Isolation, screening and characterization of bacteria from Rhizosphere soil of root crops for different plant growth promotion (PGP) activities: an *in vitro* study

Konda Lakshmi Jahnavi, Sai Jaya Sri I. and Srilekha V.*
Department of Biotechnology, Chaitanya (Deemed to be University), Himayat Nagar(V), Moinabad (M), Ranga Reddy, 500075, Telangana, INDIA
*srilekamicrobiology@gmail.com

Abstract

The use of plant growth-promoting rhizobacteria (PGPR) to enhance productivity may serve as a viable alternative to organic fertilizers. The primary objective is to mitigate pollution and conserve the environment through ecological agricultural principles. Plant growth-promoting rhizobacteria (PGPR) influence plant growth through various direct and indirect mechanisms. The bacteria inhabiting the plant's rhizosphere are referred to as plant growth-promoting rhizobacteria (PGPR). PGPR bacteria are free-living microorganisms that colonize plant roots and enhance plant growth. This study aimed to identify and select the most effective PGPR and assess their efficacy in zinc solubilisation, HCN generation and ACC deaminase activity.

This research assessed 19 isolates from several root crop fields for their plant growth-promoting characteristics. The detected bacterial strains, especially the KJ02 isolate, exhibit favourable PGPR traits, rendering them suitable for biofertilizer production to enhance zinc availability in soil, thereby promoting the growth and development of root crops. KJ02, derived from the rhizosphere soil of roots, can be employed in soils deficient in zinc or where insoluble zinc is abundant. In this investigation, three isolates showed positive results in the HCN synthesis assay, evidenced by a colour change from yellow to dark brown, with KJ02 displaying the most significant colour shift.

Keywords: Bacteria, Molecular identification, Rhizobacteria, PGPR, Zinc solubilization.

Introduction

In contemporary agriculture, the primary focus is on enhancing and augmenting agricultural productivity. A significant achievement has been realized in this sector due to the green revolution and its impact on farmers' lives. However, the reliance on excessive chemical fertilizers and pesticides in agricultural fields significantly undermines sustainable farming practices. This has degraded soil quality, diminished the availability of mineral nutrients and adversely affected groundwater. In these challenging circumstances, organic farming can help to mitigate the situation and can reverse the degradation of the ecosystem. Consequently, bio-fertilizer, as a blend of growth-specific nutrients, offers a promising solution for contemporary, growth-oriented agriculture, fostering sustainable crop production while safeguarding and preserving environmental conditions[26].

Plants establish mutualistic associations with soil bacteria during their growth and development. It is believed that they exude 20–50% of their photosynthetically derived carbon into the soil rhizosphere[12]. Soil bacteria exploit these exudates, along with shed root cells, as a nutrient source, thereby positively influencing the host plant[15]. The organisms encompass plant growth-promoting rhizobacteria (PGPR), which aid plant growth by nitrogen fixation and the synthesis of plant growth regulators such as indole-3-acetic acid (IAA) and siderophores which provide essential iron to plants[2,3,9]. These rhizobacteria produce cyanide to exert antagonistic effects on phytopathogenic soil microbes, together with 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which regulates ethylene levels in developing plant roots[1,7,17,24].

PGPR may facilitate the solubilization of phosphate and potassium, the breakdown of environmental contaminants and the synthesis of antibiotics and/or lytic enzymes that inhibit plant infections, in addition to heavy metal detoxification and salinity tolerance. PGPR activities significantly contribute to soil fertility[4,13,21,29]. Their engagement with plants has been commercially exploited and their potential significance for sustainable agriculture has sparked increased scholarly attention[14].

The rhizosphere microbiomes of numerous crop plants have been extensively reported regarding the identification of plant growth-promoting rhizobacteria (PGPR) and their processes of plant growth promotion (PGP)[27,30,31]. Nevertheless, grass species that could serve as homes for new PGPR are still largely unexamined[18]. This study aimed to discover, characterise and evaluate the bacterial isolates in the rhizosphere of root crops in the Warangal region of Telangana, India, that enhance plant growth. This study aims to identify a potent strain that improves understanding of PGPR and its role in promoting plant growth.

Material and Methods

Sample Collection from Rhizosphere Soil: Rhizospheric soil samples were taken from the Fort Warangal fields (17.957273S, 79.614976N), Warangal, Telangana, India. The soil was excavated to a depth of approximately 5-30 cm, preserving the roots. The soil attached to the origins was

dislodged by gently shaking them, after which the roots were collected in a clean polyethene bag. The sample was either utilized immediately or stored at 4°C until use. The soil's moisture content was determined using the oven-dry method.

