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Pseudomonas Aeruginosa VJ003: A Potential PGPR for Rhizosphere Colonization, Plant Growth Promoting Traits, and Biofilm Formation

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ABSTRACT:
Plant growth-promoting rhizobacteria (PGPR) are a broad group of soil bacteria that play a significant role in stimulating plant growth through a variety of advantageous actions. This is mostly accomplished by PGPR's successful rhizosphere colonization. In natural as well as stressed situations, the creation of biofilms by PGPR is thought to be a survival strategy that outperforms the planktonic mode of growth. Different biotic and abiotic factors impacting survival, colonization, and functions might cause inconsistent performance of microbial inoculants under field circumstances. As a result, it is anticipated that rhizobacteria with numerous PGP properties and an effective colonization capacity will perform better. We hypothesized that PGPR's capacity to create biofilms on plant roots would facilitate rhizosphere invasion. We have therefore selected a promising isolate of PGPR from the soybean (Glycine max) rhizoplane using a random screening technique. The ability to form biofilms and a range of PGP activities, such as the production of ammonia, hydrogen cyanide, phosphate solubilization, indole acetic acid, and biocontrol activity, were taken into consideration throughout the selection process. Pseudomonas aeruginosa VJ003 was identified as the selected isolate by the use of 16s rRNA partial gene sequencing. Soybean seedling roots harbored a strong biofilm formed by strain VJ003. Exopolysaccharide synthesis (1610.36 ± 1.20 µg/ml), alginate (142.34 ± 1.12 µg/ml), and cell surface hydrophobicity (45%) were among the biofilm-related characteristics displayed by VJ003. Therefore, a further criterion to choose a better rhizosphere colonizer and promote plant growth could be the production of biofilms on plant roots by promising PGPR.

1. Introduction

Rhizobacteria are known to produce biofilms as a prevalent mode of life in the natural environment, which protects them from many biotic and abiotic stressors. The relationship between different rhizobacteria that promote plant growth has primarily been studied concerning the planktonic growth mode. On the other hand, knowledge of PGPR's behavior in biofilm mode on the soil and root surface is still developing. Although most rhizobacteria can create biofilm in vitro, little research has been done on root-associated biofilm formation in situ because soil-plant root systems have differing circumstances. To further our understanding of the relationship between plants and microbes, a thorough examination of the biofilm formation on the root surface and rhizosphere colonization is therefore required. Biofilms are collections of bacteria that have formed extracellular polymeric substances (EPS) to enclose them in it. Bacteria can thrive in harsh environments thanks to a protected growth mode called biofilm found in nature. Many characteristics of *Pseudomonas* sp. and other bacteria, such as EPS synthesis, cell surface hydrophobicity, and alginate production, are linked either directly or indirectly to the establishment of biofilms (Angus et al., 2013).

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The biofilm may affect the competition for resources, the synthesis of chemicals that limit growth, or the level of tolerance to abiotic stressors (Rao et al., 2005). Benefitful plant growth-promoting rhizobacteria (PGPR) attached to plant root surfaces are well known to increase plant production (Nadeem et al., 2014). Plant growth promotion by diverse PGPR has well-documented indirect (lytic enzymes and antibiotics, induced resistance, HCN synthesis, and competition) as well as direct (production of plant hormones, nitrogen fixation, phosphate solubilization, and iron sequestration) processes (Kloepper et al., 1980, Ahmad and Khan, 2008 and Bernard, 2012). On the other hand, environmental factors that affect rhizosphere colonization and survival were crucial in maintaining PGPR performance constancy. As compared to their planktonic counterparts, biofilms have advantages such as higher rates of horizontal gene transfer, improved resistance to antibacterial compounds, and enhanced protection against desiccation and protozoan predation (Davey and O'toole, 2000, Jefferson, 2004 and Sørensen et al., 2005). It is known that biofilm plays a crucial function in preserving ecological balance and promoting plant growth in natural soil (Burmølle et al., 2012). It is known that the PGPR clings to or colonizes the rhizosphere, where it promotes plant development and inhibits plant diseases (Normander and Prosser, 2000). Compared to non-biofilm-formed inoculants, there are earlier reports that inoculation with biofilm-forming PGPR promotes plant development more effectively (Singh et al., 2014). In a different study, Lee et al., 2014) showed how the biofilm growth mode's resistance mechanism shielded the plant from stress. Given the significance of biofilm as a bacterial survival tactic, we postulated that a promising strain of PGPR with a robust capacity for biofilm formation could serve as a potent root/rhizosphere colonizer. In order to evaluate Pseudomonas sp. VJ003's capacity to form biofilms and colonize rhizospheres as a potential soybean PGPR, a promising novel isolate of the bacterium was chosen for this investigation.

