Arab Universities Journal of Agricultural Sciences
(2024) 32 (2) 199-203
Website: http://ajs.journals.ekb.eg

Isolation and Identification of Bacteria Producing Indole From Rhizospheric Plant

Dina MS Hassan[1*], Mohamed E El-Demerdash[1], Yasmin MR Abdellatif [2], Enas A Hassan[1]

1- Agricultural Microbiology Dept, Fac of Agric, Ain Shames Univ, P.O. Box 68, Hadayek Shubra 11241, Cairo, Egypt

2- Agricultural Botany Dept, Fac of Agric, Ain Shames Univ, P.O. Box 68, Hadayek Shubra 11241, Cairo, Egypt

*Corresponding author: dinamondy@agr.asu.edu.eg

https://doi.org/10.21608/AJS.2024.305171.1577

Received 17 July 2024; Accepted 25 September 2024

Short Communication

Keywords:

Indoles,
Rhizosphere,
Kocuria,
PGPR,
16S rRNA

Abstract: Forty-six bacterial isolates were obtained from seven rhizosphere samples using nutrient agar and glucose agar media. These isolates were morphologically categorized into long rods, short rods, and cocci, comprising 52%, 17%, and 31% of the total count, respectively. The collected isolates from the rhizosphere were assessed for their capability to produce indole-3-acetic acid (IAA). The initial qualitative screening revealed diverse abilities among isolates for IAA production, with notable variations in productivity levels. Quantitative analysis of the top ten isolates revealed that cocci-shaped isolates produced the highest IAA levels (4.80 to 6.30 mg/100 mL), with the SS1 isolate achieving the maximum value (6.30 mg/100 mL). Genetic identification of the highest IAA producer (SS1) through 16S rRNA gene sequencing showed that it belongs to the genus *Kocuria*. Phylogenomic analysis confirmed a high similarity to *Kocuria rosea,* with 98% identity.

1 Introduction

Microscopic organisms, including bacteria, fungi, actinomycetes, protozoa and algae, dominate in the soil. The rhizosphere of the soil is where microscopic life typically resides and interacts with plants. The interaction between microorganisms and plants in the rhizosphere affects soil fertility and plant health. Plant-growth-promoting rhizobacteria (PGPR) are a type of bacteria that colonize plant roots and stimulate their development (Patel et al 2015).

Growth regulators are categorized into three classes based on their source: synthetic substances, endogenous compounds made by plants (known as phytohormones) and products of microbial production. Growth regulators derived from microorganisms are also known as phytohormones because they share similarities with substances produced by plants, such as gibberellins, abscisic acid, auxins and cytokinins (Pirog et al 2018).

Plant hormones are signaling molecules that function as chemical messengers to regulate plant growth and development. These hormones are synthesized in small quantities in specific plant regions and can be

transported to other areas to trigger various biochemical, physiological, and morphological responses. They are crucial for enhancing plant tolerance to various biotic and abiotic stresses (Zhang et al 2023).

Growth regulators are used in agriculture to increase yields, protect against specific diseases, activate vegetative growth, accelerate flowering and maturation, and stimulate seed germination. According to Pirog et al (2018), using these regulators in agricultural production can significantly reduce the usage of chemical plant protectors.

Auxins are low molecular weight hormones, extensively used plant growth regulators having an aromatic ring structure (Patel et al 2015). Auxin is produced near the tips of growing shoots and transported down the main stem by the polar auxin transport mechanism. It is involved in organogenesis, apical dominance, elongation and, cell division (Leyser 2018). Many microorganisms that are connected to soil and plants can manufacture IAA. Plant-growth regulator, IAA, is thought to be produced by 80% of rhizospheric bacteria (Patten and Glick 1996). *Kocuria* are Gram-positive cocci bacteria often seen in tetrads or irregular clusters. These bacteria are non-spore-forming and non-encapsulated. *Kocuria* species, including the type species *K. rosea*, are commonly found in soil (Shchyogolev et al 2024) and also exist as endophytes within various plant species. They can be isolated from the rhizosphere of many plants.

The indole-producing bacteria have been reported in various genera of PGPR including *Azotobacter*, *Arthrobacter*, *Azospirillum*, *Acinetobacter*, *Bradyrhizobium*, *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Pantoea*, *Rhodococcus*, *Rhizobium*, *Streptomyces*, *Serratia*, and *Strenotophomonas* (Ozdal et al 2017, Morales-Cedeño et al 2021). This study aims to isolate and identify bacteria in soil rhizosphere that are highly efficient for indole production.

