Original Research Article

Functional characterization of rhizobia for multiple plant growth promoting activities

Abstract

This research focuses on the functional characterization of 19 rhizobia strains isolated from Southern Rajasthan's Udaipur and Dungarpur districts, with an emphasis on their multiple Plant Growth Promoting (PGP) activities. Through a series of tests encompassing Indole-3-Acetic Acid (IAA) production, siderophore production, phosphate solubilization, ammonia production, and hydrogen cyanide (HCN) production, this study elucidates the diverse PGP potential of these rhizobia strains. The results showcase variations in IAA production, highlighting their ability to enhance root development and overall plant growth, while also revealing strains capable of siderophore production, indicating potential iron uptake facilitation. Furthermore, some strains exhibit phosphate solubilization, ammonia production, and HCN production capabilities, signifying their potential to improve nutrient availability and protect plants from pathogens. These findings underscore the promise of harnessing the PGP activities of these rhizobia strains for sustainable agricultural practices in Southern Rajasthan and similar agroecological regions, with implications for enhanced crop yields and soil health.

Keywords: Rhizobia, Functional Characterization, Agricultural Sustainability, Microbial Diversity, Plant Growth Promoting Activities

Introduction

The pea, known by its botanical name *Pisum sativum*, belongs to the Fabaceae family (Dobrovolna *et al*., 2022). This popular legume species traces its origins to the Mediterranean region and Southwestern Asia (Zohary *et al*., 2012). Taxonomically, it falls under the Kingdom Plantae, Division Magnoliophyta, Class Magnoliopsida, Order Fabales, and Family Fabaceae. In the context of its symbiotic relationship with rhizobia, *Pisumsativum* forms nodules on its roots where compatible strains of rhizobia, particularly *Rhizobium leguminosarum* (Laguerre *et al*., 2007), establish a mutually beneficial association. Within these nodules, the rhizobia fixes atmospheric nitrogen, converting it into a form that is readily available for plant nutrition, thereby contributing significantly to the pea plant's growth and nitrogen nutrition. Plant growth promotion refers to the enhancement of plant growth, development, and overall health through the interactions between plants and specific microorganisms present in the soil (Hayat *et al*., 2010). These microorganisms, often termed as plant growth-promoting microorganisms (PGPMs), encompass a diverse group of bacteria, fungi, and actinomycetes (Richardson *et al*., 2009). The significance of plant growth promotion in agriculture cannot be overstated, as it offers a sustainable approach to improving crop productivity and reducing the reliance on synthetic chemical fertilizers and pesticides (Ramakrishna *et al*., 2019). PGPMs play a vital role in increasing nutrient availability to plants by solubilizing minerals, such as phosphates, making them more accessible for root uptake (Teotia *et al*., 2016). Moreover, they can produce phytohormones, such as indole-3-acetic acid (IAA), that stimulate root growth and enhance nutrient absorption (Kumar *et al*., 2015). Additionally, PGPMs are involved in the synthesis of siderophores, molecules that facilitate the uptake of essential nutrients like iron (Ma *et al*., 2016). The antagonistic activity exhibited by these microorganisms against plant pathogens further contributes to plant health and disease resistance. As modern agriculture seeks more sustainable and eco-friendly practices, exploring and harnessing the potential of plant growth promotion through PGPMs presents a promising avenue for achieving higher agricultural yields while minimizing environmental impact.