2. Materials and Methods

2.1 Microbial Strains Isolation and their Characterisations

Soil sampling sites, soil treatment, isolation procedure and a total 13 isolates with exopolysaccharide-producing ability from the collected soil samples, their characteristics, morphological and biochemical identification were already reported in the previous study (Jadhav et al., 2022). Further experiments to detect their PGP potential are continued in this investigation.

2.2. Screening Assay for PGP Attributes

Using the approach of (Brick et al., 1994), the generation of indole acetic acid (IAA) was qualitatively estimated. The quantitative estimation of IAA generation in the presence of 500 µg/ml tryptophan was conducted using the method of Loper et al., 1986, which was used by Ahmad and Khan, 2005. In peptone water, ammonia was produced using the procedure described in (Dye, 1962). The siderophore was quantitatively estimated using a modified version of (Reeves et al., 1983). By looking for a halo zone surrounding the bacterial colony developing in the Pikovskaya medium, the qualitative measurement of phosphate solubilization was carried out (Gaur, 1990). As previously mentioned, tricalcium phosphate was quantitatively dissolved in a liquid media (Ahmad and Khan, 2005). By monitoring the clear zone surrounding a bacterial colony growing in PDA medium (Hi-media), antifungal activity was ascertained (Riungu et al., 2008).

2.3. Quantitative Assessment of the Production of Exopolysaccharides (EPS)

The procedure for EPS extraction and quantification followed the instruction of Mody et al., 1989. The pre-inoculum was cultured for *Pseudomonas aeruginosa* VJ003 for an entire night at 28˚C ± 2˚C in KB broth (Hi-media). 500µl of pre-inoculum was introduced to a conical flask, containing fresh 50 ml culture medium, and the mixture was allowed to grow for five days at 120 rpm in a rotatory shaking incubator at 28˚C ± 2˚C. A 200 ml culture volume was centrifuged for 20 minutes at 4˚C at 800 rpm. A 0.45 µm pore size nitrocellulose filter was used to filter the supernatant. After adding three liters of refrigerated ethanol to the final filter, EPSs were precipitated, and the solution was left at 4˚C overnight to precipitate exopolysaccharides. Post-drying the precipitate at at 80˚C for 48 h, the weight of the precipitated EPS was measured.

2.4. Alginate Quantification Assay

Test isolates underwent alginate extraction using a 48-hour-grown culture. Following incubation, the culture was centrifuged at 10,000 rpm for 10 minutes to extract the cell-free supernatant. Deacetylated alginate was isolated from culture supernatants by introducing a

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same quantiy of isopropanol and storing it in a static environment for a day. Centrifugation was used for 10 minutes at 10,000 rpm to collect the precipitate. One milliliter each of 70% and 96% ethanol was used to successively wash the resulting pellet. After 15 minutes of drying at 37˚C, the pellet was dissolved in 1 milliliter of sterilized dd'H2O. To quantify, 100 µl of the suspension was put into new test tubes, and Milli-Q water was added to get the volume up to 1 ml. Following the addition of 30 µl of new carbazole reagent, one milliliter of freshly made borate sulfuric acid solution was added and carefully mixed. After allowing the combination to sit at room temperature for 15 minutes, the absorbance was measured at 500 nm using a reagent blank, and the result was expressed in µg/mg of wet biomass (Wozniak et al., 2003).

