Arab Universities Journal of Agricultural Sciences
(2023) 31 (1) 111-118
Website: http://ajs.journals.ekb.eg

Molecular Identification of Some *Rhizobium* and *Serratia* Isolates as Potential Producers of Indole-3-Acetic Acid

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https://doi.org/10.21608/AJS.2023.160088.1494

Received 1 September 2022; Accepted 14 November 2022

Keywords:

Rhizobium,
Serratia,
Phytohormones,
PGPR,
Indole-3-Acetic Acid (IAA)

Abstract: In order to reduce the use of chemical fertilizers, the use of biological inoculants has been increased. Beneficial microorganisms are used to increase crop yields by stimulating plant growth through the production of phytohormones. In the present study, Indole Acetic Acid (IAA) production was analyzed in 18 bacterial isolates with Plant Growth-Promoting (PGP) capabilities; 15 of these isolates were *Rhizobium*, and three were *Serratia*. All isolates have been characterized morphologically and biochemically, and their IAA production in the presence of tryptophan, a precursor for IAA biosynthesis, has been evaluated. IAA production was detected using the Salkowski reagent with CT-2200 spectrophotometer at 530 nm. The levels of IAA production varied between the different isolates. The top two IAA producers were selected for genetic identification using 16SrRNA primers (27F and 1492R). One of the *Rhizobium* isolates (NRC-R2) shared 95% sequence similarity with *Rhizobium sp*., according to a Blastn search in GenBank and *Rhizobium leguminosarum*, whereas the *Serratia* isolate (Ain Shams Center for Genetic Engineering and Biotechnology (ACGEB)-S2 was 99.5% similar to *Serratia sp.* Accordingly, these two isolates could serve as biological sources for IAA.

1 Introduction

Beneficial soil microorganisms have a potential role in sustainable agriculture due to their ability to enhance plant growth. They can manage soil fertility and reduce the use of synthetic chemical pesticides (Rashid et al 2019). Consequently, the use of these microorganisms aids in the removal of pollutants and promotes plant growth (Verma et al 2019). Plant growth-promoting *Rhizobacteria* (PGPR), which stimulate plant growth, can also colonize the root, fix nitrogen, and boost crop yields. Furthermore, PGPR increases iron availability and phytohormone production in the plant (Kundan et al 2015). One of the most well-known examples of PGPR is *Rhizobium*, which can form a symbiotic relationship with its host plant and increase its ability to produce a higher yield (Miransari 2016). Besides nitrogen fixation, *Rhizobium* produces IAA and Gibberellic Acid in the root nodules, which are two of the most commonly desired auxins in the industry (Purwaningsih et al 2021). In addition to *Rhizobium*, *Serratia* is an effective plant growth promoter (Khan et al 2017) and a biological control agent (Ordentlich et al 1987). *Rhizobium* and *Serratia* can also produce plant hormones (also known as phytohormones) that regulate plant growth, including auxins, gibberellins, cytokinins, ethylene, and abscisic acid (Patel et al 2015). Auxins play a crucial role in plant growth development and regulate a vast array of

biological processes, including cell division, elongation, differentiation, and fruit development (Parvin et al 2020).

IAA is one of the auxins that are considered secondary metabolites in plants and is a byproduct of L-tryptophan metabolism. It is known to be produced by various microorganisms, including PGPR (Mohite 2013). L-tryptophan is a known physiological precursor of auxin production in both plants and microorganisms (Lebrazi et al 2020). However, the physiological effects of bacterial IAA production on plants and their potential role as a phytohormone in plant-microbe interaction have been studied (Parvin et al 2020). Therefore, this study aims to increase crop yields by supplying phytohormones through soil bacteria in order to maintain ecological balance.

2 Materials and Methods

2.1 Bacterial isolates

2.1.1 Isolates collection

The NRC and ACGEB yielded a total of 18 isolates, of which 15 were *Rhizobium* and three were *Serratia*. This research was conducted at the ACGEB laboratories, Faculty of Agriculture, Ain Shams University, Cairo, Egypt.

