CHAPTER: 4

Isolation, Screening and Molecular Identification of the Plant Growth-Promoting Rhizobacteria-PGPR

4.1 Isolation and screening of the Rhizobacteria

The rhizospheric soil samples of groundnut plants were collected from various agricultural fields in the Saurashtra region, Gujarat. The samples collected were labeled as RG for Rajkot, RGK for Jamnagar, RGV for Virnagar, and RGKP for Amreli districts. A total of eighty-four rhizobacteria were isolated from rhizospheric soils and were analyzed for their plant growth-promoting traits.

Indole-3-acetic acid (IAA) is a crucial phytohormone that plays an essential role in the growth and development of plants. It is particularly important for cell division and differentiation, which are fundamental processes in plant development. Additionally, IAA supports root elongation, contributing to the formation of robust root systems that are vital for nutrient and water uptake and overall plant health and growth (Chen et al., 2021). In this study, the production of IAA by isolates was assessed. The results in Figure 4.1 (a) shows the results for IAA production. The IAA production levels were quantified using spectrophotometric analysis, as shown in Figure 4.2.

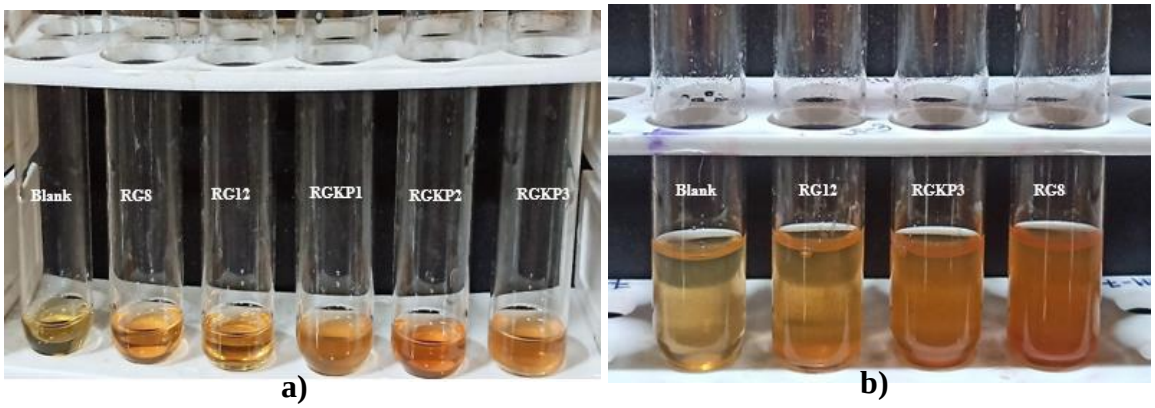


Figure 4.1: (a) Quantitative analysis of IAA production of isolates; and (b) Quantitative analysis of ammonia production of isolates

The results indicated that thirty-five isolates produced IAA in the range of 20.7–133 µg/mL. The isolates RG11, RG21, and RGV2 produced a maximum concentration of IAA,

which was 87% higher than the least IAA production by RG9. The potent KP3 produced IAA at a significantly higher level (42.8 µg/mL), compared to IAA production reported in the literature.

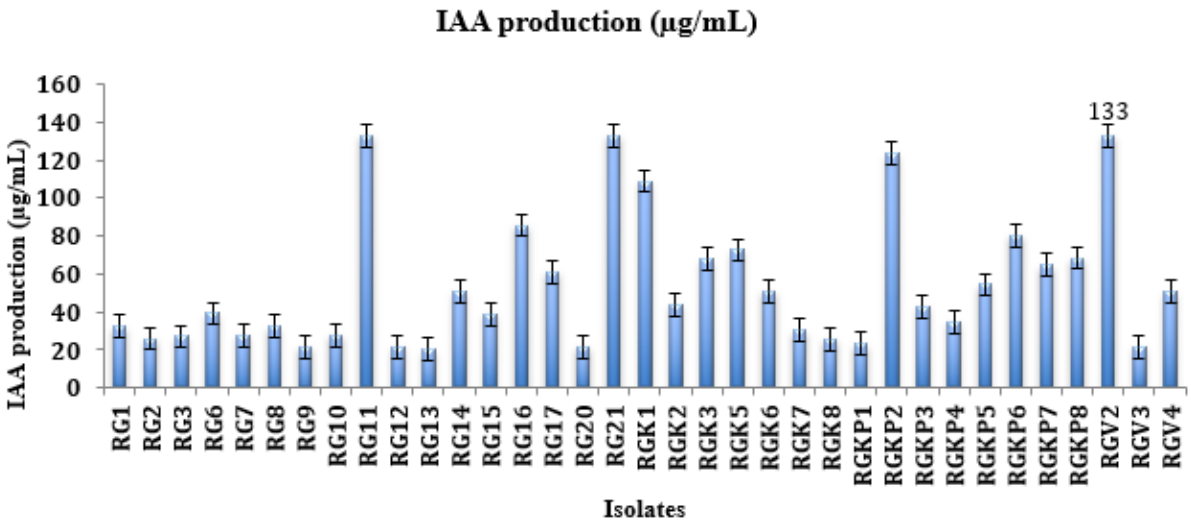


Figure 4.2: Quantification of IAA production of 35 positive isolates (Bar represents standard error)

The production of ammonia by Plant Growth-Promoting Rhizobacteria (PGPR) plays an indirect yet significant role in enhancing plant growth and development by providing a vital nitrogen source essential for plant nutrition.