2 Materials and Methods

2.1 Isolation of indole-producing bacteria

To isolate indole-producing bacteria, soil samples were collected from the rhizosphere of various plants, *Corchorus olitorius* (Mallow), *Solanum lycopersicum* (Tomato), *Zea mays* (Maize), *Trifolium alexandrinum* (clover), *Brassica oleracea* (cabbage), *Abelmoschus esculentus* (Okra) and *Brassica rapa* (Turnip), grown in Giza and Qalyubia Govermmenates. The isolation of indole-producing bacteria was performed by serial ten-fold dilution according to Timonin (1940) using nutrient agar in a Petri dish and then incubated at 30˚C/48h; every bacterial colony was isolated and purified to obtain a pure culture.

2.2 IAA Qualitative Assay

The tested isolate was spot inoculated in the center of Petri dishes containing nutrient agar medium supplemented with L-tryptophan (0.2 g L^{-1}) and then incubated at 30˚C/48 hours. The detection of indole-producing bacteria was performed by soaking Whatman paper in Salkowski reagent (12 g/L $FeCl_3$ in 429 ml/L H_2SO_4) and then applying a few drops of the reagent to the bacterial colony. The development of pink color indicates a positive result for indole production (Bric et al 1991). The efficiency of the isolates is categorized into four levels of production: -, +, ++, +++.

2.3 Indole-3-acetic acid (IAA) quantitative assay

The indole production was determined for selected isolates by nutrient broth and Salkowski's reagent (Gutierrez et al 2009). The tested isolate was grown in nutrient broth supplemented with L-tryptophan (1 mM) and incubated at 30℃/5 days in the dark condition; cell-free culture filtrate was taken after centrifugation for 10 minutes at a speed of 8000 rpm. Two milliliters of Salkowski's reagent were added to 2.0 mL of cell-free culture (Glickman and Dessaux 1995) and then incubated in the dark condition at room temperature for 0.5-3 hours. The density of the pink color was read at 530 nm using a T60 UV-visible spectrophotometer (PG Instruments). The concentration of indole in the supernatant was calculated from a standard curve (5-100 µg/ml) and expressed in mg/100 ml.

2.4 Identification of Bacterial Isolate

The morphological characters of the selected isolate were tested microscopically. Molecular identification was also performed by extracting the genomic DNA using the Gene JET genomic DNA purification kit according to the manufacturer's protocol. Amplification of 16S rDNA genes by polymerase chain reaction (PCR) was performed using universal primers of bacteria: forward primer (27F) and reverse primer (1492R) (Sherpa et al 2018). The PCR products were purified and sequenced using an Applied Biosystems ABI 3500 Genetic Analyzer (Japan) with the previously mentioned universal bacterial primers from Sigma. The National Center for Biotechnology Information (NCBI) nucleotide blast tool was used to identify the sequence,

and MEGA v.10 software was used for creating the phylogenetic tree. The identified sequence for the selected isolate was deposited in the NCBI gene bank with an accession number.

3 Results and Discussion

3.1 Isolation of Indole-Producing Rhizobacteria

Forty-six bacterial isolates were isolated from a total of seven rhizosphere samples using nutrient agar and glucose agar medium. They were classified according to their morphological shape into long rods, short rods and cocci. The corresponding percentages for each group were 52, 17 and 31% of the total count, in the same above–mentioned respective order, as shown in **Fig 1**.

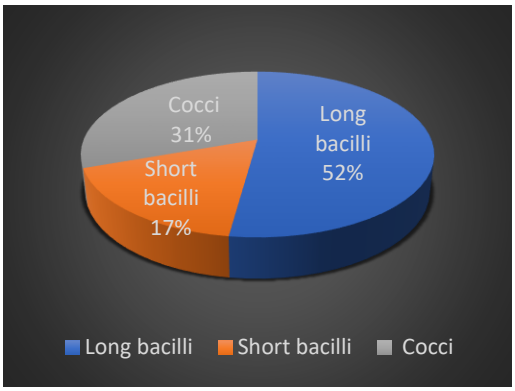

Fig 1. The distribution percentages of the collected rhizobacteria isolated from rhizosphere samples according to morphological shape.

3.2 IAA Qualitative and Quantitative Assays

Initially, the qualitative assessment was performed to evaluate the efficiency of collected isolates in their ability to produce plant hormones. All bacterial isolates were tested to assess their productivity for indole production according to Bric et al (1991). It was found that 25 isolates do not produce indoles (-), 11 isolates (9 bacilli, 2 cocci) have low production (+), 8 isolates (4 bacilli, 4 cocci) have moderate productivity (++), while only two isolates (2 cocci) exhibited high efficiency (+++), based on the diameter of the pink halo surrounding bacterial growth **Fig 2**. Afterwards, the ten highest productivity isolates were evaluated quantitatively according to Glickman and Dessaux (1995). **Table 1** presents that cocci-shaped isolates (6 isolates) recorded the highest productivity ranging from 4.80 to 6.30 IAA mg/100 mL and bacilli–shaped isolates produced 2.06-4.14 mg/100mL IAA. The SS1 isolate achieved the highest quantity of IAA (6.30 mg/100mL) and thus it was selected for further study. The SS1 isolate was Gram-positive, cocci, non-spore-forming and non–capsulated.