2.1.2 Isolates characterization

Gram staining morphologically confirmed all isolates (Smith and Hussey 2005). *Rhizobium* and *Serratia* were cultivated at 28°C for 48 h on yeast extract mannitol (YEM) medium containing 0.0025% (w/v) Congo red as an indicator (Lebrazi et al 2020) and King's medium (Venil et al 2009), respectively. Individual colonies were distinguished by colony form, margin, elevation, color, and mucosity (Nagalingam et al 2020). Catalase and indole tests were performed (Giammanco and Pignato 1994).

2.1.3 Catalase Test

A drop of 3% hydrogen peroxide was applied to a 48 h old bacterial colony that was placed on a clean glass slide and stirred to test isolates for catalase enzyme production (Kumar et al 2012).

2.2 IAA production screening

2.2.1 Indole test

Salkowski reagent (0.5 M ferric chloride ($FeCl_3$) and 35% perchloric acid ($HClO_4$)) were used to test isolates for IAA production (Kumar et al 2012).

2.2.2 IAA standard curve preparation

YEM and King's medium were utilized to formulate an IAA solution (0, 5, 10, 20, 50, and 100 µg/ml). In both, the amount of IAA was calculated by mixing 1 ml of the bacterial culture with 2 ml of Salkowski reagent; this mixture was incubated for 30 min. at RT in the dark. After incubation, the absorbance of the mixture was measured using a CT-2200 spectrophotometer (ChromTech, Taiwan) at 530 nm. Using the standard curve of IAA concentration vs. absorbance, the unknown IAA concentrations were estimated (Gang et al 2019).

2.2.3 Quantification of IAA production in the presence and absence of tryptophan

Isolates were inoculated in YEM and King's media, respectively ± tryptophan (0.1%), for 48 h at 28°C and 100 rpm shaking, and a non-inoculated broth served as a negative control. The supernatant was saved after 20 min of centrifugation at 4,000 rpm to eliminate bacterial cells. In the dark, 1 ml of supernatant was combined with two milliliters of Salkowski reagent. The IAA production was determined by plotting the absorbance against the standard curve at 530 nm using the CT-2200 spectrophotometer (ChromTech, Taiwan) (Mohite 2013).

2.3 Molecular Analysis

The isolates with the highest IAA production were chosen to be partially 16SrRNA gene sequenced with universal primers 27F (5'-AGAGTTTGATCCTGGCT CAG-3') and 1492R (5'-GGTTACCTTGTTACGAC TT-3') (James 2010), PCR products were purified using the Montage PCR Clean-up kit (Millipore), and the purified amplicons were sequenced at Macrogen using (MiSeq's sequencer) and was performed on the isolates with the highest IAA production (Macrogen, Inc., Seoul, Korea). The codon Code Aligner program version 9.0 (Codon Code Corporation, Dedham, MA, USA) was used to view chromatograms and generate contigs. NCBI's GenBank database was queried using Blastn (Altschul et al 1990) to retrieve the highest score and E-values. Using CLUSTAL W (Thompson et al 1994), multiple sequence alignment was performed, and pairwise distance was computed. The phylogenetic tree was generated using MEGA version 5.2.2 (Tamura et al 2011) with the Neighbor-Joining method and 1000 bootstrap replicates (Saitou and Nei 1987).

3 Results and Discussion

3.1 Isolates Characterization

All 15 isolates of *Rhizobium* displayed spherical, white, elevated, and sticky colonies on YEM plates. Similar results were reported by Nagalingam et al (2020) used YEM as an indicator medium for eight bacterial isolates extracted from the root nodules of *Cajanus cajan* to characterize its morphology and biochemistry. They determined that they were *Rhizobium*.