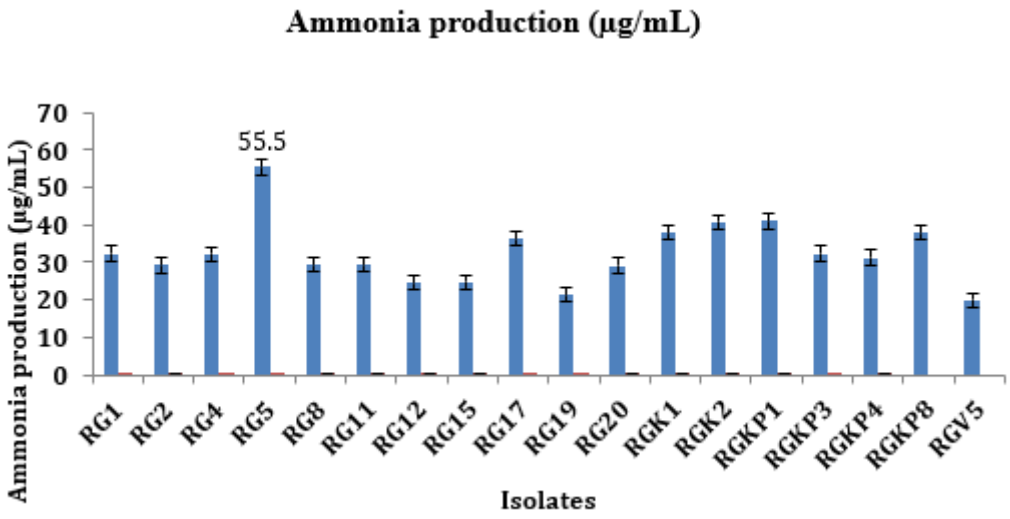


Figure 4.3: Quantification of ammonia production of 18 positive isolate (Bar represents standard error) isolates

PGPR facilitates this process by breaking down peptones into ammonia, which is released into the soil and absorbed by plants as nutrients, thereby improving soil fertility and supporting sustainable agriculture (Nehra et al., 2016). This study examined ammonia production in eighteen isolates using Nessler's reagent, as depicted in Figure 4.1(b), with spectrophotometric analysis quantifying the resulting brown coloration. Figure 4.3 highlights that isolate RG5 exhibited the highest ammonia production (55.5 µg/mL), while RG19 showed the lowest. The other isolates produced ammonia in the range of 21.4–55.5 µg/mL. Notably, these findings support those of Singh and Lal, (2013) who reported a Ammonia production was detected in *Bacillus* (100%), *Pseudomonas* (85%) and *Rhizobium* (70%) compared to control.

Some microorganisms produce HCN, which plays a key role in bioremediation and controlling harmful organisms. HCN helps improve plant growth and protects plants by suppressing harmful pathogens in the soil. This makes it useful for promoting healthy crops and reducing soil-borne diseases.

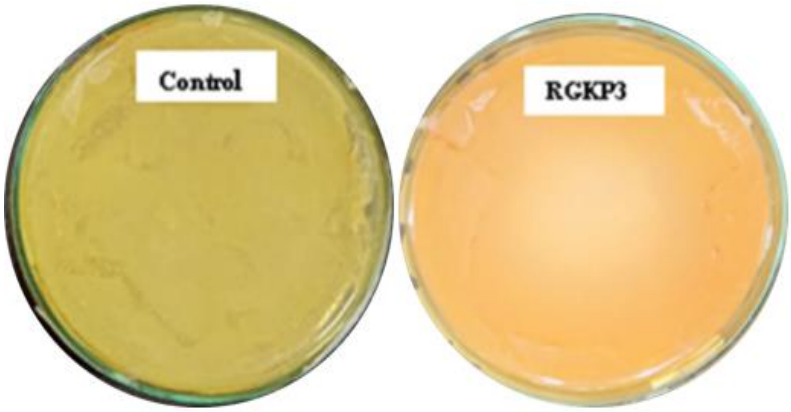


Figure 4.4: Qualitative analysis of HCN production of all positive isolates

In this study, HCN production was analysed qualitatively using filter paper soaked in sodium picrate solution. A positive result was indicated by a colour change from yellow to orange-brown.

Twenty-six isolates demonstrated HCN production, as evidenced by the yellow to orange-brown coloration, indicating their potential for biocontrol and plant growth promotion, as shown in Figure 4.4.