Utilizing multiple screening methods to identify potent plant growth-promoting microorganisms (PGPMs) holds significant importance in modern agricultural practices. The diverse array of growth-promoting activities exhibited by PGPMs necessitates a comprehensive approach to their identification. By employing a combination of screening methods, researchers can more effectively uncover microorganisms that possess a wide range of growth-promoting traits, enhancing their potential for agricultural applications. One key advantage of using multiple screening methods is the ability to capture various mechanisms by which PGPMs contribute to plant growth enhancement (Soumare *et al*., 2021). These methods might encompass nutrient solubilization, production of phytohormones like auxins, phosphorous mobilization, and even the synthesis of compounds that antagonize plant pathogens. Relying on a single screening method might overlook microorganisms with specialized capabilities that are not adequately represented through that particular approach. Furthermore, the use of multiple screening methods helps to mitigate the limitations of individual techniques. Some microorganisms may excel in one activity but exhibit modest or negligible effects in others. By integrating data from diverse assays, researchers can discern a more comprehensive profile of each microorganism's growth-promoting potential. This holistic understanding enables the selection of microorganisms with synergistic activities, maximizing their effectiveness in promoting plant health and yield. In a practical context, the agricultural industry benefits from this approach by identifying PGPMs that can address specific challenges faced by crops, such as nutrient deficiencies or disease susceptibility. By incorporating a variety of screening methods, researchers can tailor their selections to match the unique requirements of different crops and environmental conditions. This adaptability increases the likelihood of successful implementation and contributes to sustainable and resilient agricultural systems.

The primary purpose of this research is to systematically screen and evaluate a collection of microorganisms for their potential as plant growth-promoting agents. The study aims to identify and characterize microorganisms that exhibit multiple growth-promoting activities, including nutrient solubilization, phytohormone production, phosphate mobilization, siderophore synthesis, and antagonistic effects against plant pathogens. By comprehensively assessing these various traits, the research seeks to uncover microorganisms with a holistic and multifaceted approach to enhancing plant growth and health. The objectives of the study aim to establish correlations and potential synergies among different growth-promoting activities exhibited by these microorganisms. By understanding how these traits might interact and reinforce each other, the research aims to provide insights into the mechanisms underlying successful plant-microbe interactions. Ultimately, this research aims to contribute to the development of sustainable agricultural practices by providing a deeper understanding of how microorganisms can positively influence plant growth and health. By systematically identifying and characterizing microorganisms with multiple growth-promoting activities, the study endeavors to pave the way for more effective biofertilizers and biopesticides that can enhance crop productivity while reducing the environmental impact of conventional agricultural practices.

Material method:

Functional characterization of rhizobia for multiple PGP activities: Various direct or indirect mechanisms of PGPR influence plant growth.

IAA production: Bacterial cultures were grown for 3 days in YEMA broth at 28°C.

Fully grown cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 ml) was transferred to a fresh tube and mixed with 4ml of the Salkowski reagent (50ml, 35% of perchloric acid, 1 ml 0.5 MFeCl3 solution) (Brick *et al.,*1991 and Petten and Glick, 1996). The development of pink color indicates IAA production, the absorbance was read in a spectrophotometer after 30 minutes at 535nm.

Siderophore production: Siderophore production was estimated qualitatively. Chrome AzurolS (CAS) Agar medium (Schwyn and Neilands 1987). For the detection of siderophores, each rhizobium isolate was grown in a synthetic medium, containing 0.5 µM of iron and incubated for 24 hours on a rotary shaker at room temperature.ChromeAzurol S (CAS) assay is used to detect the siderophores. The CAS plates were used to check the culture supernatant for the presence of siderophores. Culture supernatant was added to the wells made on the CAS agar plates and incubated at room temperature for 5-7 days. The formation of a yellow to orange colored zone around the well indicates siderophore production. This zone was measured and recorded.

Phosphate solubilization: Phosphate Solubilization Bacterial isolates were evaluated for their ability to solubilize inorganic phosphate. Pikovskaya’s agar medium containing calcium phosphate as the inorganic form of phosphate was used in this assay. A loop full of bacterial culture was placed on the plates and kept for incubation at 28 °C for 7 days. The presence of a clear zone around indicated phosphate solubilization. The isolates giving clear zones were considered phosphate solubilizers.

Ammonia production: The isolates were tested for ammonia production by inoculating the isolates into 10 ml of pre-sterilized peptone water in the test tubes. The tubes were incubated for 48-72h at 36±2°C. Nessler’s reagent (0.5 ml) was added in each tube. The change in color of the medium from brown to yellow color was taken as a positive test for ammonia production.