Similar to Su et al (2016) research, where he and others isolated *Serratia surfactantfaciens sp.* nov. YD25T, our three *Serratia* isolates exhibited bright red color and smooth and sticky colonies on King's medium. *Serratia surfactantfaciens sp.* nov. YD25T is used to investigate prodigiosin's biosynthesis, regulation, and production. Similar to **Fig 1.**, the catalase test was positive for all isolates, as indicated by the results (Kumar et al 2012). This study aimed to isolate and characterize bacteria from Rhizospheric soil for various plant growth promotion activities, i.e., symbiosis, IAA synthesis, phosphate solubilization, and HCN synthesis. **Fig 2.** IAA production in the form of qualitative colorimetric results turned the isolates red, which were the same results as those of Rahman et al (2010), who used Salkowski's reagent test as a primary screening index for functionalities of *Rhizobacteria* isolated from wild Dipterocarp samples in Indonesia in order to detect indolic substances.

3.2 IAA production screening

3.2.1 Standard curve of IAA

All isolates were screened for IAA production by comparing the stranded curve of known IAA concentrations (0, 5, 10, 20, 50, and 100 µg/ml) with the Salkowski reagent; *Rhizobium* isolates were tested for IAA production in YEM, as illustrated in **Fig 3**. The testing of *Serratia* isolates in King's is illustrated in **Fig 4**. There was a strong correlation between the red color intensity and the IAA concentration, as determined by the validity coefficient and illustrated by (R^2 values) in **Figs 3** and **4**. Lebrazi et al (2020) used various concentrations of IAA (0, 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 µg/ml) to create a standard curve for measuring IAA production in twenty-one isolates of *Rhizobium leguminosarum* (IAA production). We used some of the known concentrations of IAA and a higher concentration (100 µg/ml) to increase the production's scope of coverage.

3.2.2 Quantification of IAA production

The spectrophotometric quantification of *Rhizobium* and *Serratia* isolates' produced IAA revealed that the amount of IAA varied among isolates. **Table 1.** showed significant variance between IAA concentrations in the isolates, which ranged between 22.02 - 1079 when grown on a medium supplemented with 0.1% L-tryptophan. The maximum IAA production was detected in NRC-R2 (1079 µg/ml) and ACGEB-S2 (357 µg/ml). In a medium devoid of L-tryptophan, IAA concentrations ranged from 0 to 1079 µg/ml (333 - 4.637). ACGEB-S2 produced (333 µg/ml) while NRC-R2 produced (4.637 µg/ml). Although it was a low concentration of IAA, Lebrazi et al (2020) reported that L-tryptophan induced the biosynthesis of IAA in 21 *Rhizobium* isolates extracted from several leguminous plants (*Vicia faba* and *Lens culinaris*). However, our results were consistent with those of Ahmad et al (2008), and Datta and Basu (2000), who reported that the production of IAA increased when the medium was supplemented with up to 3% L-tryptophan, indicating that *Rhizobium* isolates thrive in the presence of L-tryptophan at concentrations as low as 5 mg/ml and as high as 3000 mg/ml.

Table 1. Screening of IAA concentrations produced by the *Rhizobium* and *Serratia* isolates ± L-tryptophan

Isolates	IAA concentrations (µg/ml) in isolates without Trp.	IAA concentrations (µg/ml) in isolates with Trp.
R1	6.955	936.2
R2	4.637	1079
R3	10.05	835.8
R4	30.91	995.8
R5	35.55	922.3
R6	11.98	945.5
R7	8.114	878.3
R8	26.66	874.4
R9	37.48	981.5
R10	10.43	22.02
R11	12.36	32.84
R12	20.48	984.2
R13	21.25	23.96
R14	10.85	42.24
R15	12.01	136.81
S1	5.65	98.26
S2	333	357
S3	---	77.39

