Original Research Article

Study of endophytic bacteria in pearl millet [*Pennisetum glaucum* (L.) R. Br.] roots

ABSTRACT

Aims: The current investigation aims to isolate and identify the root endophytic bacteria from Pearl millet (*Pennisetum glaucum*) variety MSH-1970, as well as evaluate the qualities that promote plant growth.
Study design: Thestudy involves isolating endophytic bacteria, performingbiochemical and molecular characterization, and evaluating their ability to stimulate plant development through biochemical tests.
Place and Duration of Study: DepartmentofBioscience and Biotechnology, Banasthali Vidyapith, Tonk, Niwai, Rajasthan, 2022–2023.
Methodology: Pearl millet MSH-1970 was planted in pots in the greenhouse of the Department of Bioscience and Biotechnology and endophytic bacterial strains were isolated. The isolates' morphological features were recorded using Bergey'sManual of Systemic Bacteriology, and biochemical testing was done. 16s rRNA gene PCR was used to do molecular characterization of the potential endophyte, and the PCR product was sequenced. Ammonia production, phosphate solubilization, and HCN production were used to identify the properties that promote plant development.
Results: In the initial culture of endophytic bacteria isolated from the pearl millet variety MSH-1970yielded 14 colonies on Nutrient agar medium. One of the colonies, BVCA-16 could solubilize phosphate, and generate hydrogen cyanide, and ammonia. Using 16f-16r and 63f-1244r primers, a molecular analysis of BVCA16 revealed 99.92% similarity with *Pseudomonas sp.* AUS22.The sequence was submitted to GenebankNCBIas*Pseudomonas sp*. strain BVCA-16, accession numberOR350932.
Conclusion: In the Pearl millet variety MSH-1970, the endophytic bacteria*Pseudomonas* sp. strain BVCA-16 was identified and it showedpotential to promote plant growth.

Keywords:Endophytic bacteria, pearl millet, Pseudomonas, phosphate solubilisation.

1. INTRODUCTION

Pearl millet (*Pennisetum glaucum* (L.) R. Br.), a crucial staple crop in arid and semi-arid regions, plays a significant role in global food security. Interest in understanding the complexities of pearl millet's plant-microbe interactions has grown due to its unusual capacity to adapt to harsh climatic circumstances, such as drought and high temperatures (Numan etal., 2021). One important but less-studied aspect of the plant microbiome is the endophytic bacteria found in the root tissues. Endophytic bacteria are important regulators of plant development and health (del Carmen etal., 2022). They frequently confer a variety of advantages from their symbiotic interactions with host plants, such as the ability to absorb nutrients and withstand stress (Chaudhary et al., 2022). *Aerobacter*, *Aeromonas*, *Agrobacterium*, *Bacillus*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Mycobacterium*, *Flavimonas*, *Pseudomonas*, *Sphingomonas*, and other endophytic bacteria have been

identified in bean (Struz et al., 1997). Although endophytic bacteria have been investigated in great detail in a variety of plant species, little is known about their functions and diversity in the context of pearl millet (Verma et al., 2023; Watts et al., 2023).
A dynamic microbial community inhabits the rhizosphere, the zone where plant roots and soil meet, with endophytic bacteria playing a critical role in supporting plant health. A thorough investigation of the endophytic bacteria associated with pearl millet roots is necessary to determine their functional capacities, taxonomic diversity, and their agronomic uses.

2. MATERIAL AND METHODS

2.1. Isolation of endophytic bacterial from pearl millet roots

2.1.1 Pearl millet root collection

The roots of pearl millet MSH-1970 were collected from the plants grown in greenhouse at the Department of Bioscience and Biotechnology, BanasthaliVidypapith, Rajasthan.

2.1.2 Isolation of endophytic bacteria

The surface of the roots was sterilized in a series of three solutions: 2.6% (w/v) sodium hypochlorite solution for 2 min, 75% (v/v) ethanol, and then 75% (v/v) ethanol sequentially for about half a min and then thoroughly cleaned with sterile distilled water.The roots were placed on nutritional agar (NA) plates and incubated at 28±2°C for 3 days to check for and surface contamination.
1g root sample was ground up into a homogeneous paste in a pestle and mortar with 10 ml of sterile, distilled water and left to stand for 20 min.10µl of the diluted supernatant was spread over NA plates and incubated at (28± 2)°C and examined at periodic intervals. Bacterial colonies that were classified based on visualobservation and colony characteristics were noted (Kersters and Vancanneyt, 2005). Pure cultures were established. For subsequent studies, the bacterial plates were stored at (4±1)°C and glycerol stock were prepared for long term storage.