HCN production: All the isolates were screened for the production of hydrogen cyanide (HCN)by adapting the method. The nutrient broth was amended with 4.4 g/l glycine and bacteria were streaked on a modified agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed into the test tube. Tubes were sealed with parafilm and kept on a rotary shaker at 100 rpm for 4 days. The development of orange to red color indicated HCN production.

Statistical analysis: Relevant Statistical studies were done to find out the spatial variability in the rhizobia count in the sampling data and measures of dispersion such as CV(%), SD, SEm, and mean were estimated.

Result and Discussion:

Screening for multiple plant growth promoting activities: The main characteristic functions of rhizobial strains are IAA production, siderophore production, phosphate solubilization, ammonia production, and HCN production which are described below:

IAA production: Rhizobial strains were tested for qualitative production of IAA. The media was supplemented with 100 ug/ml of tryptophan. Tryptophan is a key precursor in the synthesis of IAA. IAA production by different strains is affected by cultural conditions, and growth stage substrate availability. All strains of *Rhizobia were* shown to have positive production of IAA. Rhizobial isolate R14.2 had maximum IAA production (487 ug/ml), followed by R12.2 (432 ug/ml) and R3.3 also had moderate amounts of IAA production (313ug/ml).

Table. 1 IAA productivity tests on different strains of *Rhizobia*

S. No.	Strain	IAA Production (ug/ml)
1.	R1.1	12
2.	R1.2	16
3.	R3.1	376
4.	R3.3	313
5.	R4.1	222.5
6.	R4.4	247
7.	R4.5	16.5
8.	R5.1	17.5
9.	R6.1	97.5
10.	R10.2	310
11.	R12.2	432
12.	R13.2	32
13.	R14.2	487
14.	R15.2	20
15.	R16.2	13.5
16.	R17.2	49.5
17.	R18.2	762.5
18.	R19.2	423
19.	R20.2	18

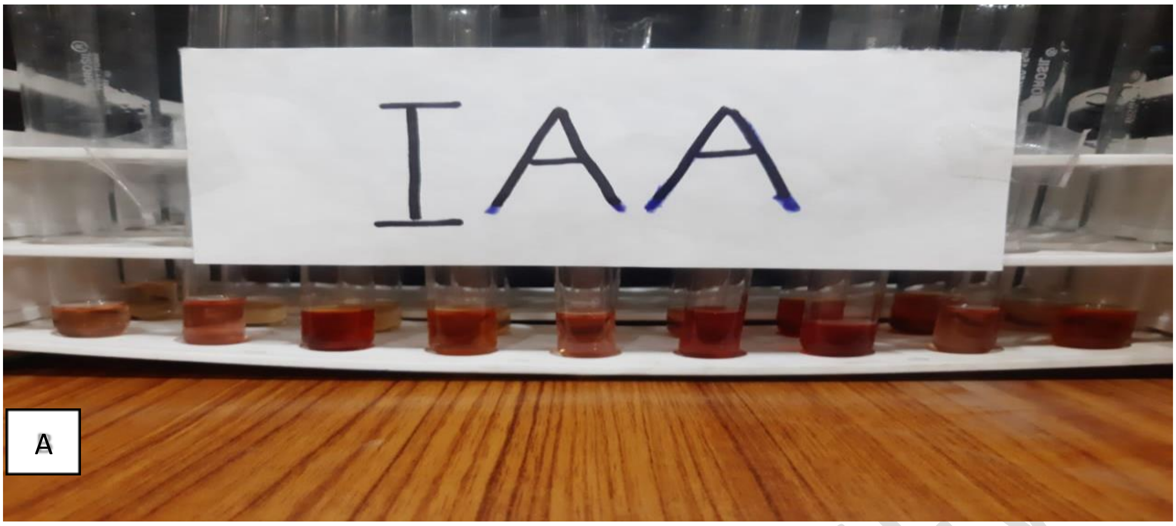

Figure.1 IAA production in 19 Rhizobial strains.