Fig 1. Catalase test

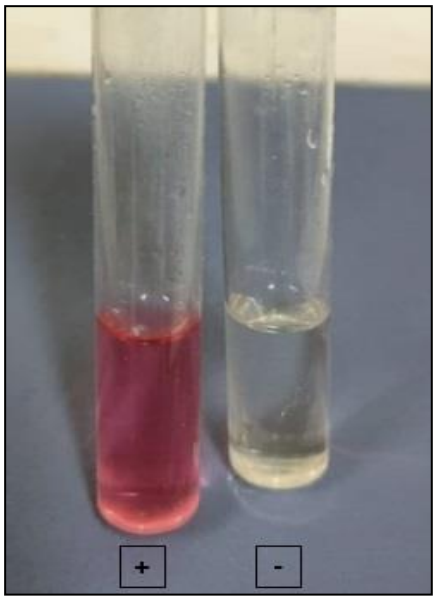

Fig 2. Indole test

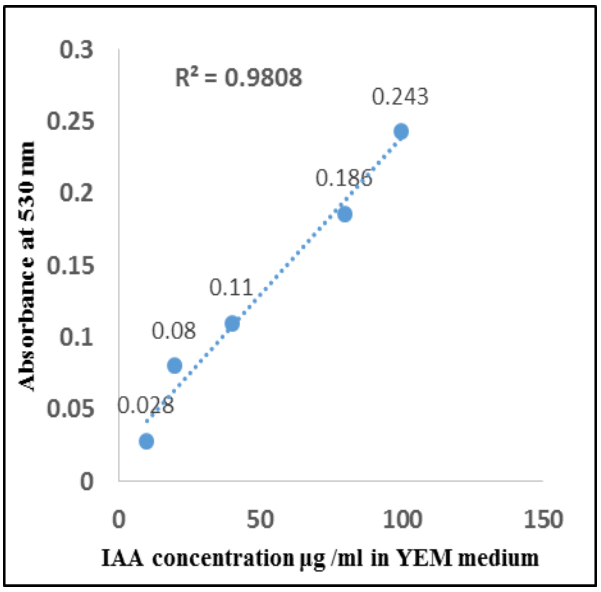

Fig 3. IAA Standard curve for *Rhizobium* isolates

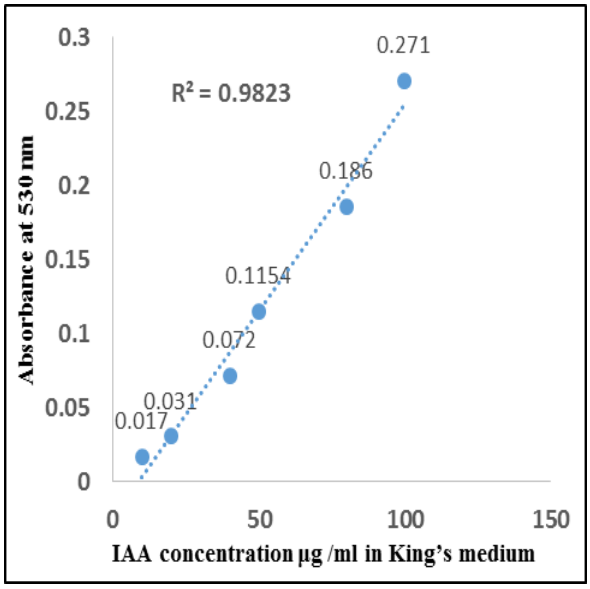

Fig 4. IAA Standard curve for *Serratia* isolates

3.3 Molecular analysis

Based on their highest IAA production, NRC-R2 and ACGEB-S2 isolates were chosen to be partially sequenced using universal primers 27F and 1492R (James 2010, Duangkhet et al 2018). The contigs of both isolates were generated using Codon-Code Aligner. The contig was (1079 bp) for NRC-R2 and (1151 bp) for ACGEB-S2.