It was reported that the rhizosphere isolates are more efficient indole producers compared to the isolates collected from soil (Sarwar et al 1995) as 80% of the isolated bacteria from the soil can produce indole (Patten and Glick 1996).

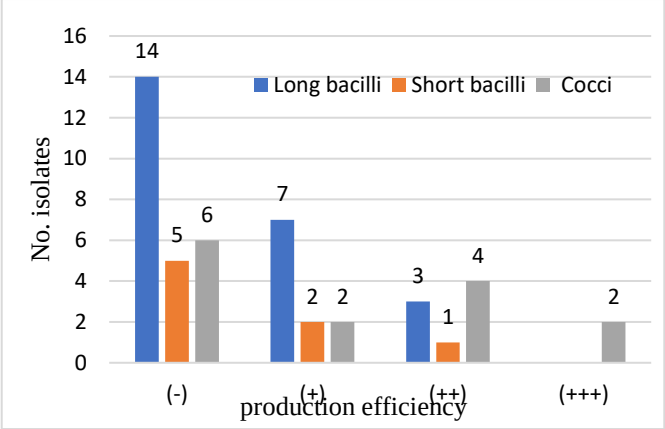

Fig 2. The efficiency of collected bacterial isolates for IAA production represented under four degrees of production: - high (+++) moderate (++) low (+) non (-)

Table 1. Determination of IAA concentration produced by selected bacterial cultures.

Isolate's code	Morphological shape	IAA Conc. (mg/100ml)
SC 6	Cocci (G^{+ve})	4.97
SZ 1		5.64
ST 3		5.27
SBrH 5		5.46
SS 1		6.3
SS 4		4.80
SA 2	Long bacilli (G^{+ve})	2.99
ST 4		4.14
SBrH 3		2.06
SBrH 13	Short bacilli (G^{-ve})	2.1

3.3 Molecular Identification of Bacterial Isolate

The 16 S rRNA gene sequence analysis of SS1 isolate shows that it belonged to genus *Kocuria* after analysis and comparison with nucleotide sequences of NCBI as presented in **Fig 3**. The obtained resulting genomes were employed for phylogenomic and comparative genomic analysis with reference to the Gene Bank database of the NCBI using the BLAST function; subsequently, a phylogenetic analysis was performed. The analyzed genome of isolate SS 1 showed a high degree of similarity to *Kocuria rosea* with 98% and it was accessed in the gene bank with accession No. OR944054.

Ozdal et al (2017) and Karnwal (2019) confirmed that only a small number of Gram-positive bacteria belonging to *Kocuria* (Karnwal 2019) and *Arthrobacter* (Ozdal et al 2017) are known for indole production. Some *kacuria* species possess characteristics of PGPR because of their capability to produce indole and other phytohormones, as well as enhance plant resistance to stress (Li et al 2020). The addition of L-tryptophan in the culture medium enhanced the IAA biosynthesis, by indole-producing bacteria, since it is a precursor of indole production (Patten and Glick 1996). Bacterial phytohormones can be used to improve the efficiency and success rates of tissue-cultured plants.

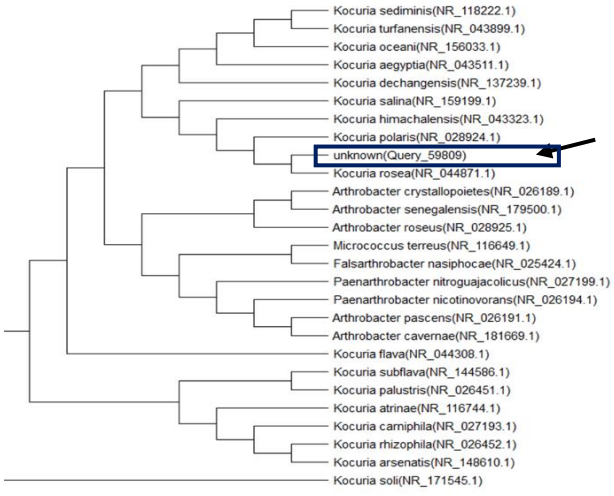

Fig 3. Phylogenetic tree of partial sequence of forward direction of the PCR product of 16S rRNA gene from *Kocuria* SS1 isolate and the related universal bacterial strains

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