Statistical analysis: The assessment of Indole-3-Acetic Acid (IAA) production among 19 rhizobia strains revealed a notable variation in their ability to produce this phytohormone, with production ranging from 12 µg/ml to a remarkable 760 µg/ml. These strains were categorized into four groups based on IAA production levels: 10 strains fell within the 0-190 µg/ml range, five strains in the 190-380 µg/ml range, three strains in the 380-570 µg/ml range, and one strain exhibited the highest IAA production at 760 µg/ml. The statistical analysis of these data unveiled a mean IAA production of 285.25 µg/ml, with a standard deviation (SD) of 124.16, a standard error of the mean (SEM) of 35.45, and a coefficient of variation (CV) of 43.60%. So this data showed that rhizobia, produce phytohormones, such as indole-3-acetic acid (IAA) which helps in plant growth and development (Susilowati *et al*., 2018).This wide range of IAA production levels among the rhizobia strains underscores their significant potential for influencing plant growth, making them valuable candidates for further investigation in the context of plant-microbe interactions and their application in agriculture for enhanced crop productivity.

Phosphate solubilization: Phosphorus is an important macronutrient and found in insoluble form in the rock system. All isolates of *Rhizobia* were tested on Pikovskaya's agar and phosphate solubilization was determined based on producing holo-transparent zones. The zones measured between a range of 0.5 cm to 2.5 cm among different phosphate solubilizing bacteria (PSB). R3.1, R3.3, R4.1, R4.4, R6.1, R10.2, R12.2, R13.2, R14.2, R17.2, R18.2 and R19.2 had shown transparent zones around bacterial spots.

Table. 2 Phosphate solubilization tests on different strains of *Rhizobia*

S. No.	Strain	Phosphate solubilization
1.	R1.1	-
2.	R1.2	-
3.	R3.1	+
4.	R3.3	+
5.	R4.1	+
6.	R4.4	++
7.	R4.5	-
8.	R5.1	-
9.	R6.1	++
10.	R10.2	+
11.	R12.2	+
12.	R13.2	+
13.	R14.2	+
14.	R15.2	-
15.	R16.2	-
16.	R17.2	+
17.	R18.2	+
18.	R19.2	+
19.	R20.2	-