Subsequently, the nearest species and subspecies referenced sequences were imported from GenBank and added to the query to perform multiple sequence alignments with CLUSTAL W and computing pairwise distances with maximum composite likelihood. A phylogenetic tree was generated using the Neighbor-Joining method with 1000 bootstrap values. NRC-R2 in **Fig 5.** belonged to the Family *Rhizobiaceae*, and it was most closely related to members of Genus *Rhizobium*, including *Rhizobium leguminosarum* strain ALD 2.7 with (94.96%) sequence similarity, *Rhizobium leguminosarum* strain MNF-EM-R4 (92.92%), *Rhizobium sp.* BGC8 (95.42%), *Rhizobium sp.* BGM6 (95.42%), *Rhizobium sp.* GGVW1 (95.42%), *Rhizobium sp.* GGC10 (95.42%),

Rhizobium sp. SOY7 (95,42%) and *Rhizobium leguminosarum* strain MNF-EM-R17 (94,20%) with an E-value of 0.0. As of ACGEB-S2 in **Fig 6**, it belonged to the Family *Enterobacteriaceae*, and it was most closely related to members of the Genus *Serratia*, including *Serratia marcescens* strain L1 with sequence similarity of (99.65%), *Serratia marcescens* strain 15-SYU (99.65%), *Serratia marcescens* strain A4 (99.51%), *Serratia marcescens* strain BLPS3 (99.58%), *Serratia marcescens* strain SerEW01 (99.58%), *Serratia marcescens* strain Se1 (99.65%), *Serratia marcescens* strain 7-SYU (99.44%), *Serratia marcescens* strain 11-SYU (99.51%), and *Serratia marcescens* strain TWV301 (99.51%) with E-value of 0.0.

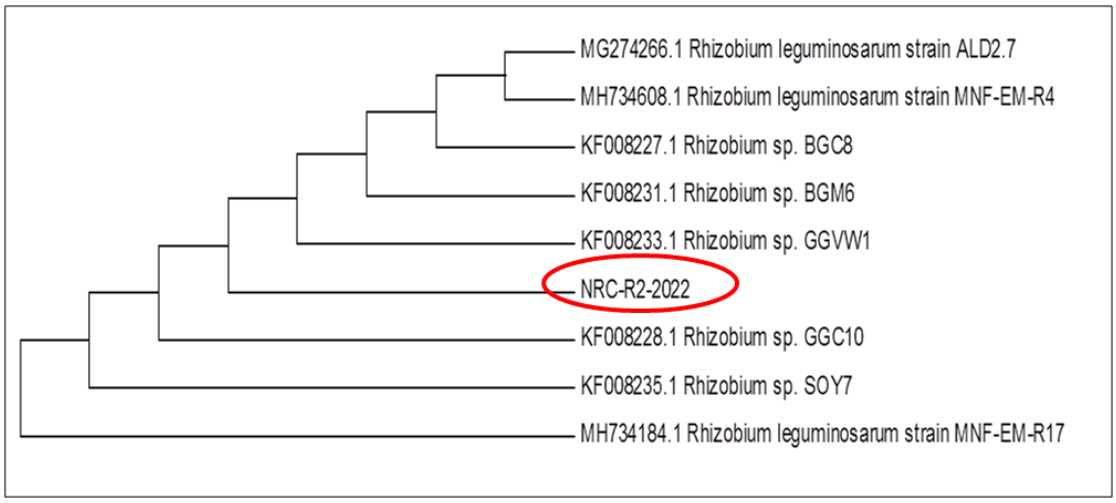

Fig 5. Phylogenetic tree of NRC-R2 (*Rhizobium*) using Neighbor-Joining with 1000 bootstrap