Isolation of the Rhizobacteria from the Soil Sample Collected: Ten grams of collected soil were dissolved in 90 milliliters of sterile deionized water and the soil particles were suspended by stirring for 10 minutes. One milliliter of the suspension was combined with nine milliliters of deionized water to achieve a one-fold dilution; similarly, serial dilutions were performed for 10^{-2}, 10^{-3} and 10^{-4}. Twenty microlitres of each dilution were inoculated into nutrient agar plates, yeast extract mannitol agar plates and NAM glycerol. Among these three media, the YEMA medium exhibited significant growth. Bacterial isolates were selected and sub-cultured based on their morphological traits until pure cultures were achieved (Figure 1). The pure isolates were stored for future use in an 80% glycerol stock at -60°C.

Biochemical Characterization: The colony morphology including color, transparency, elevation, margin and form of the bacterial isolates, was documented. Biochemical assays, including Gram staining, motility assessment, catalase analysis, citrate utilization evaluation, methyl red test, gelatin and ureases were conducted (Table 1 and 2).

Figure 1: Pure cultures of isolated pigmented bacteria

Table 1
Morphological characteristics of bacteria isolated from soil of root crops

Isolate	Motility	Shape	Gram Strain	Surface	Margin	Pigmentation
KJ01	Non motile	Cocci	+ve	Smooth	Entire	white
KJ02	Motile	Rod	-ve	Smooth	Entire	Red
KJ03	Non motile	Cocci	+ve	Smooth	Entire	Red
KJ04	Non motile	Cocci	-ve	Smooth	Entire	Cream
KJ05	Motile	Cocci	-ve	Smooth	Entire	cream
KJ06	Motile	Cocci	+ve	Smooth	Entire	white
KJ07	Motile	Rod	+ve	Smooth	Entire	Orange
KJ08	Motile	Rod	+ve	Smooth	Entire	Red
KJ09	Motile	Rod	+ve	Smooth	Entire	white
KJ10	Motile	Rod	+ve	Smooth	Entire	Yellow
KJ11	Motile	Rod	-ve	Smooth	Undulate	Thick Orange
KJ12	Motile	Cocci	+ve	Smooth	Entire	white
KJ13	Motile	Rod	-ve	Smooth	Entire	white
KJ14	Motile	Rod	-ve	Smooth	Entire	Red
KJ15	Non motile	Rod	-ve	Smooth	Entire	Light cream
KJ16	Non motile	Rod	+ve	Smooth	Entire	cream
KJ17	Non motile	Cocci	+ve	Smooth	Undulate	cream
KJ18	Motile	Cocci	+ve	Smooth	Entire	cream
KJ19	Motile	Cocci	+ve	Smooth	Entire	white

Table 2
Represents the biochemical assay of the bacterial isolates

Isolate	Citrate	Indole	MR Test	VP Test	Gelatin	Urease
KJ01	+	-	+	+	-	-
KJ02	+	+	-	-	+	-
KJ03	+	-	-	-	-	+
KJ04	+	-	-	-	-	-
KJ05	+	+	+	-	-	-
KJ06	+	+	+	-	-	-
KJ07	+	-	+	-	+	-
KJ08	+	-	+	-	+	-
KJ09	-	-	+	-	+	-
KJ10	-	-	+	-	+	-
KJ11	-	-	+	-	+	-
KJ12	-	+	+	-	+	-
KJ13	-	+	-	-	+	-
KJ14	+	+	-	-	-	-
KJ15	+	+	-	+	-	-
KJ16	+	+	-	+	-	-
KJ17	+	-	-	+	-	-
KJ18	+	-	-	+	-	-
KJ19	+	-	-	+	-	-

Screening of Isolates for Zinc Solubilization by Plate Assay: A loop of bacterial culture isolates was individually diluted in sterile distilled water using the serial dilution procedure and thereafter disseminated onto Petri plates containing liquid salt agar medium with insoluble sources of ZnO and $ZnCO_3$ respectively. Following incubation, the diameters of the bacterial colonies and the halo zones surrounding them were measured and the values were computed using the solubilisation index formula: SI= (Colony 54 diameter + Halozone diameter / colony diameter)[5].