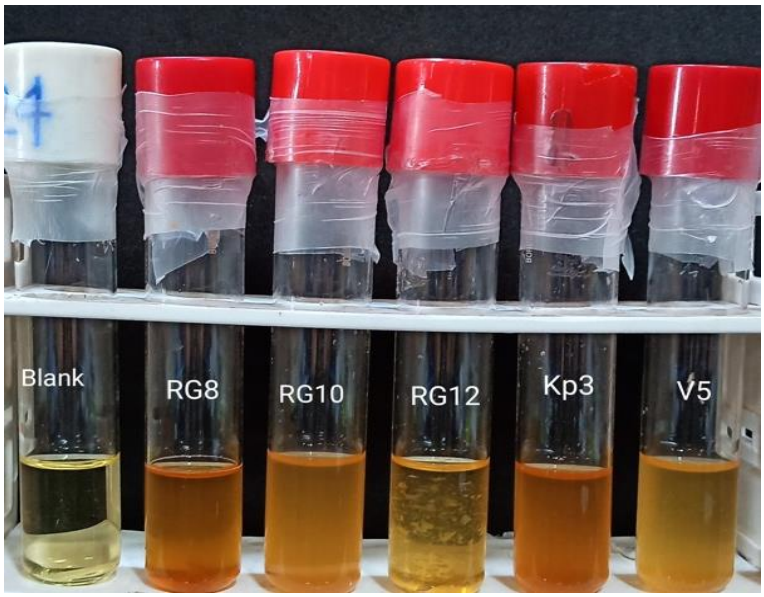


Figure 4.5: Quantitative analysis of HCN production

These findings supported the work of Jadav et al. (2020), who reported only four HCN-producing isolates from rhizosphere bacteria associated with *Limonium stocksii*.

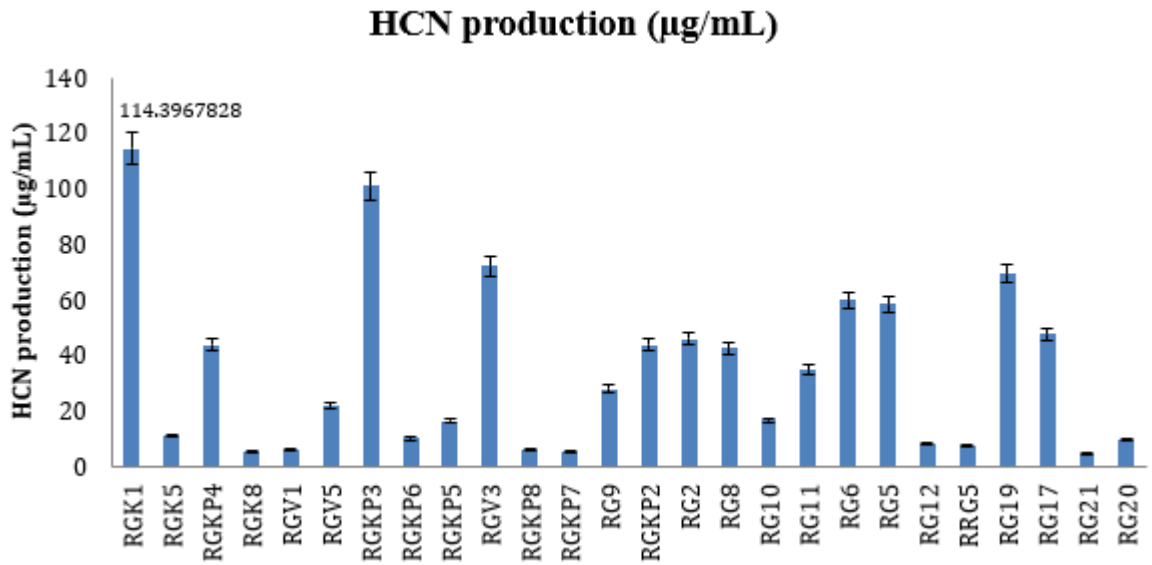


Figure 4.6: Quantification of HCN production of twenty-six positive isolates (Bar represents standard deviation)

Phosphorous is the second-key nutrient after nitrogen for plant growth (Gyaneshwar et al., 1998). The results indicated that out of all isolates, only 4 isolates demonstrated the

capacity to solubilize phosphorous from an insoluble phosphate source present in the media.

