IAA PRODUCING PGPR ISOLATION, QUALITATIVE ANALYSIS AND PARTIAL PURIFICATION

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) benefit plants through a variety of mechanisms, including I the production of secondary metabolites such asantibiotics, cyanide, and hormone-like substances; (ii) siderophores production; (iii) resistance to soilborne root pathogens; (iv) phosphate solubilization; and (v) di-nitrogen fixation.

IAA, a common byproduct of L-tryptophan metabolism, translocated carbohydrates during its synthesis and regulates important physiological processes such as cell growth, tissue differentiation, and tactic responses in its naturally occurring form (Etesami and Beattie, 2018). It has also been reported that IAA promotes cell elongation, flowering, and fruiting through increased osmosis and protein synthesis, while delaying abscission (Zhao et al., 2018). IAA is produced by a variety of microorganisms, but it also serves as a growth factor for bacteria.Many bacteria have been found to produce IAA. It is even assumed that more than 80% of rhizosphere bacteria can synthesis IAA (Patten and Glick 1996; Khalid et al. 2004).

Plant-microbe interactions occur through chemical communications in the rhizosphere, and a three-part interaction mechanism has been noticed between plants, pathogenic microbes, and plant-beneficial microbes. However, full knowledge on rhizosphere communications between plants and microbes, tripartite interactions, and the biochemical impact of these interactions on the plant metabolome is limited and poorly understood. Furthermore, the molecular and biological processes causing PGPR impacts on induced systemic resistance (ISR) and priming in plants remain unknown. (Mashabela MD, Piater LA, Dubery IA, Tugizimana F, Mhlongo MI 2022).

1. Introduction

Plants constantly interact with a number of microbes in the rhizosphere micro-habitat, including bacteria, fungus, and viruses (Wang, H., Liu, R., You, M. P., Barbetti, M. J., & Chen, Y.2021). Interactions between beneficial microorganisms and pathogens have attracted a lot of attention because they are important for plant health and growth (Gupta, G., Parihar, S. S., Ahirwar, N. K., Snehi, S. K., & Singh, V. 2015). However, most study has concentrated only on the interaction of a single pair of interacting species. (i.e., one pathogen and one antagonist),skipping the enormous microbial diversity within these functional communities coexisting around or in plant roots. As a result, such investigations are really not important to natural soil conditions and oppose the idea that various species coexist in microbial communities (Van der Putten, W. H., Vet, L. E., Harvey, J. A., & Wäckers, F. L. 2001).

Plant Growth Promoting Rhizobacteria are those microorganisms which colonizes plant roots and helps them in growth and improves fertility of the soil. (Priyanka, 2020) There are two kinds of mechanisms involved in beneficial activity of PGPR which is direct mechanism and indirect mechanism. The direct mechanism includes phytohormones production, mineral solubilization and nitrogen fixation. While in indirect mechanism involves antagonistic activity against pathogens, secondary metabolites production, fungicides involvement and siderophore production. (Meena et al., 2020) Hence Plant Growth Promoting Rhizobacteria are more effective on crop enhancement and for sustainable environment.(Meena et al., 2020) One of the mechanism for growth enhancement in crops is the production of phytohormones such as Indole acetic acid (IAA), Gibberellic acid and Cytokines. (Bhattacharyya & Jha, 2012).

Indole acetic acid is the most active and commonly present in all the plants which helps and regulate in growth of the plant. (Kannojia et al., n.d.) Indole acetic acid (IAA) is the phytohormone involves in root initiation, root elongation and cell division. The effect of indole acetic acid is mainly depended on the sensitivity of the plant towards IAA and the concentration of IAA produced by plant associated bacteria. (Khan et al., 2014).