2.3. Characterization of plant growth-promoting activities

2.3.1.Phosphate solubilization

To perform the phosphate solubilization test, Pikovskaya's agar (Pikovskaya, 1948)was prepared and inoculated with isolated bacteria for 5-7 days at 28±2^{o}C.The plates were periodically checked for clear zones around the bacterial growth (Karpagam and Nagalakshmi, 2014).

2.3.2.Test for drought tolerance capacity of the bacterial strain:

Drought tolerance was assessed in bacterial isolates that solubilized phosphate (as described in section 2.3.1). Nutrient agar media (NAM) was prepared with varying concentrations of PEG6000 for the drought tolerance assay.The corresponding concentrations (10%, 15%, 18%, 21%, and 24%) of polyethylene glycol (PEG_{6000}) were then added (Muscolo et al., 2014).100µl of a 24h pre-culture (OD540nm=0.2, 108cfu/ ml) was added to PEG-supplemented broth, and was continuously shaken at 150 rpm on a rotary flask shaker at 30°C. The absorbance was noted at till 78h (6-12h intervals) at 540 nm using a spectrophotometer. The bacterial isolate(s) that perform better in the drought tolerance test were taken forward for further experiments.

2.3.3Ammonia production test

Ammonia production was tested by adding a bacterial suspension to 10 mL of peptone water, which was then incubated for 3–5 days at (28±2)^{o}C(Cappuccino and Sherman, 1992).After incubation, 0.5ml ofNessler's reagent, was added drop by drop from the side of the wall to the bacterial suspensionafter the medium became turbid (Chaudharyetal., 2021).

2.3.4. HCN test

To perform the HCN test, Glycine (4.4 g/ l) was added to the nutrient agar medium (Lorck, 2004). The spreading plate technique was used to inoculate the bacterial culture onto the plate and after growth at (28±2)^{o}C, for 4-6 days, a sterile Whattman filter paper soaked in sodium bicarbonate (2%) and picric acid solution (0.5%) was placed over the culture and observed for change in colorfrom yellow to brown-red.

2.4. Biochemical characterization of isolated endophytic bacteria

The bacterial isolate(s) selected to have growth promoting activities from the above mentioned tests (2.3.1, 2.3.2, 2.3.3, 2.3.4) were taken and the following biochemical tests performed:indole test, MR-VP test, motility test, citrate test, catalase test, urea test, nitrate test, H_2S test (Aneja et al., 2007).

2.5 Molecular characterization

The Sigma-Aldrich Cat. No. NA2110 GenElute Bacterial Genomic DNA Kit was used to extract bacterial DNA. The following universal 16S rRNA primer set (Weisburg et al., 1991)was used in PCR to amplify DNA:fd1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rd1 (5'-AAGGAGGTGATCCAGCC-3').

In another set, the forward primer 63f (5'-AGGCCTAACACATGCAAGTC-3') and the reverse primer (5'-CTCGCTGCCCACTGTCAC-3') was also used to amplify the 16S rRNA gene (Srivastava et al., 2008).

A PCR tube containing 2.5mM dNTPs, 1XTaq buffer, 0.2µM primers, 0.5U/µLTaq polymerase, and 125ng/µg of DNA was used to conduct the reactions. Applied Biosystems' Verity Thermal Cycler was used for PCR amplification (Weisburg et al., 1991). 1.5% agarose gel containing ethidium bromide was used for gel electrophoresis, and the results were shown using a gel documentation system. The PCR product was sent for sequencing to NCMR-NCCS, Pune and Xcelris Genomics, Ahmedabad, India. EZTaxon (https://www.ezbiocloud.net/) and nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/) was used to analyze the partial sequences.

3. RESULTS AND DISCUSSION

A total of 14 bacterial endophytes was successfully isolated in pure culture from the pearl millet roots. Out ofthe 14 bacterial isolates, only 6 (BVCA 14, BVCA15, BVCA16, BVCA 17, BVCA18, BVCA19) showed phosphate solubilization capacity by formation of distinct clear zone around the bacterial colony(Fig. 1a), demonstrating the capacity to dissolve insoluble phosphate compounds. Phosphates are a critical component of soil fertility and plant nutrient and by increasing plants' accessibility to insoluble phosphates, this characteristic raises agricultural output (Kumar et al., 2021).

Using a NAM medium supplemented with different PEG concentrations, the growth of (BVCA 14, BVCA15, BVCA16, BVCA 17, BVCA18, BVCA19) bacterial isolates was monitored at 540nm for 72h at regular intervals.

The absorbance value steadily decreased as the PEG concentration increased, the bacterial strains were susceptible to PEG at high concentrations. The growth suppression observed was consistent with research showing that chemicals such as PEG 6000 could cause osmotic stress, which can hinder the development of bacteria (Marulanda et al., 2009).

It was observed that among the six isolates, BVCA-16 showedvisible growth in even higher PEG concentrations, whereas the other strains were not able to survive. As such a detailed analysis of the bacterial isolate BVCA-16 was done and the data is represented in Fig.2.