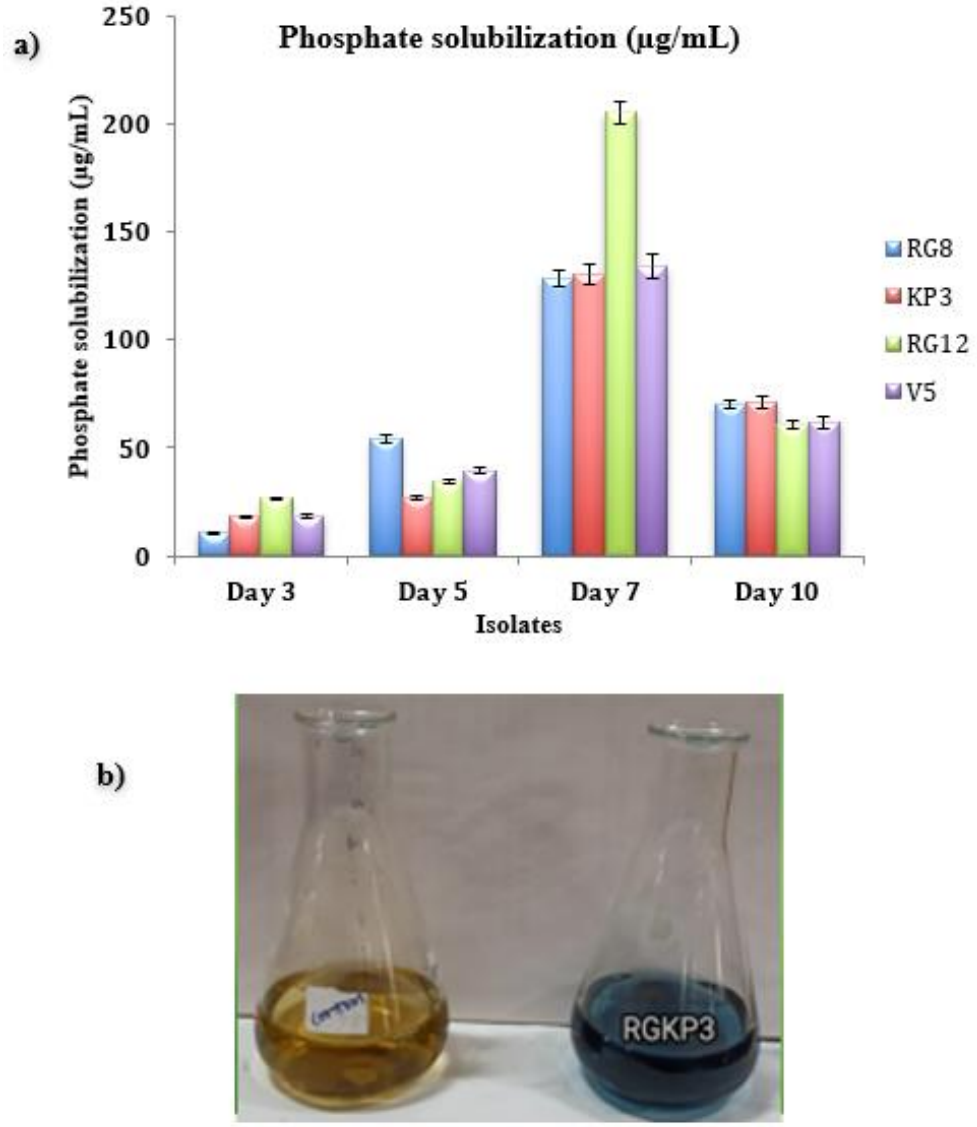


Figure 4.7: (a) Quantitative analysis of phosphate solubilization control with positive result of isolate RGKP3 (Bar represents standard deviation) and (b) Quantification of phosphate solubilization of positive isolates

Furthermore, 4 positive isolates were studied for quantitative estimation of phosphorous using the colorimetric method. Phosphate solubilizing isolates show a blue color compared to yellow colored control on the addition of chlorostannous reagent. On the fifth day of the assay, RG12 demonstrated the highest solubilization capacity. The 4 isolates had the potential to solubilize phosphate from Pikovskaya's media in a range of 11.06–205 µg/mL.

On the 3rd and 5th days, phosphate is solubilized in a range of 11.04–53.96 µg/mL, and on the 7th day, isolate RG12 had 205 µg/mL phosphate solubilization. The standard curve of TCP was plotted in the range of 50–500 µg/mL. Figure 4.7 shows that the RGKP3 had maximum solubilization after 10 days. Tahir et al., (2013) supported our findings reporting, that the *Azospirillum* strain WS-1 solubilized 218.1 µg/mL phosphate, which is 13% less than our findings.

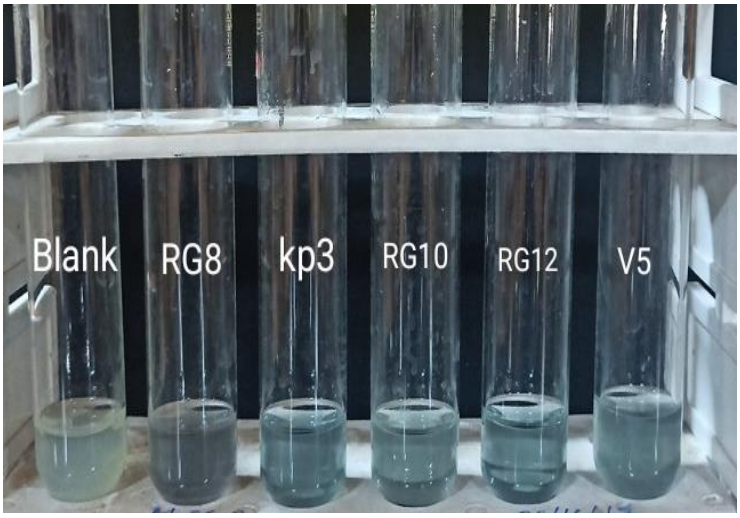


Figure 4.8: Quantitative analysis of Gibberellins (GA) production with compared to control