(-)=Noproduction;(+)=Weakproduction;(++)=Moderateproduction

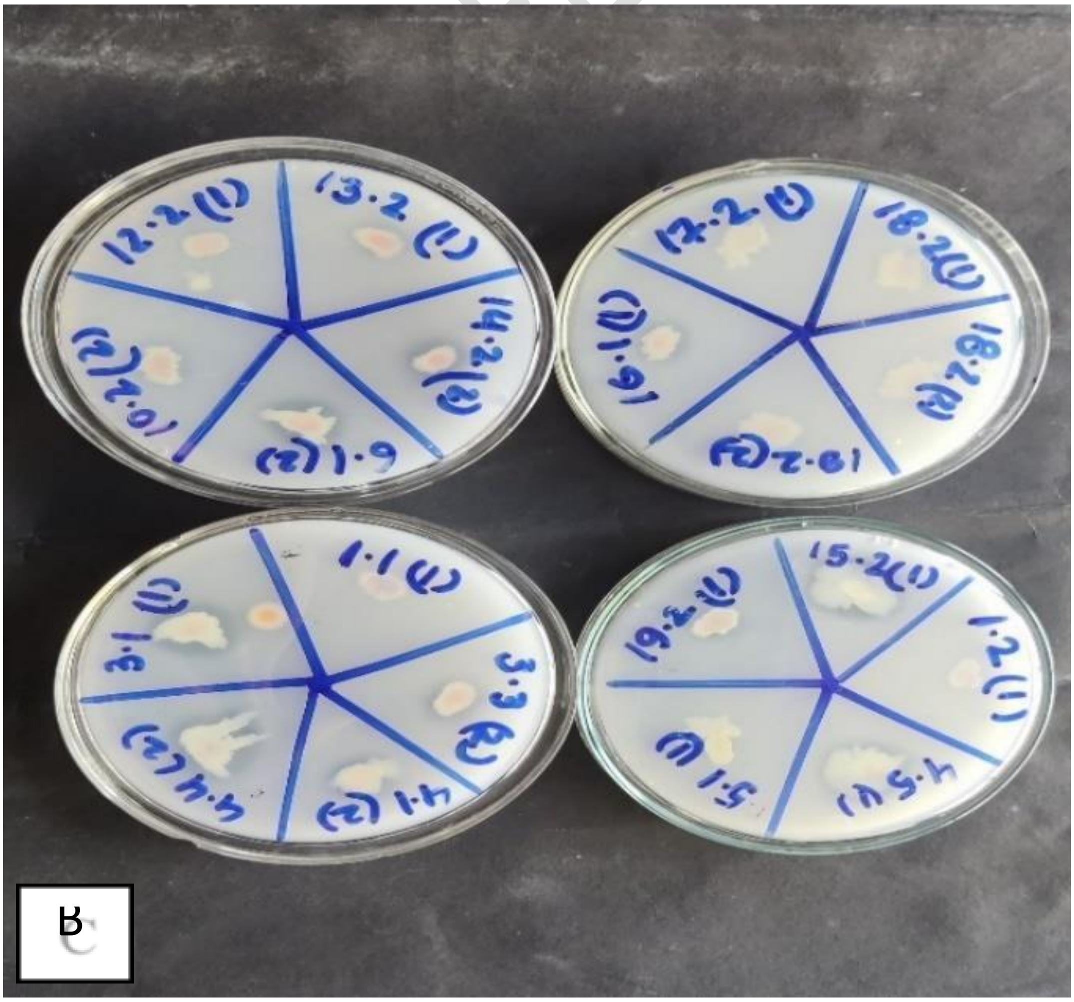

Figure. 2 Phosphate solubilization in 19 Rhizobial strains.

Statistical analysis: The evaluation of Phosphate Solubilization capability among the 19 rhizobia strains revealed varying degrees of solubilization. Among these strains, 7 exhibited no solubilization, 10 showed a moderate level of solubilization, and 2 displayed a high level of solubilization. Notably, none of the strains exhibited the highest level of solubilization. The statistical analysis of these data indicates a mean phosphate solubilization value of approximately 1.74, with a standard deviation (SD) of approximately 0.64 and a standard error of the mean (SEM) of roughly 0.15. These findings highlight the variability in the phosphate solubilization potential among the tested rhizobia strains, signifying their potential utility in enhancing nutrient availability in soils, which is crucial for promoting plant growth and crop productivity. Further investigations into the mechanisms underlying this variability and its practical applications in agriculture are warranted.

Siderophore production: Siderophores are produced by micro-organisms under iron-deficient conditions. The Chromo-AzurolS (CAS) supplemented agar medium was used for determining siderophore production in all 19 strains. 12 strains *viz.* R3.1, R3.3, R4.1, R4.4, R6.1, R10.2, R12.2, R13.2, R14.2, R17.2, R18.2, R19.2 showed development of orange zones against dark blue background after incubation of the treated agar plates at 48-72 hours.