2.5. Cell Surface Hydrophobicity Assay

The cell surface hydrophobicity (CSH) was measured using the microbial adhesion test to hydrocarbons (MATH), according to Rosenberg et al., 1980. Following the initial, second, and fourth days of incubation, the bacterial cultures' hydrophobicity was evaluated in nutritious broth. After centrifuging 5 ml of a culture that had grown for 24 hours at 8000 rpm for 10 minutes, the absorbance at 400 nm was measured using a UV-visible spectrophotometer (Shimadzu UV-visible spectrophotometer–1800). The pellets were then resuspended in phosphate-magnesium buffer (pH = 7.4) in order to determine the percentage of hydrophobicity. Using a cyclomixer, five milliliters of culture were combined with 0.2 milliliters of hexadecane and thoroughly mixed. The absorbance at 400 nm, which is known as the final concentration in the aqueous phase, was measured after the aqueous phase was separated. The hydrophobicity percentage was calculated using the following formula:

Percent hydrophobicity (%) = $[1-(A_1/A_0)]$ x 100

Where A_1 denotes an initial bacterial suspension absorbance while absorbance of the aqueous phase is denoted by A_0.

2.6. Pot study to determine the PGP activity:

To find out how the isolates affected germination and seedling vigor, five soybean seeds inoculated with each isolate were grown in 9-cm petri dishes on two layers of moist filter paper. As a control treatment, seeds that were only given water as opposed to a bacterial inoculum were also seen. To guarantee that the Petri plates had enough moisture for germination, 5 milliliters of distilled water were added to the seeds every other day while they were being cultivated in a climate control growth chamber. Every 24 hours for seven days, the germination percentage was recorded. One week later, the length of the roots and shoots were measured. With five replications for each isolate, the experiment was designed using a fully randomized methodology.

Germination rate (%) = number of seeds germinated / total number of seeds x 100

Vigour index = % germination x total plant length

3. Results

3.1. Characterization of Plant Growth growth-promoting activities, Biofilm Screening, and Identification of Test Bacteria

Thirteen bacterial strains (VJ001 to VJ013) were extracted from the soil samples. The isolates underwent characterization, and the most exopolysaccharide-producing isolate (VJ003) was found to be *Pseudomonas aeruginosa*, which has previously been documented in a study published by Jadhav et al. (2022).

3.2. Quantitative Estimation of PGP Attributes

Pseudomonas aeruginosa VJ003 strain produced the most IAA (142.34 ± 1.12 µg/ml), according to the quantitative estimate of IAA production in the presence of an exogenous source of tryptophan (500 µg/ml). Furthermore, Table 1 illustrates the different levels of IAA generation exhibited by additional isolates.

3.3. Alginate and EPS Quantification Assay

The strain VJ003 produced the most alginate, measuring 117.62 ± 1.12 µg/ml. As Table 1 illustrates, additional isolates did, however, exhibit differing degrees of alginate synthesis. The VJ003 strain was shown to produce higher exopolysaccharides (EPS) (1610.36 ± 1.20 µg/ml). However additional isolates also displayed different levels of EPS generation (Table 1).

3.4. Cell Surface Hydrophobicity

Pseudomonas aeruginosa VJ003 in this investigation demonstrated a 63% hydrophobicity and adherence to hydrocarbons. Nevertheless, the remaining isolates underwent hydrophobicity testing as well, and Table 1 displays the different patterns in their percentage of adherence to hydrocarbon.

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Table 1. Features of the Pseudomonas strains under research that produce biofilms and are stress-tolerant and promote plant development.

PGP Strains	VJ001	VJ003	VJ010
IAA (µg/ml)	127.22 ± 1.76	142.34 ± 1.12	119.65 ± 1.05
Phosphate Solubilization (µg/ml)	+ve (78.53 ± 1.32)	+ve (89.61 ± 1.18)	+ve (63.74 ± 1.13)
Siderophore (µg/ml)	+ve (11.29 ± 1.02)	+ve (15.18 ± 1.46)	+ve (11.82 ± 1.27)
Antifungal Activity	++	+++	+
Biofilm formation	+++	++++	++
Cell Surface Hydrophobicity	34%	45%	32%
EPSs Production (µg/ml)	1283.19 ± 1.89	1610.36 ± 1.20	1089.98 ± 1.44
Alginate Production (µg/ml)	109.25 ± 1.05	117.62 ± 1.12	111.16 ± 1.08
Identification	-	*Pseudomonas aeruginosa* VJ003	-