Gibberellins are important plant hormones that regulate various growth processes, including seed germination and stem elongation, as noted by Xia et al. (2022). Recent research suggests that certain bacteria have evolved an independent biosynthetic pathway to produce gibberellins, providing new insights into their role in plant-microbe interactions (Kang et al., 2012). In this study, only six bacterial isolates were found to produce gibberellins in measurable quantities, with concentrations ranging from 10.2 µg/mL to 112.16 µg/mL. Among these, isolate RGK7 demonstrated the highest gibberellin production, a concentration of 112.16 µg/mL. In contrast, isolate RGV3 exhibited the lowest gibberellin production, with a concentration of 10.2 µg/mL. These findings highlight the variability in gibberellin production among different bacterial isolates.

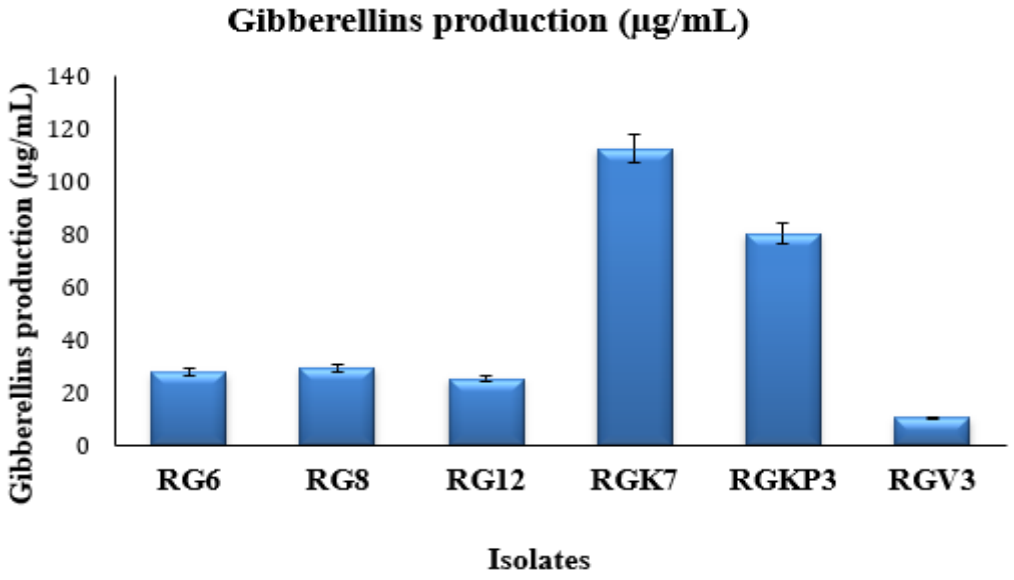
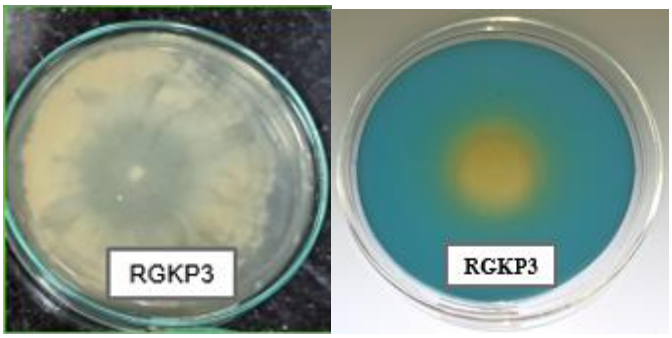


Figure 4.9: Quantification of Gibberellins (GA) production of isolate**s** (Bar represents standard deviation)

Chitinase is an enzyme produced by endophytic microorganisms, aiding in the degradation of plant cell walls to facilitate their entry into plants through natural openings such as pores on roots, stems, flowers, cotyledons, and injuries (Grover et al., 2012). In this study, seven isolates demonstrated chitinase activity, evident by the formation of clear zones (Fig. 4.10a) resulting from chitin hydrolysis.



a) b)

Figure 4.10: (a) Chitin hydrolysis with a positive result of isolate RGKP3; and (b) Siderophore production with a positive result of isolate RGKP3

This enzymatic activity plays a dual role in plant-microbe interactions. It not only assists endophytes in colonizing plant tissues but also provides a protective function by breaking down chitin, a major component of the cell walls of pathogenic fungi, thereby inhibiting

their growth in the rhizosphere. Bhatt et al. (2020) also highlighted the antifungal role of chitinase, reporting that out of thirty-two isolates, only five PGPRs demonstrated similar chitinase activity, supporting the findings of this study.