Test for hydrogen cyanide (HCN) Production: To ascertain the synthesis of HCN, a loopful of overnight culture was inoculated onto nutrient agar plates enriched with glycine (4.4 g/L). The Petri dish was thereafter flipped and a sterile piece of Whatmann filter paper no. 1, saturated with a yellow picric acid solution (0.5% picric acid in 2% sodium carbonate), was positioned on the lid. The Petri dish was sealed with parafilm and incubated at 28ºC for 96 hours. The successful generation of HCN was evidenced by the emergence of an orange-to-red hue on the paper following the incubation time[6].

Isolation and screening for ACC deaminase activity: Soil samples from the rhizosphere of root crops were obtained at Fort Warangal Fields, Telangana, India and tested for ACCD-positive bacteria according to Glick et al[9] with minor modifications. The minimal medium ($CaCO_3$ 4.0gm, Glucose 2.0gm, Sodium citrate 2.0gm, Potassium gluconate 2.0gm in 1000 ml Distilled water) and minimal ACC media agar plates with a final ACC content of 3.0 mmol l^{-1} were formulated as outlined by Penrose and Glick[21]. Bromothymol blue (BTB) at 0.005% and phenol red at 0.008% were individually included as pH indicator dyes into minimum ACC plates.

Diluted soil samples (10^{-5}) were inoculated onto plates and incubated at 37 °C for 24 hours. Isolated colonies were cultivated on minimal ACC medium agar plates and preliminary characterisation and identification were conducted using Bergey's Manual with pH indicator dye + minimal ACC medium agar plates. Preliminary bacterial identifications were then validated using 16S rRNA sequencing analysis.

Molecular Characterization of the Bacterial Isolate: Based on the data obtained, the most significant isolate, KJ02, was selected and submitted to Barcode Biosciences in Bengaluru, Karnataka, for molecular characterization via 16S rRNA gene sequencing. Bacterial genomic DNA was isolated and subjected to electrophoresis on 1% agarose gel. Fragments of the 16S rRNA gene were amplified via Polymerase Chain Reaction (PCR). The 16S rRNA F and 16S rRNA R primers were used as forward and reverse primers respectively, utilizing the Bacillus direct testing (BDT) v3.1 cycle sequencing kit on the ABI 3730xl genetic analyser.

The consensus sequences derived from the sequencing data are aligned using alignment tools and analyzed with the Basic Local Alignment Search Tool (BLAST) to discover potential matches. Ten sequences were chosen based on the highest identity score, followed by a multiple sequence alignment of 70 using CLUSTAL W. The phylogenetic tree was ultimately generated utilizing Molecular Evolutionary Genetics Analysis (MEGA) 10 software[22].

Results and Discussion

Isolation and identification: Rhizospheric soil samples were collected from the Fort Warangal fields (17.957273, 79.614976), Warangal, Telangana, India. The samples were designated KJ01-KJ19 according to their collection locations. The soil samples were serially diluted and spread-plated onto a nutrient agar medium, resulting in the isolation of 19 morphologically distinct bacterial colonies designated as KJ01 through KJ19. The morphological distinctions among the isolates were validated through a comprehensive study of colony morphology. The isolates were selected for further investigation to determine their potential plant growth-promoting features, including the synthesis of HCN, ACC deaminase and zinc solubilization.

Test for zinc solubilisation: In the current study, the strain KJ02 exhibited the largest zone of solubilisation (Figure 2). KJ02 was identified as a powerful zinc-solubilizing bacterium, exhibiting a solubilisation zone of 28 mm. Goteti et al[11] similarly stated that the highest halozone in zinc oxide and zinc carbonate was observed by Bacillus sp. Saravanan et al[25] observed that the solubilization potential of the bacterial isolate Pseudomonas sp. was greater in zinc oxide-supplemented media than in zinc sulfate-supplemented media. They have also documented a maximum of 20 cm halozone in zinc oxide and 14 cm in zinc carbonate medium. The current work corroborates prior findings, demonstrating that both RRT19 and RRT34 can significantly solubilise insoluble zinc in a liquid salt solution, hence facilitating their application in soil to promote plant growth and development.

Test for HCN production: Bacteria can synthesise HCN which has a significant function in defence against harmful fungi, weeds, or insects. Certain *Bacillus species* contain the HCN synthase gene which facilitates the manufacture of HCN. This promotes plant growth by sequestering metals in the soil, so indirectly rendering soil nutrients accessible to the plants. It is generated through oxidative decarboxylation of amino acids including methionine, glutamate and glycine.