Tryptophan-Independent Pathway Plants can synthesise IAA without the tryptophan precursor, according to mutants in the tryptophan biosynthetic pathway, and the branch point for this tryptophanindependent pathway occurs at indole-3-glycerol phosphate or indole-3-glycerol phosphate. However, no genes or proteins involved in this pathway have been identified in plants as of yet (Woodward and Bartel 2005). A tryptophan-independent route was suggested to occur inbacteria (A. brasilense) by feeding studies with tagged tryptophan (Prinsen et al. 1993). However, in this situation, no clear genetic or molecular evidence has been offered to support this pathway.

Our major goal is to isolate bacteria that produce the most IAA from the substrate via a Tryptophan-dependent pathway and see if the IAA can be partially purified.

2. Material and Methods

2.1. Collection of Sample and Isolation of Rhizobacteria

Rhizospheric soil samples were collected from four different agriculture fields of Kotdapitha, District Rajkot, Kalawad, from the Saurashtra, Gujarat. Soil samples were screened for isolation of Rhizobacteria. Briefly, the groundnut plants were uprooted and shoots were cut

off and roots along with the rhizosphere soil was stored aseptically in sample bags. The soil samples were stored at 4°C until further use. The samples were serially diluted in the range 10^{-4} to 10^{-7} and colonies with morphological variations were isolated.

2.2. Characterization of the Isolates for IAA Production

Rhizospheric soil samples were collected from Four different agriculture fields of Kotdapitha, Virnagar, District Rajkot, Kalawad, District Jamnagar from the Saurashtra, Gujarat. Soil samples were screened for isolation of Rhizobacteria. Briefly, the groundnut plants were Uprooted and shoots were cut off and roots along with The rhizosphere soil was stored aseptically in sample Bags. The soil samples were stored at 4°C until Further use. The samples were serially diluted in the Range 10-3 to 10-8 and colonies with morphological Variations were isolated.

A selected medium of Nutrient Broth supplemented with L-Tryptophan (0.001g/ml) (pH –6.23-7.00) serves as a substrate culture for the isolated bacteria to produce IAA. A loop full of culture was added to the 50ml Culture broth and incubated at room temperature IAA production was monitored at an interval of 24 h till 120 h (Sarwer and Kremer, 1995).

2.3 IAA (indole-3-acetic acid) Production

Salkowski's reagent (1 ml of 0.5M FeCl3 in 50 ml 35% HClO4 with continuous stirring) was used to measure IAA generation, described by Gordon and Weber (1951). 5 ml of culture suspension was collected and centrifuged for 20 minutes at 1000 rpm. 1 mL of supernatant and 1 mL of Salkowski's reagent were mixed together. Shaking the ingredients, they were allowed to stand in the dark at room temperature for 30 minutes until the pink colour developed. Spectroscopic absorbance data at 536 nm were used to estimate IAA quantitatively. As a control, uninoculated liquid broth was used.

2.4 Extraction of crude IAA

The isolated bacterial cells were centrifuged at 10,000 rpm for 30 Minutes. The pH of the supernatant was adjusted between 2.5-3 with 1N HCL. Add ethyl acetate twice with same amount of Acidified supernatant. Then, placed the total solution in rotary evaporator at 60c dried and evaporated ethyl acetate fraction in the solution. the insoluble fraction was dissolved in methanol.

2.5 Thin layer chromatography of extracted IAA

Ethyl acetate fractions (10-20 microlitre) were plated on TLC plates (Silica gel G f254, thickness 0.25 mm) and developed with isopropanol: ammonia: water as the mobile phase (16:3:1). Pink spots with Rf values identical to true IAA were discovered under UV light (254 nm) and by spraying Salkowsky's reagent, and their Rf values were compared to Rf values of pure IAA as a positive control (Patel and Saraf, 2017).

2. Results

Table 1 summarises the findings of colony characteristics of potential IAA secreting bacteria.IAA biosynthesis was examined in a selective medium enriched with 3% Nutrient broth and 1% L- Tryptophan, and IAA activity was measured over a 72-hour period. Plants'

protective reactions towards biotic and abiotic stressors are regulated by phytohormones. It was also observed that both the development and tolerance for different environmental stresses (Ryu and Cho, 2015).