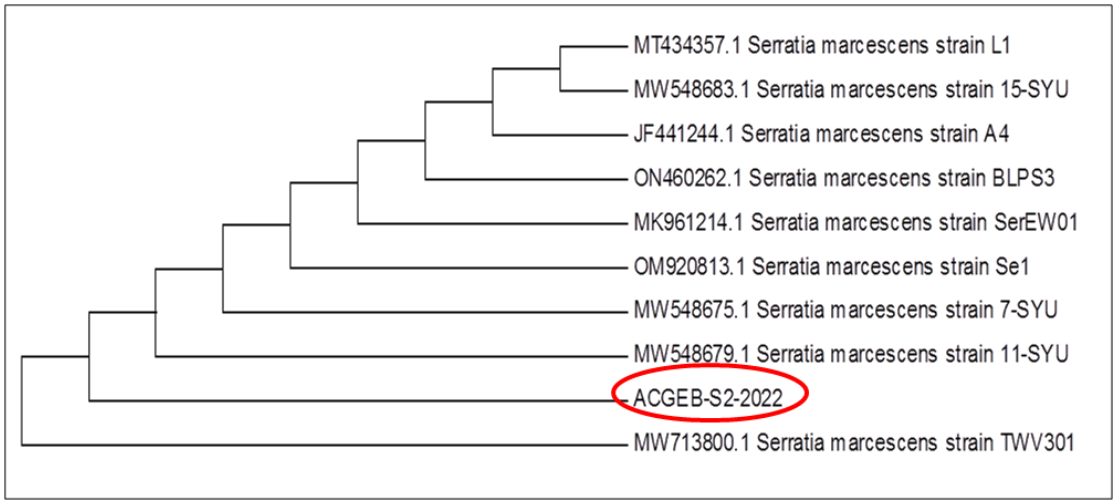

Fig 6. Phylogenetic tree of ACGEB-S2 *(Serratia)* using Neighbor-Joining with 1000 bootstrap

Gehlot et al (2012) used MEGA version 5.0 for tree generating with Neighbor-Joining algorithm and bootstrap values calculated for 1,000 replications for molecular characterization of the *Rhizobia* in India's Thar Desert. Sequence analysis and phylogeny of 16 selected isolates were aligned and compared with 16SrRNA of Rhizobial type strains available on GenBank. The results showed that his isolates from *Mimosa hamata* (JNVU MH3a & JNVU MH8) and *Acacia jacquemontii* (JNVU AJ10) exhibited 99.8% similarity to *Sinorhizobium sp.* ORS 1085 (AJ295078) was extracted from *Acacia tortilis ssp.* nodules. *Raddiana* grew in the Sahara region of Africa. In addition, Ghosh et al (2015) genetically characterize five *Rhizobium* isolates extracted from healthy nodules of *Neptunia oleracea* grown in a YEM medium supplemented with L-tryptophan. The phylogenetic analysis revealed that the isolates were identified as *Rhizobium undicola*, which can produce a substantial amount of IAA in the presence of L-tryptophan Ghosh et al (2015).

4 Conclusion

Indole-3-Acetic Acid is one of the most important physiological hormones regulating several crucial plant activities. In this study, the ability of 18 bacterial isolates to synthesize IAA in the presence and absence of L-tryptophan was evaluated. One isolate from each species (NRC-R2 and ACGEB-S2) exhibited a significant increase in IAA production and was selected for genetic classification using 16SrRNA with universal primers and NJ phylogenetic analysis. These isolates shared a high degree of similarity with other *Rhizobium sp.* and *Rhizobium leguminosarum* with 95% sequence similarity, whereas ACGEB-S2 displayed a high degree of sequence similarity with *Serratia sp.* (99.5%). These two isolates resemble an excellent indicator for acquiring one of the most vital phytohormones (IAAs) that bacteria can produce. Moreover, their ability to produce IAA could be used as bio-fertilizer inoculants to replace chemical fertilizers for crops such as wheat, maize, groundnut, soybean, and other vegetable crops in order to enhance their growth and provide a clean, sustainable environment.

Acknowledgment

The research team would like to thank Dr. Ashraf Gamil Attallah, Professor of Molecular Genetics, Biotechnology Research Institute, Microbial Genetics Department at the National Research Center, for providing some of the *Rhizobium* isolates used in this research study.