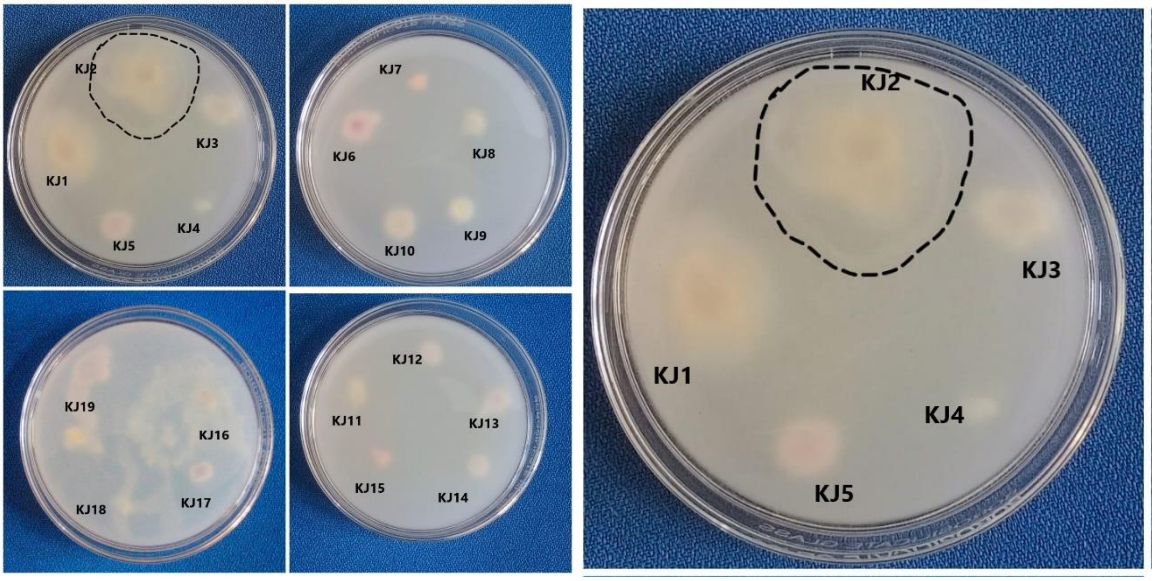

Figure 2: Representing the Zinc solubilization halo of the isolate KJ02

Figure 3: Representing the HCN Production of Isolate KJ02

HCN is recognised for its efficacy in managing various soil-borne diseases. It was demonstrated that Bacillus spp. Enhances plant growth and exhibits the capacity for HCN synthesis[28]. A further investigation has shown the capacity for HCN breakdown in *Pseudomonas stutzeri* and *B. subtilis*[19]. The *B. subtilis* ER-08 (BST) strain has been documented for its capacity to produce HCN [16]. In a separate study, *B. cereus* CUAMS116 was found to possess the capability to produce HCN[23]. In the current investigation, three isolates exhibited promising results in the HCN synthesis assay, as evidenced by a colour change from yellow to dark brown with KJ02 displaying the most significant colour alteration (Figure 3).

The production of ACCD is a crucial biomarker of the activity of plant growth-promoting rhizobacteria (PGPR). The use of 1-aminocyclopropane-1-carboxylate (ACC) as the exclusive nitrogen source in the minimum medium is a prevalent and widely employed technique for screening ACC-degrading microorganisms[10]. This approach does not provide a primary indication of the effective ACC deaminase activity of any putative microbial isolate. Consequently, to address this deficiency and enhance the efficacy of ACCD producer screening, we have devised a straightforward technique. The catalysis of ACC-by-ACC deaminase produces ammonia.

The quantity of ammonia emitted indicates the relative activity of ACCD. Ammonia can additionally be identified using pH indicator dyes such as bromothymol blue and phenol red. This establishes a foundation for developing a sufficiently sensitive ACCD detection system. The optimal concentrations of BTB (0.005%) and phenol red (0.008%) to be incorporated into the minimal medium with ACC were established by calculating the colony-forming units (CFU) of rhizosphere soil samples. To evaluate the appropriateness of dyes in screening microorganisms that generate ACC deaminase, serially diluted rhizosphere soil samples (10^{-5} dilution) were streaked onto control and test plates and monitored for microbial growth.