Table 1: Colony characteristics of isolates

Isolates	Colour	Size	Elevation
RJK 1	Brown	Small	Small
RJK 2	Grey	Small	-none
RJK 3	Yellow	Medium	-none
RJK 4	Green	Small	Medium
RJK 5	Yellow	Medium	Small
RJK 6	Yellow	medium	Small
RJK 7	Grey	Large	-none
RJK 8	Brown	Medium	Small
RJK 9	White	Small	Small
RJK 10	Orange	Large	Small
RJK 11	Yellow	Small	Small
RJK 12	yellow	Medium	Small
RJK 13	Grey	Large	large
RJK 14	White	Small	Small
RJK 15	Yellow	Small	Large
RJK 16	Yellow	Medium	Small
RJK 17	White	Small	-none
RJK 18	Grey	Large	-none
RJK 19	Yellow	Small	Small
RJK 20	Yellow	medium	small

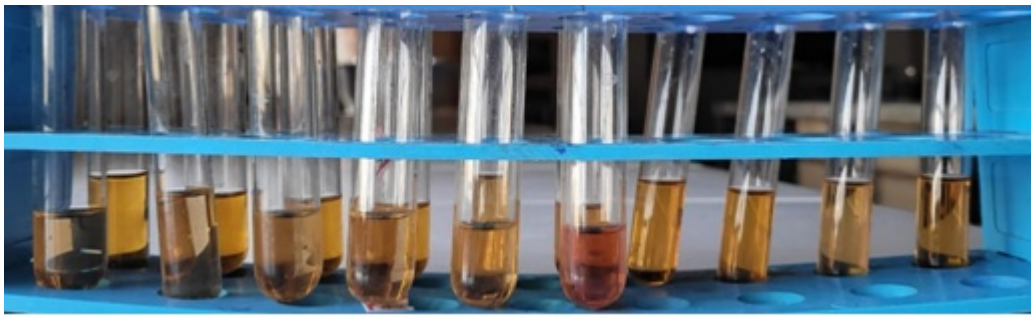
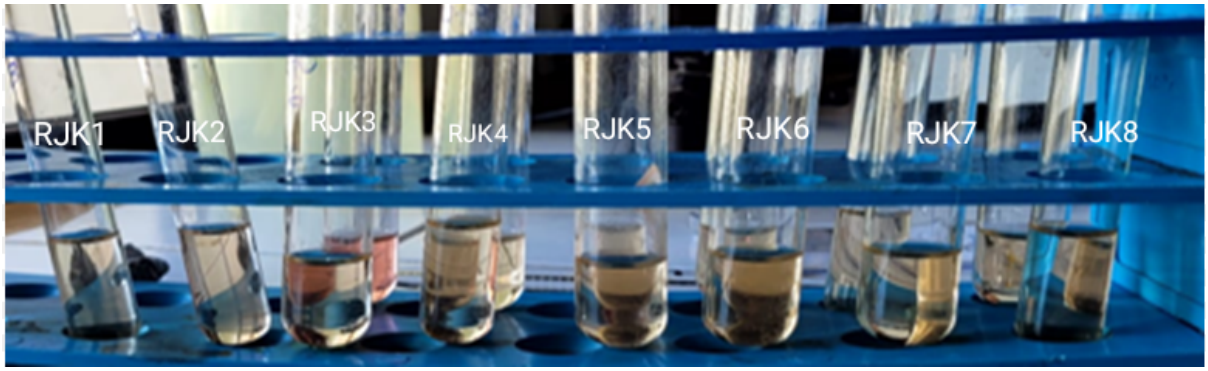


Fig 1: (a) IAA production (pink orange) by selected isolates after 72 hours.



(b) IAA production by selected isolates after 30 Minutes Incubation.

Bacterial isolates were chosen for further IAA biosynthesis testing based on their unusual colony shape.IAA producing bacterial isolates were chosen from among a total of 20 distinct isolates grown. Under 1% L-Tryptophan concentration, all the bacteria were found to be positive for IAA secretion. Figure 1(a) incubated tubes after 72 Hours and 1(b) IAA production (pink) by selected isolates.