All rhizobacterial isolates were screened for siderophore production using the CAS agar method. 4 isolates showed clear zones (Fig 4.10 b), indicating positive siderophore production. The isolates RGKP4, RGKP3, and RG18 exhibited the largest clear zones. These findings highlight the potential of these isolates to enhance iron availability in the soil, which can contribute to improved plant growth and development.

Potassium-solubilizing bacteria (KSB) play a vital role in enhancing soil fertility by converting insoluble forms of potassium into soluble K^+ ions, making them more accessible for plant uptake (Olaniyan et al., 2022). In this study, all isolates, except RGKP7, tested positive for their potassium solubilization efficiency. The isolates show halo zones ranging from 0.5 to 4.5 cm in diameter. Among the isolates, RGKP4 exhibited the highest potassium solubilization activity, with a halo zone diameter of 4.5 cm. In contrast, RG2 and RG18 displayed the lowest activity, a halo zone of only 0.5 cm.

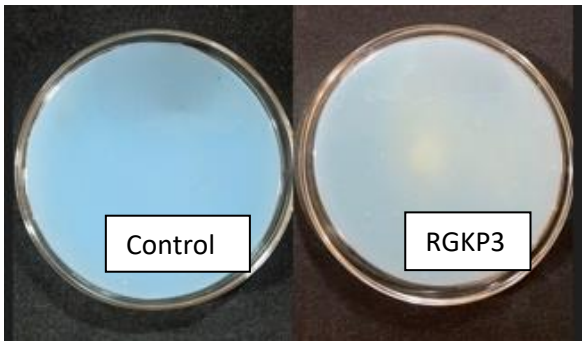


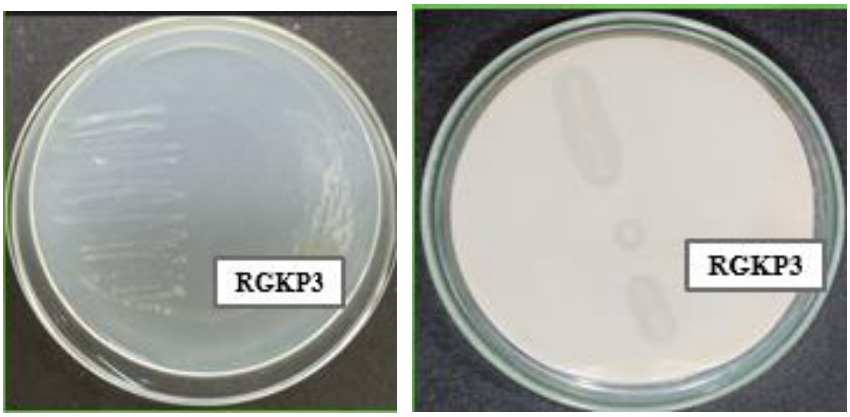
Figure 4.11: Potassium solubilization of isolate RGKP3

Youssef et al. (2010) observed that applying calcium nitrate combined with potassium sulfate, ammonium nitrate, and feldspar, along KSB, significantly increased potassium availability in soils, leading to improved yield components of crops like peanuts and sesame. Similarly, Parmar and Sindhu (2013) reported that KSB isolates from wheat fields were most effective at solubilizing potassium at a neutral pH of 7.

Zinc is an essential micronutrient required by plants, playing a crucial role as a component of various enzymes that drive key metabolic functions (Umair et al., 2020). These enzymes

are vital for maintaining proper growth and development in plant tissues, ensuring the efficient functioning of physiological processes. In this study, the role of zinc-utilizing plant growth-promoting rhizobacteria (PGPR) was investigated. Out of all the isolates tested, only six PGPR exhibited clear zones (Fig 4.12 b) on the medium after three days of incubation, indicating their ability to utilize zinc. A significant increase zinc content was observed in plants inoculated with *Pseudomonas fragi* (EPS 1), which was isolated from the wheat rhizosphere (Kamran et al., 2017).

Nitrogen is vital for plant growth and development as a key component of chlorophyll, enabling photosynthesis. It is also essential for amino acids, proteins, enzymes, and nucleic acids, supporting cell division and metabolism. Nitrogen deficiency causes stunted growth, and chlorosis, and can lead to plant death, highlighting its critical role in plant health (Kalaji et al., 2018). In this study, a total of 22 isolates demonstrated the ability to grow on Jensen's agar plates during the incubation period. Figure 4.12 (a), indicates their potential role in nitrogen fixation and their contribution to improving nitrogen availability for plant use. These findings highlight the importance of nitrogen-fixing microorganisms in supporting plant health and productivity (Mahmud et al., 2020).



a) b)

Figure 4.12: a) Nitrogen fixation of isolate RGKP3; and b) Zinc solubilization of isolate RGKP3

4.2 Identification of Potent PGPR

The most promising rhizobacterial isolates demonstrated multiple positive PGP traits, highlighting their potential for agricultural applications. To further characterize these isolates, Gram staining and the KOH method were performed, revealing variations in their

morphology. The results showed that the potent PGPR isolate RG8 is Gram-negative, whereas RG12 and RGKP3 were identified as Gram-positive bacteria. Additionally, the isolates were subjected to 16S rRNA partial sequencing for strain identification.