Table. 3 Siderophore production tests on different strains of *Rhizobia*

S. No.	Strain	Phosphate solubilization
1.	R1.1	-
2.	R1.2	-
3.	R3.1	+
4.	R3.3	+
5.	R4.1	+
6.	R4.4	++
7.	R4.5	-
8.	R5.1	-
9.	R6.1	++
10.	R10.2	+
11.	R12.2	+
12.	R13.2	+
13.	R14.2	+
14.	R15.2	-
15.	R16.2	-
16.	R17.2	+

17.	R18.2	+
18.	R19.2	+
19.	R20.2	-

(-)=Noproduction;(+)=Weakproduction;(++)=Moderateproduction

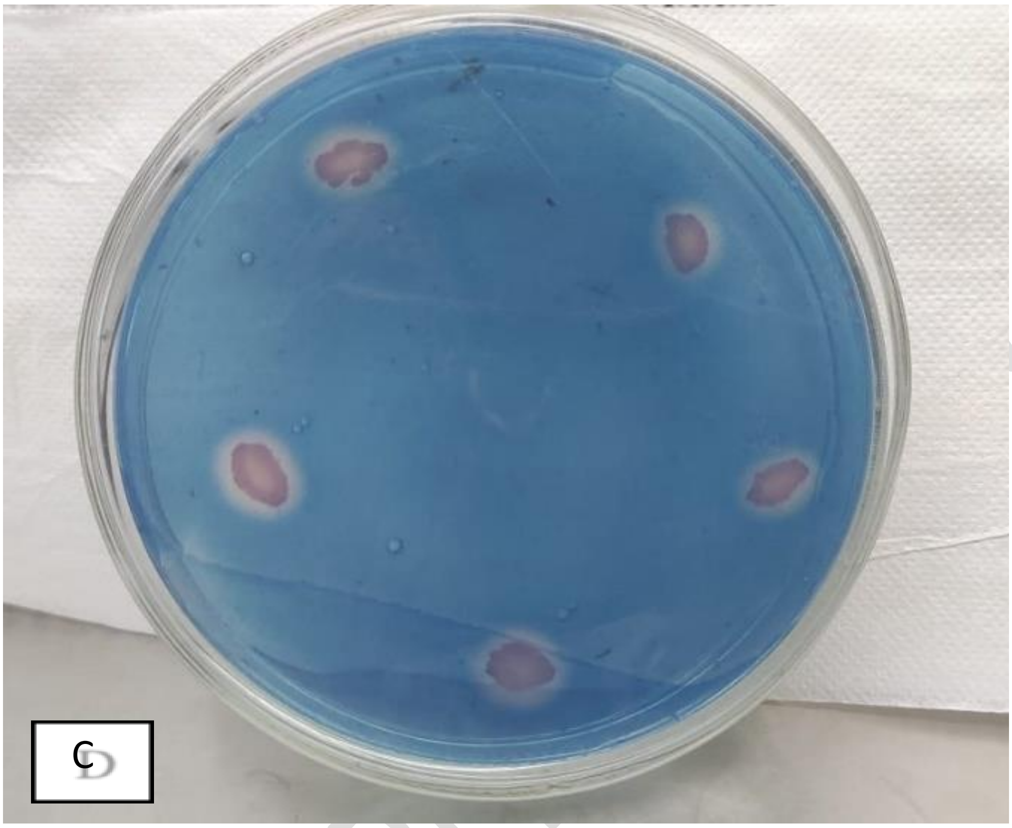

Figure. 3 Siderophore production tests on different strains of *Rhizobia*

Statistical analysis: The assessment of Siderophore Production among the 19 rhizobia strains revealed varying degrees of siderophore synthesis capability. Among these strains, 7 exhibited no siderophore production, 10 showed a moderate level of siderophore production, and 2 displayed a high level of siderophore production. The statistical analysis of these data indicates a mean siderophore production value of approximately 1.74, with a standard deviation (SD) of approximately 0.64 and a standard error of the mean (SEM) of roughly 0.15. These findings underscore the variability in the siderophore production potential among the tested rhizobia strains, reflecting their diverse capacity to facilitate iron uptake by plants, which is crucial for plant growth and overall agricultural productivity (Berni*et al.*, 2019). Further investigations into the mechanisms governing this variability and its practical implications in agriculture are warranted.