Control ACC-minimal medium plates devoid of pH indicator dyes were juxtaposed with test plates comprising ACC-minimal medium and distinct pH indicator dyes (BTB and phenol red). Few colonies appeared on both the control and test plates. Colonies on ACC-minimal medium were devoid of colour and no notable phenotypic variations in morphology, dimensions, or colony characteristics were detected among them (Figure 4).

Conversely, colonies emerged on ACC medium with a pH indicator dye, demonstrating a notable distinction among isolated colonies with corresponding zones of colour alteration surrounding them. Some colonies were colourless and lacked a surrounding colour zone, while others exhibited faint to dark colour zones. Additionally, some colonies were pigmented but did not display any colour shift in their vicinity. Therefore, it is acceptable to assert that a metabolic gradient exists in the ACC deaminase activity of microorganisms, likely due to the ACC minimum media. Our suggested assay offers an instantaneous presumptive differentiation between moderate and high ACC deaminase producers by only analysing bacterial colony morphologies on plates with indicator colours.

This medium could markedly diminish pseudo-positive samples and those exhibiting low ACC deaminase activity. The vast, untapped and unidentified microbial resources globally warrant thorough screening for microbial species with significant ACC deaminase production capability. The utilisation of effective microorganisms to enhance agricultural productivity, consequently generates nutritious, healthful and pesticide-free food is imperative[10]. This approach is cost-effective and necessitates only fundamental microbiology experimental apparatus. The isolation of ACC-encoding genes and their associated enzymatic characteristics can be compared and assessed for potential applications. Following the extensive biochemical characterisation, the bacterial isolate KJ02 underwent molecular characterisation techniques. The sequencing of the 16S rRNA gene indicated that the isolate KJ02 was a pigmented bacterium.