References

Ahmad F, Ahmad I, Khan MS (2008) Screening of free-living Rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research* 163, 173-181. https://doi.org/10.1016/j.micres.2006.04.001

Altschul SF, Gish W, Miller W, et al (1990) Basic local alignment search tool. *Journal of Molecular Biology* 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Datta C, Basu PS (2000) Indole acetic acid production by a *Rhizobium* species from root nodules of a leguminous shrub, *Cajanus cajan*. *Microbiological Research* 155, 23-127. https://doi.org/10.1016/S0944-5013(00)80047-6

Duangkhet M, Chikoti Y, Thepsukhon A, et al (2018) Isolation and characterization of Rhizobia from nodules of *Clitoria ternatea* in Thailand. *Plant Biotechnology* 35, 123-129. https://doi.org/10.5511/plantbiotechnology.18.0402a

Gang S, Sharma S, Saraf M, et al (2019) Analysis of Indole-3-acetic Acid (IAA) production in *Klebsiella* by LC-MS/MS and the Salkowski Method. *Bio-Protocol* 9, e3230. http://doi.org/10.21769/BioProtoc.3230

Gehlot HS, Panwar D, Tak N, et al (2012) Nodulation of legumes from the Thar desert of India and molecular characterization of their rhizobia. *Plant and Soil* 357, 227-243. http://doi.org/10.1007/s11104-012-1143-5

Ghosh PK, Kumar De T, Maiti TK (2015) Production and metabolism of indole acetic acid in root nodules and symbiont (*Rhizobium undicola*) isolated from root nodule of aquatic medicinal legume *Neptunia oleracea* Lour. *Journal of Botany* 2015, 575067. http://doi.org/10.1155/2015/575067

Giammanco G, Pignato S (1994) Rapid identification of microorganisms from urinary tract infections by β-glucuronidase, phenylalanine deaminase, cytochrome oxidase and indole tests on isolation media. *Journal of Medical Microbiology* 41, 389-392. https://doi.org/10.1099/00222615-41-6-389

James G (2010) Universal Bacterial Identification by PCR and DNA Sequencing of 16SrRNA Gene. In: Schuller M, Sloots T, James G, Halliday C, Carter I (Eds.), PCR for Clinical Microbiology. Springer, Dordrecht, pp 209–214. https://doi.org/10.1007/978-90-481-9039-3_28

Khan AR, Park GS, Asaf S, et al (2017) Complete genome analysis of *Serratia marcescens* RSC-14: A plant growth-promoting bacterium that alleviates cadmium stress in host plants. *PLoS ONE* 12, e0171534. https://doi.org/10.1371/journal.pone.0171534

Kumar A, Kumar A, Devi S, et al (2012) Isolation, screening and characterization of bacteria from Rhizospheric soils for different plant growth promotion (PGP) activities: an *In vitro* study. *Recent Research in Science and Technology* 4, 1-5. https://updatepublishing.com/journal/index.php/rrst/article/view/851

Kundan R, Pant G, Jadon N, et al (2015) Plant Growth Promoting *Rhizobacteria*: Mechanism and Current Prospective. *Journal of Fertilizers and Pesticides* 6, 1000155. https://doi.org/10.4172/2471-2728.1000155

Lebrazi S, Fadil M, Chraibi M, et al (2020) Screening and optimization of indole-3-acetic acid production by *Rhizobium* sp. strain using response surface methodology. *Journal of Genetic Engineering and Biotechnol*ogy 18, 21. https://doi.org/10.1186/s43141-020-00035-9

Miransari M (2016) Soybeans, Stress, and Plant Growth-Promoting *Rhizobacteria*. In*:* Miransari M (Ed), Environmental Stresses in Soybean Production: Soybean Production Vol. 2. San Diego Academic Press, pp 177-203.