Ammonia production: Rhizobium strains were quantitatively tested for their ability to produce ammonia using Nessler’s reagent. Based on colour intensity, isolates were categorized into three groups, *viz.*, weak, moderate, and high ammonia producers. Out of

19 strains of *Rhizobia*, 14 were shown to have a positive result for ammonia production which was indicated by the development of yellow-orange to brown colour.

Table. 4 Ammonia production tests on different strains of *Rhizobia*

S. No.	Strain	Ammonia production
1.	R1.1	+
2.	R1.2	-
3.	R3.1	++
4.	R3.3	+++
5.	R4.1	+
6.	R4.4	++
7.	R4.5	+
8.	R5.1	-
9.	R6.1	++
10.	R10.2	+
11.	R12.2	++
12.	R13.2	+
13.	R14.2	+
14.	R15.2	-
15.	R16.2	-
16.	R17.2	+
17.	R18.2	+
18.	R19.2	++
19.	R20.2	-

(-)=Noproduction;(+)=Weakproduction;(++)=Moderateproduction;(+++)=Highproduction

Statistical analysis: The assessment of Ammonia Production among the 19 rhizobia strains revealed diverse capabilities in ammonia synthesis. These strains exhibited varying levels of ammonia production, with 5 strains showing minimal ammonia production, 8 strains exhibiting moderate ammonia production, and 5 strains displaying a high level of ammonia production. The statistical analysis of these data demonstrated a mean ammonia production value of approximately 2.11, with a standard deviation (SD) of approximately 0.85 and a standard error of the mean (SEM) of roughly 0.20. These findings emphasize the considerable variability in ammonia production potential among the tested rhizobia strains, suggesting their diverse capacity to contribute to nitrogen availability in soils, a pivotal factor influencing plant growth and agricultural productivity(Kumar *et al.*, 2012). Further research is needed to explore the underlying mechanisms of this variability and its practical implications for agricultural applications.

Hydrogen Cyanide (HCN) production: HCN is recognized as a biocontrol agent, because of its toxicity against plant pathogens and among one of the main attributes of plant growth-promoting rhizobacteria. The picric acid assay was done to investigate the activity of *Rhizobia* for the production of HCN. There were weak, moderate, and strong HCN production activities. All bacterial isolates except R4.5 and R5.1 were showing positive results for HCN production.

Table 5 : **Hydrogen Cyanide (HCN) production**

S. No.	Strain	HCN production
1.	R1.1	+
2.	R1.2	+
3.	R3.1	++
4.	R3.3	+++
5.	R4.1	++
6.	R4.4	++
7.	R4.5	-
8.	R5.1	-
9.	R6.1	++
10.	R10.2	++
11.	R12.2	+
12.	R13.2	+++
13.	R14.2	++
14.	R15.2	+
15.	R16.2	+
16.	R17.2	++
17.	R18.2	+
18.	R19.2	+++
19.	R20.2	+

(-)=Noproduction;(+)=Weakproduction;(++)=Moderateproduction;(+++)=High production