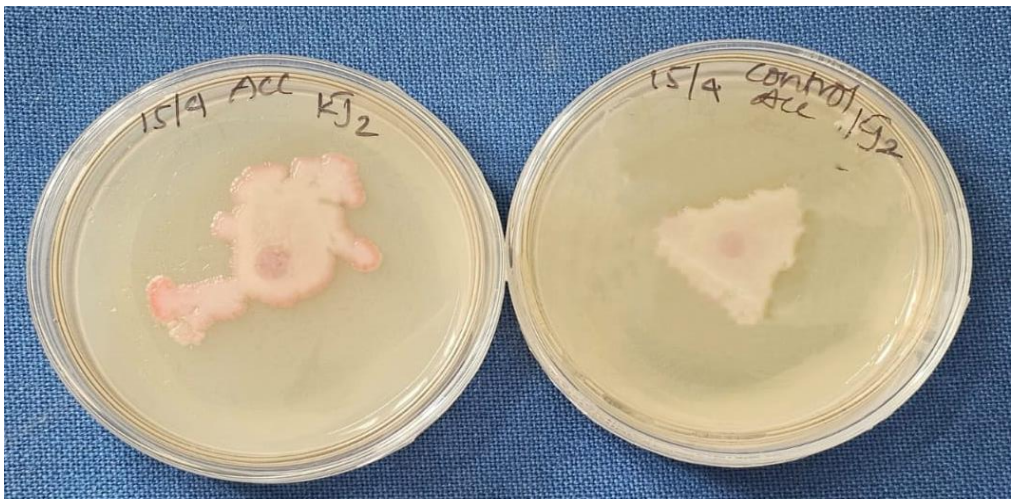

Figure 4: Representing the Acc Deaminase activity of Isolate KJ02

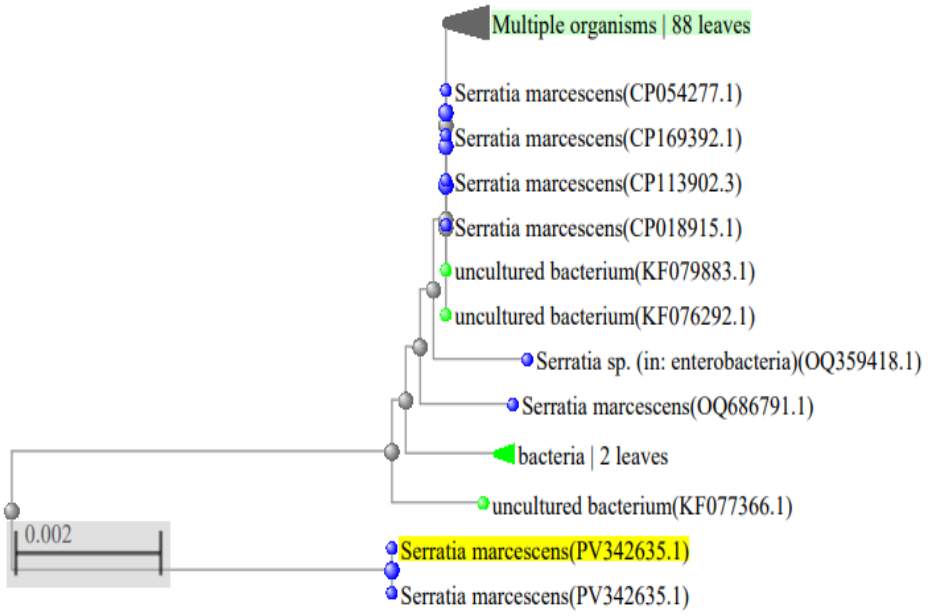

Figure 5: Representing the phylogenetic tree of Isolate KJ02

The sequences were analysed, confirmed and submitted to the GenBank database, resulting in the accession number PV342635 for *Serratia marcescens*. Phylogenetic trees were created using Clustal W for sequence alignment and MEGA 6 software for phylogenetic analysis of the strains (Figure 5).

Conclusion

This study evaluated 19 isolates from various root crop fields for their plant growth-promoting properties. The identified bacterial strains, particularly the KJ02 isolate, exhibit promising PGPR characteristics, making them suitable for development as a biofertilizer to enhance zinc availability in soil, thereby improving the growth and development of root crops. KJ02, isolated from the rhizosphere soil of root crops, can be utilised in soils lacking in zinc or where insoluble zinc is prevalent.

In the present study, three isolates demonstrated favourable outcomes in the HCN production assay, indicated by a colour transition from yellow to dark brown, with KJ02 exhibiting the most pronounced colour shift. Preliminary evidence suggests that ACC deaminase-containing PGPR strains are advantageous both in field settings and controlled laboratory environments.

References

1. Baber et al, Weed rhizosphere: a source of novel plant growth promoting rhizobacteria (PGPR), *International Journal of Bioscience*, **13**, 224–234 **(2018)**

2. Bhattacharyya P.N. and Jha D.K., Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture, *World Journal of Microbiology & Biotechnology*, **28(4)**, 1327–1350 **(2012)**

3. Dennis P.G., Miller A.J. and Hirsch P.R., Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities?, *FEMS Microbiology Ecology*, **73(3)**, 313–327 **(2010)**

4. Devine K. and Fei S., A review of impacts by invasive exotic plants on forest ecosystem services. Proceedings of the 17th Central Hardwood Forest Conference, Newtown Square, Pennsylvania, USDA Forest Service, Northern Research Station, 425–435 **(2011)**

5. Edi Premono M., Moawad A.M. and Vlek P.L.G., Effect of phosphate-solubilizing Pseudomonas putida on the growth of maize and its survival in the rhizosphere, *Indones. J. Crop Sci*., **11**, 13-23 **(1996)**

6. El-Hendawy H.H. and Abo-Elyousr K.A.M., Combination of different antagonis -tic Bacteria to control of Potato Blackleg Disease caused by Pectobacteriumatrosepticum under Greenhouse and Field Conditions, *Int J Phytopathol.*, **5**, 35–43 **(2016)**

7. Fei S., Phillips J. and Shouse M., Biogeomorphic impacts of invasive species, *Annual Review of Ecology & Evolution Systematics*, **45(1)**, 69–87 **(2014)**

8. Glick B.R., The enhancement of plant growth by free-living bacteria, *Can J Microbiol*, **41**, 109–117 **(1995)**

9. Glick et al, Biochemical and genetic mechanisms used by plant growth-promoting bacteria, London, Imperial College Press **(1999)**

10. Glick B.R., Bacteria with ACC deaminase can promote plant growth and help to feed the world, *Microbiol. Res*., **169**, 30–39 **(2014)**

11. Goteti P.K., Emmanuel L.D.A., Desai S. and Shaik M.H.A., Prospective zinc solubilising bacteria for enhanced nutrient uptake and growth promotion in maize (Zea mays), *Int. J. Microbiol*., doi: 10.1155/2013/869697 **(2013)**

12. Gouda et al, Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture, *Microbiological Research*, **206(1)**, 131–140 **(2018)**