Mohite B (2013) Isolation and characterization of indole acetic acid (IAA) producing bacteria from Rhizospheric soil and its effect on plant growth. *Journal of Soil Science and Plant Nutrition* 13, 638-649. http://dx.doi.org/10.4067/S0718-95162013005000051

Nagalingam S, Nithya TV, Gayathri D, et al (2020) Morphological, Biochemical and Plant Growth Promoting Characterization of Rhizobia Isolated from Root Nodules of *Cajanus Cajan* L. *Plant Archives* 20, 1293-1299. https://api.semanticscholar.org/CorpusID:227298368

Ordentlich A, Elad Y, Chet I (1987) Rhizosphere colonization by *Serratia marcescens* for the control of *Sclerotium rolfsii*. *Soil Biology and Biochemistry* 19, 747-751. https://doi.org/10.1016/0038-0717(87)90058-7

Parvin W, Rahman MM, Govender NT, et al (2020) Identification, Determination and Quantification of Indole-3-Acetic Acid Produced by *Pseudomonas aeruginosa* UPMP3 and Its Effect on The Growth of Oil Palm (*Elaeis guineensis* Jacq). *World Journal of Agricultural Research* 8, 75-83. http://www.sciepub.com/WJAR/abstract/11959

Patel K, Goswami D, Dhandhukia P, et al (2015) Techniques to Study Microbial Phytohormones. In: Maheshwari D (Eds), Bacterial Metabolites in Sustainable Agroecosystem. Sustainable Development and Biodiversity Springer, Cham, 12, 1-27. https://doi.org/10.1007/978-3-319-24654-3_1

Purwaningsih S, Agustiyani D, Antonius S (2021) Diversity, activity, and effectiveness of *Rhizobium* bacteria as plant growth promoting Rhizobacteria (PGPR) isolated from Dieng, central Java. *Iranian Journal of Microbiology* 13, 130-136. https://doi.org/10.18502/ijm.v13i1.5504

Rahman A, Sitepu IR, Tang SY, et al (2010) Salkowski's reagent test as a primary screening index for functionalities of Rhizobacteria isolated from wild dipterocarp saplings growing naturally on medium-strongly acidic tropical peat soil. *Bioscience, Biotechnology and Biochemistry* 74, 2202-2208. https://doi.org/10.1271/bbb.100360

Rashid MH, Kamruzzaman M, Haque ANA, et al (2019) Soil microbes for sustainable agriculture. In: Meena R, Kumar S, Bohra J, Jat M (Eds.) Sustainable Management of Soil and Environment. Springer, Singapore, pp 339-382. https://doi.org/10.1007/978-981-13-8832-3_10

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Smith AC, Hussey MA (2005) Gram stain protocols. *American Society for Microbiology* 1, 14. https://rb.gy/19ofhw https://asm.org/protocols/gram-stain-protocols

Su C, Xiang Z, Liu Y, et al (2016) Analysis of the genomic sequences and metabolites of *Serratia surfactantfaciens* sp. nov. YD25^{T} that simultaneously produces prodigiosin and serrawett in W2. *BMC Genomics* 17, 865. https://doi.org/10.1186/s12864-016-3171-7

Tamura K, Peterson D, Peterson N, et al (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution* 28, 2731-2739. https://doi.org/10.1093/molbev/msr121

Thompson JD, Higgins DJ, Gibson TJ (1994) CLUSTALW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22, 4673-4680. https://doi.org/10.1093/nar/22.22.4673

Venil CK, Kamatshi NS, Velmurugan P, et al (2009) Effect of diammonium phosphate in kings B medium for the production of prodigiosin from *Serratia marcescens* isolated from the gut of silkworm (*Bombyx mori*). *International Journal of Applied Environmental Sciences* 4, 59-70. https://rb.gy/vrsqy7

Verma M, Mishra J, Arora NK (2019) Plant Growth-Promoting *Rhizobacteria*: Diversity and Applications. In: Sobti R, Arora N, Kothari R (Eds.) Environmental Biotechnology: For Sustainable Future. Springer, Singapore, pp 129-173. https://doi.org/10.1007/978-981-10-7284-0_6