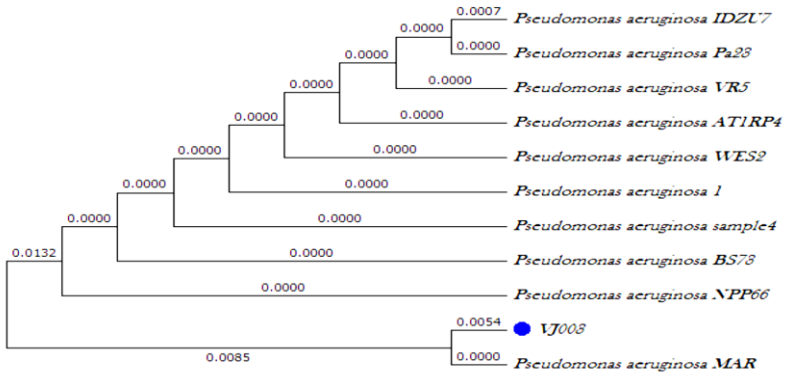

Figure 1. Phylogenetic tree of *Pseudomonas aeruginosa* VJ003 (Jadhav et al., 2022).

3.5. Pot Study for Determination of the PGP activity

Depending on the isolate, the impact of rhizobacterial therapy on soybean seed germination and vigor index varies. Compared to all the examined isolates, the VJ003 strain significantly affected the germination rate and vigour index. As treated with VJ003, seed germination improved by 84.58% ± 1.25% and the vigour index climbed to 78.25% ± 1.36% as compared to the uninoculated control. However, as Figure 2 illustrates, additional isolates also showed distinct patterns of seed germination and vigor index. Figure 3 illustrates the growth of the roots and shoots.

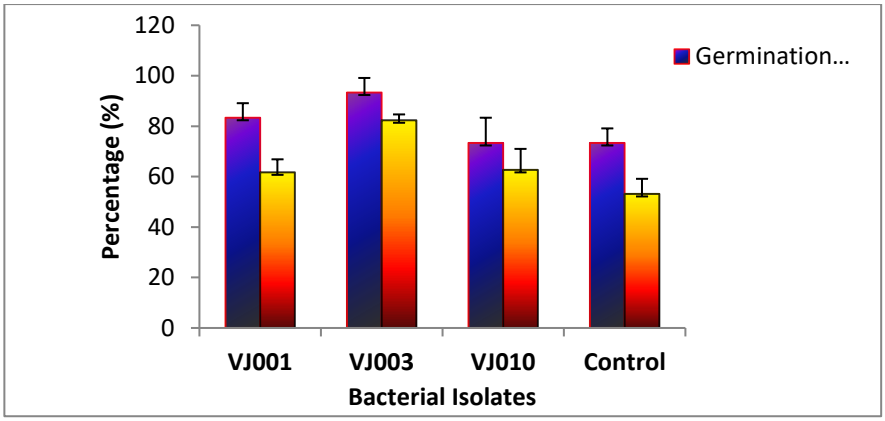

Figure 2. Effect of rhizobacterial treatment on the germination rate and seedling vigour index.

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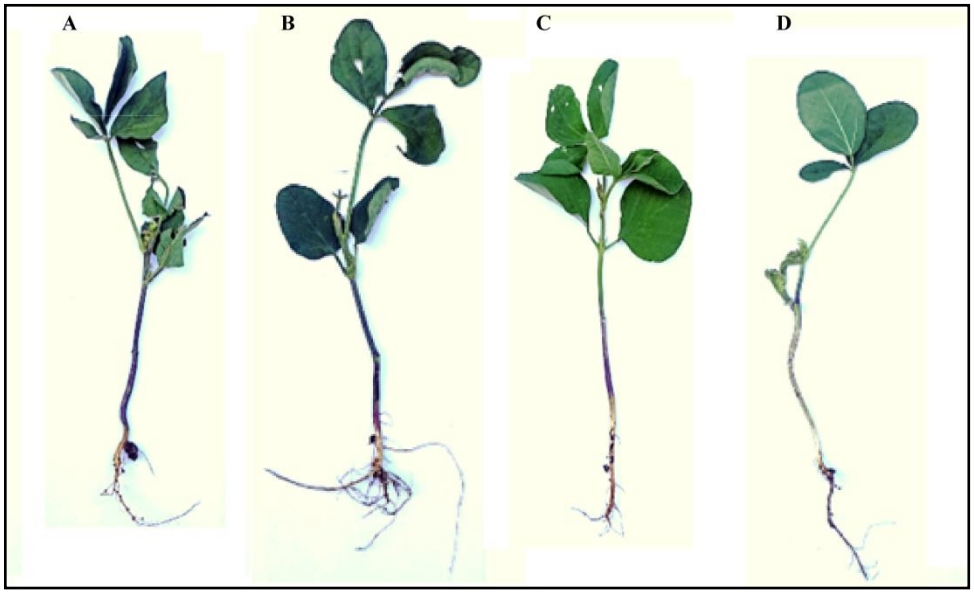