4..2.1 Gram Staining and KOH Method:

The bacterial isolate RG8 was identified as Gram-negative, while RG12 and RGKP3 were both classified as Gram-positive.

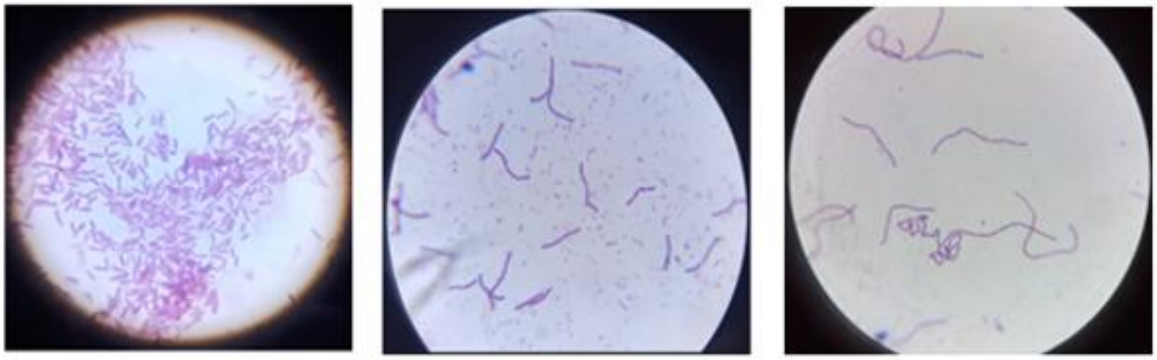


Figure 4.13: Gram staining images for all three positive isolates, a) RG8; b) RG12; and c) RGKP3

4.2.2 Molecular Identification of Potent PGPR

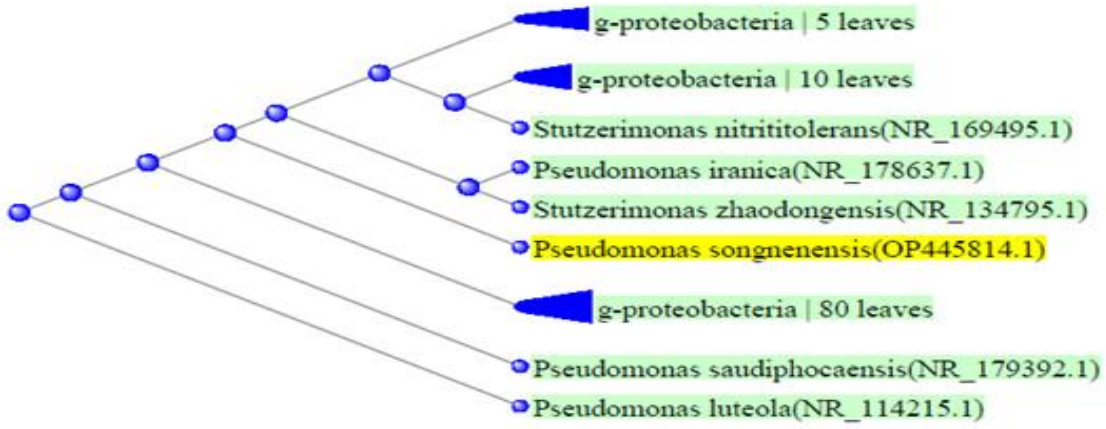


Figure 4.14: Phylogenetic tree showing the evolutionary relationship between RG8, PGPR isolates, and reference strains from the GenBank database.

The most promising rhizobacteria isolates, RG8, RG12, and RGKP3, exhibited multiple positive PGP traits, highlighting their potential for agricultural applications.

These isolates were identified through partial sequencing of the 16S rRNA gene, a widely accepted molecular marker for bacterial identification and phylogenetic analysis .Detailed phylogenetic analyses, illustrated in Figures 4.14, 4.15, and 4.16, confirmed their taxonomic placement and revealed their evolutionary relationships with other known species, aligning them with taxa recognized for beneficial PGP activities. The 16S rRNA sequences of the isolates were submitted to GenBank, where RG8 was assigned accession number OP445813, RG12 was assigned OP445814, and RGKP3 was assigned OP528743.