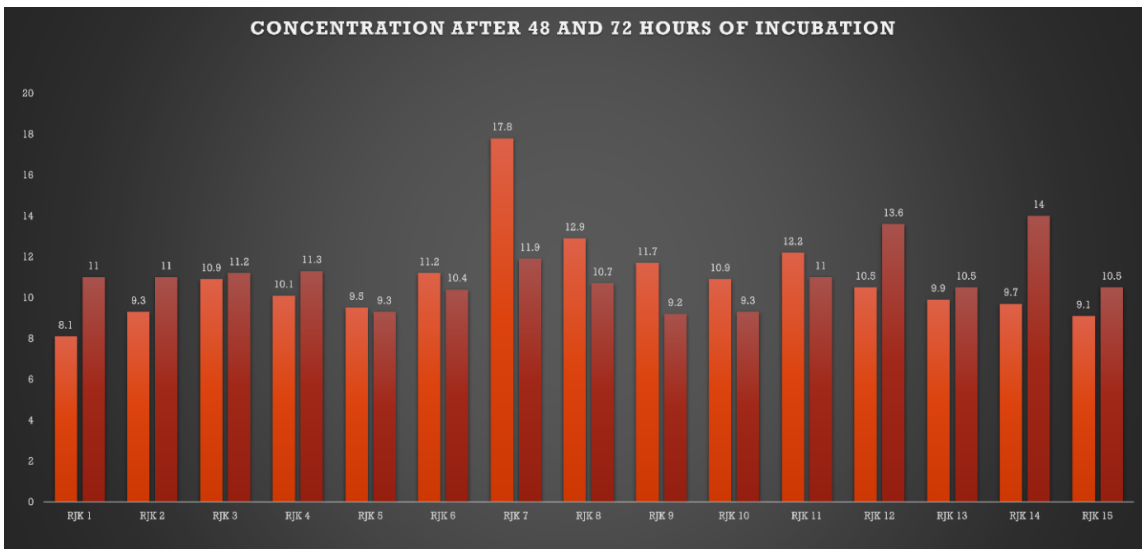


Fig. 2. Quantitative IAA Production by the all isolates

IAA secretion quantification using the spectrophotometric approach described by (Sarwar and Kremer,1995). Figure 2 depicts the outcome graphically. The intensity of the dark reddish pink colour in the tube evaluated spectrophotometrically at 536 nm after 72 hours of incubation indicates that the selected isolates synthesise a lot of IAA, as shown in Fig. 3. RJK7 has the largest IAA production (17.8 +/-0.1 µg/ml), followed by RJK 14(14 +/-0.1 µg/ml) and RJK12(13.6+/-0.1 µg/ml).

Extraction & detection of IAA by thin layer chromatography

TLC plates were loaded with IAA ethyl acetate fractions and developed with the mobile phase isopropanol: ammonia: water. (16:3:1). Salkowski's reagent, which detects the Indole

chemical, was used to identify pink dots. Figure 4 shows the results for partial purification of IAA(K.C., Bishnu & Gauchan, Dhurva & Khanal, Sanjay & Lamichhane, Janardan. (2020). Quantification of indole-3-acetic acid from Bambusa tulda Roxb. seedlings using high performance liquid chromatography. African Journal of Biotechnology.