Figure 3 Determination of Root and Shoot Length of Soybean Plant by PGP activity of Isolates A: VJ001, B: VJ003, C: VJ010 and D: Control.

4. Discussion

A crucial component of sustainable agricultural production is the use of bioinoculants to protect and enhance plant health. The intricate process of plant-microbe interaction and environmental conditions determine the effectiveness of bacterial inoculants on crop yield (Wu et al., 2009). One of the key elements in promoting plant growth is the effective colonization of the rhizosphere by bacterial inoculants and the expression of PGP characteristics, such as plant growth regulators. The subject of current research is determining how rhizobacteria's biofilm growth relates to the surface of plants (Albareda et al., 2006, Danhorn and Fuqua, 2007, Fujushige et al., 2008 and Ansari et al, 2017). According to (Altaf and Ahmad, 2017), there are differences in the intrinsic capacity of bacteria to create biofilm in vitro and in conjunction with plant roots. We still don't fully understand how plant roots interact with rhizobacterial biofilm in the rhizosphere. Instead of living in planktonic mode, the natural soil system's bacterial population mostly interacts with plant roots in biofilm mode. These interactions can be either positive or negative (Rao, 2005, Burmølle et al., 2014 and Ren et al., 2015). After primary screening, we chose the *Pseudomonas aeruginosa* VJ003 strain for this investigation. We did this based on the strain's diverse PGP features, tolerance to abiotic stress, ability to form biofilms, and knowledge of biofilm-associated functions such as EPSs, alginate, and cell surface hydrophobicity. The VJ003 strain of *Pseudomonas aeruginosa* produced a wide range of PGP characteristics, was able to withstand abiotic stress, and was able to build a robust biofilm. It is anticipated that this combination of characteristics will protect plant health by suppressing the pathogen and boosting plant development in abiotic stress situations. Additionally, the bacteria demonstrated a remarkable ability to form biofilms in vitro.

We also looked at the behaviors of the VJ003 on the root surface of ten-day-old soybean seedlings about the adhesion and growth of microcolonies/biofilm. We transplanted the treated soybean seedlings by VJ003 isolate in a sterile soil microcosm in a different experiment. The soil microcosm was kept in a growth chamber at a controlled temperature for a maximum of fifteen days. After the seedlings were transplanted into a soil microcosm, the rhizosphere and rhizoplane of the treated soybean seedlings with VJ003 demonstrated their capacity to endure, adapt, and recolonize the rhizosphere/rhizoplane region after 15 days. A similar finding was made by Islam et al., 2016. However, as a result of a successful plant-microbe interaction, PGPR is effective in colonizing plant roots and going on to multiply into microcolonies or produce biofilm. These plant-associated biofilms are highly capable of offering protection from external stress, reducing microbial competition, and giving beneficial effects to the host plant that support growth, yield, and crop quality, according to Ramey et al., 2004, Saleh-Lakha and Glick, 2006 and Lugtenberg and Kamilova, 2009).

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5. Conclusion

Based on the aforementioned research, it is possible to isolate a novel strain of PGPR with the needed diverse activity by selective screening. The production of biofilm by this strain appears to offer distinct advantages for survival and rhizosphere colonization, which would boost plant growth. Nevertheless, more research on natural soil systems is required for useful application in crop production and protection.

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