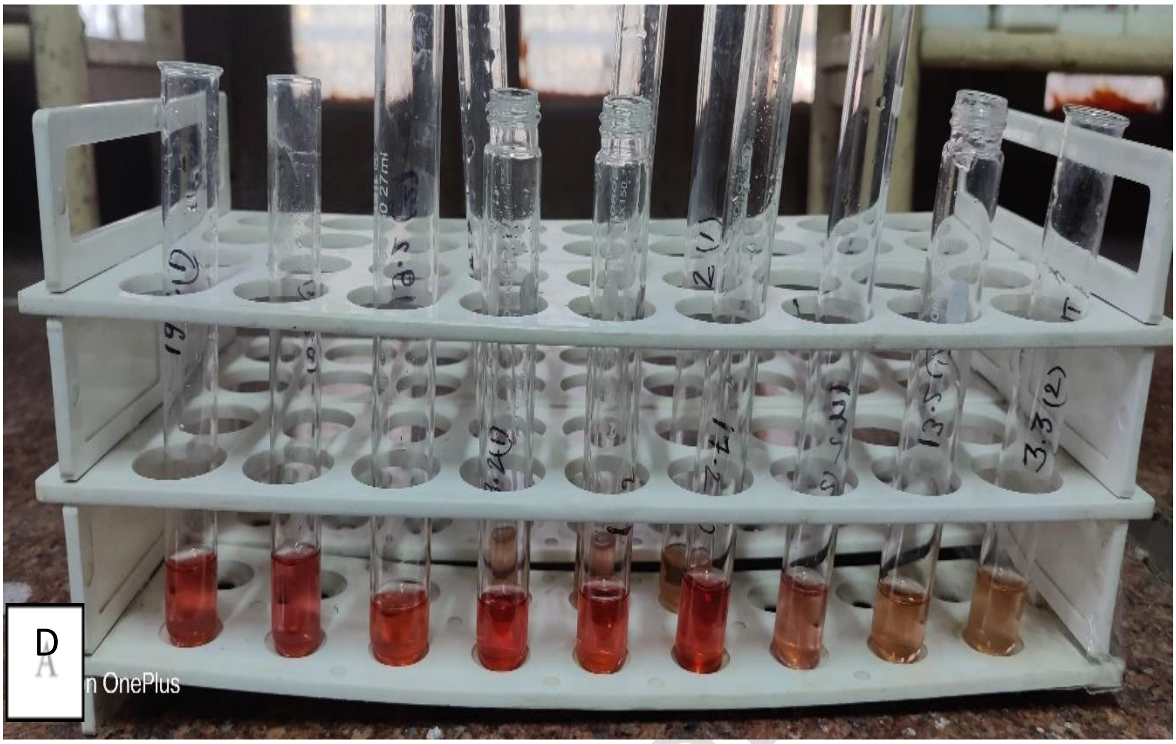

Figure. 4 HCN production tests on different strains of *Rhizobia*

Statistical analysis: The evaluation of Hydrogen Cyanide (HCN) Production among the 19 rhizobia strains revealed significant variability in their capacity to produce HCN. Among these strains, 2 exhibited minimal HCN production, 7 showed moderate HCN production, 7 displayed a high level of HCN production, and 3 strains exhibited the highest level of HCN production. The statistical analysis of these data indicates a mean HCN production value of approximately 2.58, with a standard deviation (SD) of approximately 0.88 and a standard error of the mean (SEM) of roughly 0.20. These findings underscore the considerable variation in HCN production potential among the tested rhizobia strains, highlighting their diverse capabilities in synthesizing this bioactive compound (Antoun *et al.*, 1998), which can play a role in plant defense and interactions with other microorganisms in the rhizosphere. Further investigations into the mechanisms governing this variability and its ecological significance in agricultural and soil ecosystems are warranted.

Conclusion: In conclusion, this research on the functional characterization of 19 rhizobia strains isolated from the Southern districts of Rajasthan, specifically Udaipur and Dungarpur, has provided valuable insights into their potential for multiple Plant Growth Promoting (PGP) activities. Through a comprehensive evaluation of IAA production, siderophore production, phosphate solubilization, ammonia production, and hydrogen cyanide (HCN) production, we have unveiled the diverse functional capabilities of these rhizobia strains. The findings underscore the remarkable adaptability and multifaceted nature of rhizobia in enhancing plant growth and soil fertility (Khaitov*et al*., 2016). This research not only advances our understanding of the rhizobia-plant interaction but also holds promise for practical applications in sustainable agriculture. By harnessing the PGP activities of these rhizobia strains, we can potentially revolutionize agricultural practices in Southern Rajasthan and similar agro-ecological regions, offering a path towards increased crop yields, improved soil health, and environmentally friendly farming practices. Further studies to explore the specific mechanisms and field applications of these PGP activities are warranted, offering exciting avenues for future research and the advancement of agricultural sustainability.

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