13. Gupta et al, Plant growth promoting rhizobacteria (PGPR): current and future prospects for development of sustainable agriculture, *Journal of Microbial & Biochemical Technology*, **7(2)**, 96–102 **(2015)**

14. Kong et al, Effect of Ageratina adenophora invasion on the composition and diversity of soil microbiome, *Journal of General and Applied Microbiology*, **63(2)**, 114–123 **(2017)**

15. Kumar S., Stecher G., Li M., Knyaz C. and Tamura K., MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms, *Mol. Biol. Evol*., **35(6)**, 1547-1549**(2018)**

16. Kuzyakov Y. and Domanski G., Carbon input by plants into the soil, *Journal of Plant Nutrition and Soil Science*, **163**, 421–431 **(2000)**

17. Mahmood et al, Plant growth promoting rhizobacteria and silicon synergistically enhance salinity tolerance of mung bean, *Frontiers in Plant Science*, **7(243)**, 1–14 **(2016)**

18. Niu et al, An invasive aster (Ageratina adenophora) invades and dominates forest under stories in China: altered soil microbial communities facilitate the invader and inhibit natives, *Plant and Soil*, **294(1–2)**, 73–85 **(2007)**

19. Nwokoro O. and Dibua M.E.U., Degradation of soil cyanide by single and mixed cultures of *Pseudomonas stutzeri* and *Bacillus subtilis*, *Arh. Hig. Rada Toksikol*., **65(1)**, 113-119 **(2014)**

20. Patel M. et al, *Bacillus subtilis* ER-08, a multifunctional plant growth-promoting rhizobacterium, promotes the growth of fenugreek (Trigonella foenum-graecum L.) plants under salt and drought stress, *Front. Microbiol*.,**14**, 120-143 **(2023)**

21. Penrose D.M. and Glick B.R., Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria, *Physiologia Plantarum*, **118(1)**, 10-5 **(2003)**

22. Poudel et al, Biology and management of the invasive Ageratina adenophora (Asteraceae): current state of knowledge and future research needs, *Weed Research*, **59(2)**, 79–92 **(2019)**

23. Ramanaiah M., Balakrishna M., Gouthamsri S., Sowbhagya Lakshmi M. and Jayarao K., Impact of Sodium Dodecyl Sulfate Anionic Micelles on the Complex Equilibria of Divalent Metal Ions with 5-Sulfosalicylic Acid, *Res. J. Chem. Environ.*, **28(3)**, 10-18 **(2024)**

24. Sarathambal et al, A review on weeds as source of novel plant growth promoting microbes for crop improvement, *Journal of Applied and Natural Science*, **6(2)**, 880–886 **(2014)**

25. Saravanan V.S., Subramoniam S.R. and Raj S.A., Assessing in vitro solubilization potential of different zinc solubilizing bacterial (ZSB) isolates, *Brazil. J. Microbiol*., **34**, 121–125 **(2003)**

26. Sherpa M.T., Bag N., Das S., Haokip P. and Sharma L., Isolation and characterization of plant growth promoting rhizobacteria isolated from organically grown high yielding pole type native pea (Pisum sativum L.) variety Dentami of Sikkim, India, *Current Research in Microbial Sciences*, **2**, 100068 **(2021)**

27. Sun X., Gao C. and Guo L.D., Changes in arbuscular mycorrhizal fungus community along an exotic plant Eupatorium adenophorum invasion in a Chinese secondary forest, *Journal of Microbiology*, **51(3)**, 295–300 **(2013)**

28. Wagi S. and Ahmed A., Bacillus spp.: potent microfactories of bacterial IAA, *Peer J*, **7**, 725-748 **(2019)**

29. Walsh J.R., Carpenter S.R. and Vander Zanden M.J., Invasive species triggers a massive loss of ecosystem services through a trophic cascade, Proceedings of the National Academy of Science of the United States of America, **113(15)**, 4081–4085 **(2016)**

30. Xia et al, Compositional and functional profiling of the rhizosphere microbiomes of the invasive weed Ageratina adenophora and native plants, *Peer J.*, **9**, e10844 **(2021)**

31. Zhao et al, Ageratina adenophora invasions are associated with microbially mediated differences in biogeochemical cycles, *Science of the Total Environment*, **677**, 47–56 **(2019)**.

(Received 18th July 2025, accepted 20th August 2025)