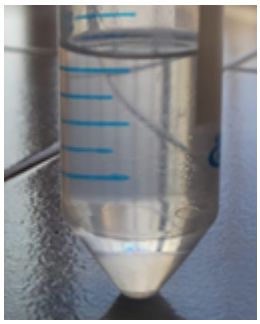
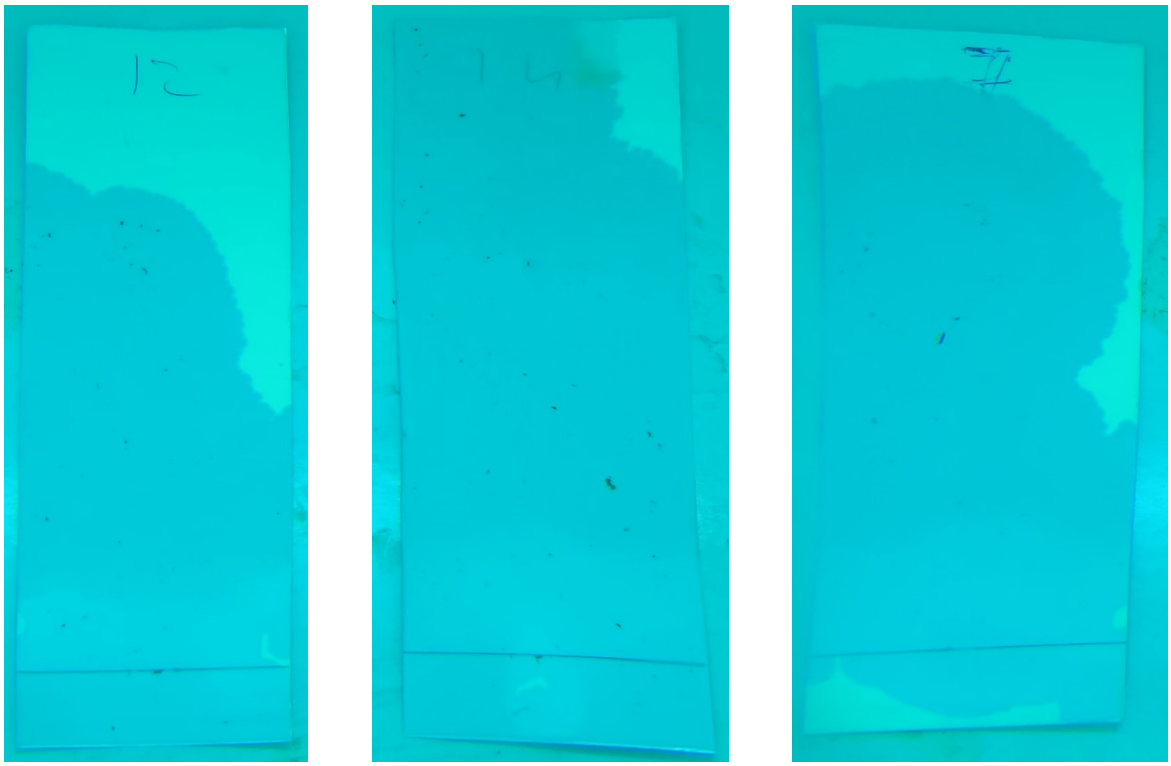


Fig. 4 : Partially purified IAA from the ethyl acetate fraction

Figure 5a, 5b, and 5c depicts the results of the TLC slides after being sprayed on with Salkowaski Reagent. R_f values of the samples were observed as RJK 7=0.73, RJK 12=0.60 and RJK 14= 0.87, which compared to standard which is 0.90.(K.C., Bishnu & Gauchan, Dhurva & Khanal, Sanjay & Lamichhane, Janardan. (2020).



5a **5b** **5c**

Figure 5a, 5b, and 5c :- Results of the TLC slides

Conclusion

The present attempt is an approach to isolating novel organisms and studying their metabolic and biotechnological potential. Agriculture and farming depend extensively on PGPR and the phytohormones generated by it, therefore the current research underlines the ability of microbes to synthesise IAA in the natural form most suited for plants and assist them withstand abiotic challenges such as salinity or drought. In the current environment, discovery of such novel bacterial isolates would be critical to increasing crop plant output.However, in terms of future applications, the isolation, identification, and cloning of genes responsible for enhanced IAA synthesis among such isolates will undoubtedly provide an advantage in developing bio-based products that can be used as bio-fertilizers or direct cloning in plant varieties for a sustainable agriculture approach.

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