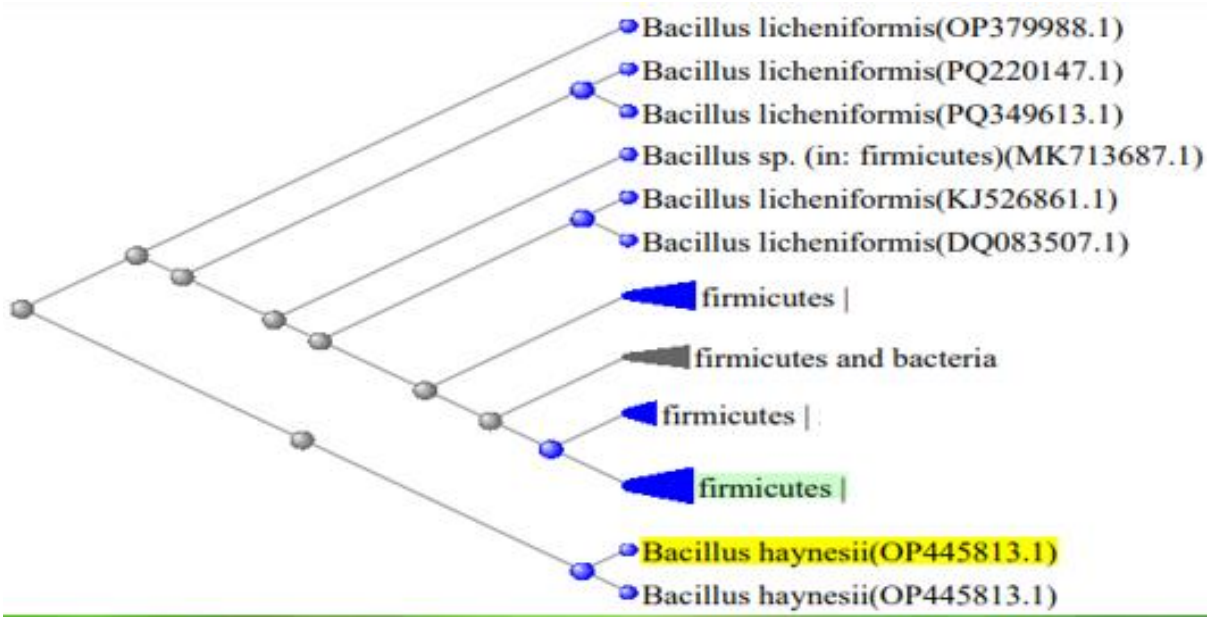


Figure 4.15: Phylogenetic tree showing the evolutionary relationship between RG12, PGPR isolates, and reference strains from the GenBank database

Comparative analysis using GenBank data revealed that RG8 shares close homology with *Pseudomonas songnenensis*, RG12 with *Bacillus haynesii*, and RGKP3 with *Priestia megaterium*, a bacterium known for its beneficial effects on plant growth. Additionally, the phylogenetic analysis provided further insights into its classification. The close homology of these isolates with well-known beneficial bacteria underscores their potential as effective

(PGPR) candidates. These findings suggest that RG8, RG12, and RGKP3 can significantly contribute to enhancing crop productivity.

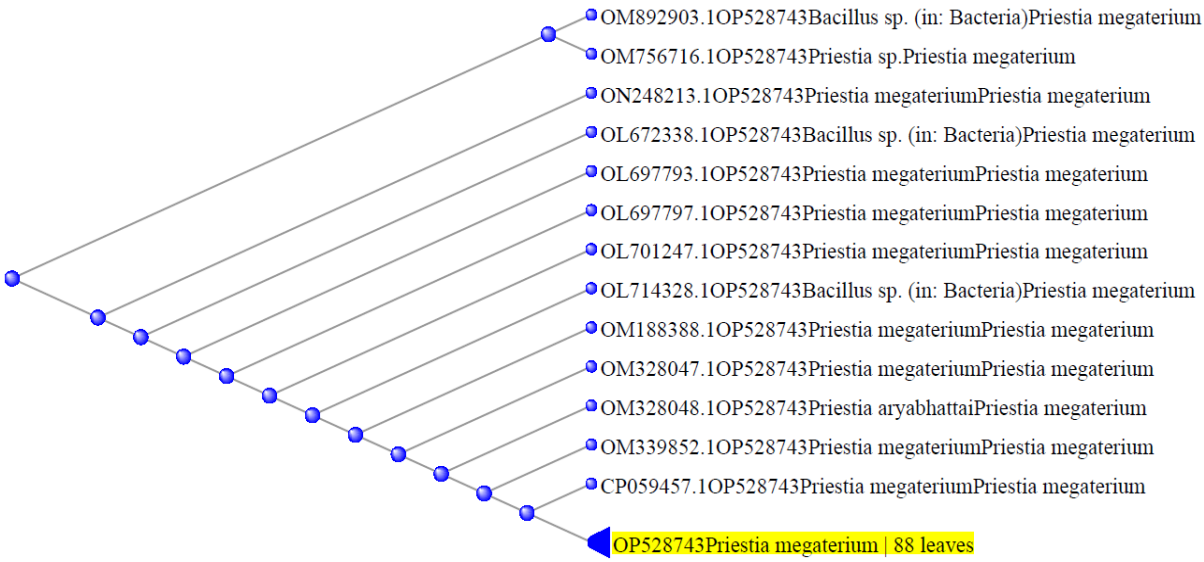


Figure 4.16: Phylogenetic tree showing the evolutionary relationship between RGKPP3, PGPR isolates, and reference strains from the GenBank database