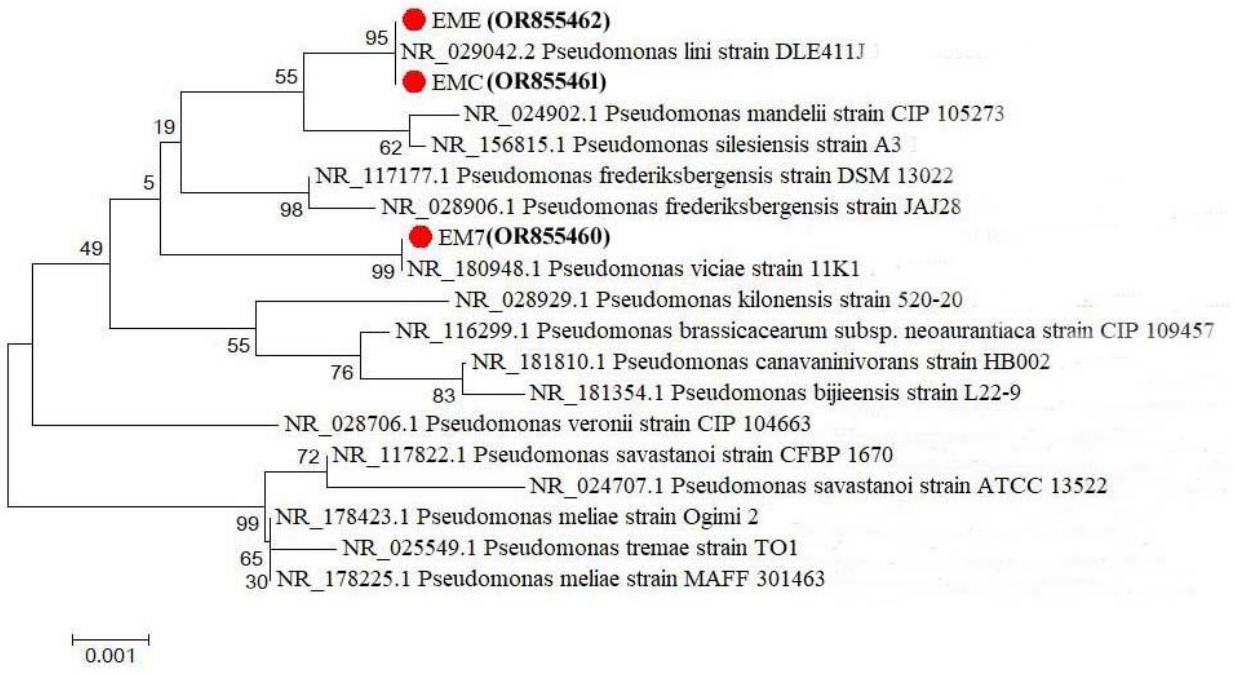


**Figure 3. Neighbor-joining phylogenetic dendrogram showing the genetic relationships between the three non-rhizobial nodules associated bacterial isolates (EMC *OR855461*, EME *OR855462* and EM7 *OR855460*) isolates and their closest strains in GeneBank database based on 16S rRNA gene sequence.**

*Pseudomonas* genus is commonly found coexisting with rhizobia in leguminous crop nodules. It could be used as a single bio-inoculant or in combination with rhizobia to improve nodulation and plant growth of leguminous crops.

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## الملخص العربي

# التوصيف والتعريف الجزيئي لعزلات بكتريا غير عقديه معزولة من العقد الجذرية لبعض النباتات البقولية في محافظه سوهاج

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قسم الوراثه، كليه الزراعة، جامعة سوهاج
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اجريت هذه الدراسة بقسم الوراثة بكلية الزراعة جامعة سوهاج لعزل وتشخيص العقيدات غير الريزوبية المصاحبة للبكتيريا في المحاصيل البقولية المختلفة (البرسيم المصري والفول السوداني والترمس) في محافظة سوهاج. تم عزل ٢٥ عزله من البكتيريا التي تكون العقد الجذرية في هذه الدراسة. تم توصيف جميع العزلات الـ ٢٥ باستخدام الجينات التكافلية (nodA) الموجودة بشكل طبيعي في Rhizobium sp. وقد وجد أن جميع العزلات التي تم اختبارها أنتجت باندات PCR واضحة عند وزن حوالي ٦٦٦ قاعده نيتروجينه (bp) باستثناء العزلات السبعة (١، ٩، ١٠ و ١٧) من البرسيم المصري و (١٩، ٢١ و ٢٢) من الترمس. من بين العزلات البكتيرية السبعة غير الريزوبية، تم تحديد ثلاث عزلات بكتيرية (عزلتان EME و EMC من البرسيم المصري وواحدة EM7 من الترمس) بواسطة تقنيات التسلسل الجيني S rRNA١٦. أشارت البيانات المتحصل عليها إلى أن جميع العزلات البكتيرية تنتمي إلى جنس Pseudomonas. وقد وجد أن العزلتين EME وEM7 أعطتا ١٠٠% من التشابه مع سلالة Pseudomonas lini DLE411J، NR_029042 وسلالة Pseudomonas viciae 11K1، NR_180948، على التوالي. بينما أعطت العزلة الثالثة (EMC) تشابهاً بنسبة ٩٧,٧٢% مع سلالة Pseudomonas lini DLE411J, NR_029042. أكدت شجرة النشوء والتطور لتسلسل جينات 16sRNA للعزلات الثلاث على النتائج السابقة. يوجد جنس Pseudomonas بشكل شائع ويتعايش مع الريزوبيا في عقيدات المحاصيل البقولية. يمكن استخدامه كتلقيح حيوي منفرد أو بالاشتراك مع rhizobia لتحسين العقد ونمو النباتات للمحاصيل البقولية.