All further experiments were performed with the bacterial isolate BVCA-16.

The ammonia production test was performed to evaluate the capacity of the bacterial isolate to metabolize amino acids and create ammonia as a metabolic byproduct (Devi and Thakur,2018). In the present study, positive results were obtainedwhen yellow medium turned brown, indicating ammonia production, which is typical of bacterial metabolism of amino acids(Fig. 3a).

Hydrogen cyanide (HCN) production is an important metabolic characteristic of certain bacteria.With implications for both microbial identification and interactions with the environment, the bacterial isolate BVCA-16 was tested for its capacity to produce hydrogen cyanide. BVCA-16 showed a positive result in the HCN test, demonstrating the existence of hydrogen cyanide production (Fig.3b). According to Bumunang et al. (2014), hydrogen cyanide can operate as an effective antibacterial agent and participate in allelopathy, which affects the manner in which the bacteria and other organisms interact.

The biochemical tests are shown in Fig. 3c-j. The reddish-pink color in the broth indicates the presence of indole indicate that the organism couldproduce the enzyme tryptophanase (Kabiraz etal., 2023). The MR-VP test yielded a negative result, showing the lack of mixed acid fermentation and acetoin synthesis (Das et al., 2022). During the motility test, the diffusion of bacterial growth away from the inoculation point in semisolid agar indicated positive motility. The capacity of the bacteria to use citrate as its sole carbon source is shown by a positive citrate test, which helps in the identification of Enterobacteriaceae (MacFaddin, 2000). The use of citrate is confirmed by the change in pH to alkaline. The existence of catalase in the bacteria was confirmed by a positive catalase test, which allows hydrogen peroxide to be broken down into water and oxygen (Wheelis etal., 2008). The urea test yielded a negative result, indicating the absence of urease activity in the isolated bacterial strain. This finding aids in the differentiation of urease-negative organisms (Clark and Dunlap, 2022). The positive result of the nitrate test indicated that the bacteria were capable of reducing nitrate to nitrite or nitrogen gas (MacFaddin, 2000). The H_2S test produced a negative result, indicating that the bacterial isolatedoes not produce hydrogen sulfide gas during the metabolism of sulfur-containing substances. This finding is pertinent to the classification and differentiation of bacteria, especially in the context of microbial identification and environmental investigations (Johnson and Patel, 2021). The liquefaction of gelatin showed a favorable result for the gelatinase test. This implied that the bacterial isolate couldproduce the enzyme gelatinase, which aids in the breakdown of gelatin into smaller peptides and amino acids.

Genomic DNA of the bacterial isolate BVCA-16 was extracted and amplified using universal primers targeting the 16S rRNA gene, a commonly used marker for bacterial identification.

EZTaxon and NCBI BLAST tools were used to examine the partial sequence BVCA-16. The sequence, exhibited >99% similarity to other strains in the BLAST analysis at NCBI portal and was submitted to the NCBI database as*Pseudomonas sp.* (BVCA-16) with the accession code OR350932.

Endophytesalong withphyllosphere microorganisms,play a crucial role in maintaining plant health. These microbes, which are predominantly bacteria and fungi, can be foundin all parts of the plant, including the roots, stems, leaves, fruits, and seeds.Among the seed endophytic bacteriaidentified in pearl millet, species such as*Kosakoniacowanii, Bacillus subtilis, B. tequilensis, B.velezensisPantoeastewartii, Paenibacillusdendritiformis and Pseudomonas aeruginosa*have been reported (Kumar et al., 2021). Additionally,*P. aeruginosa* and other diazotrophic endophytic bacteria are associated with pearl millet (Gupta et al., 2022). The soil microbiome as well as the phyllospheric and endophytic elements of the millet-associated microbiome contribute significantly to nutrient cycling, absorption, pathogen defence, and plant growth promotion. Therefore these microbes hold considerable promise for developingmicrobial formulations aimed at enriching agricultural productivity, yield and growth.

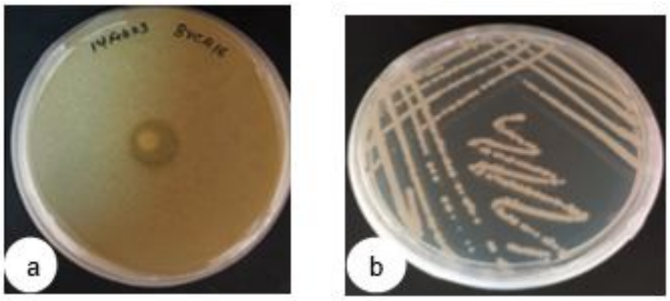

Figure 1.a) Clear zone around bacterial culture (BVCA-16) in phosphate solubilizing test, b)bacterial isolate BVCA-16 grown in NA medium.

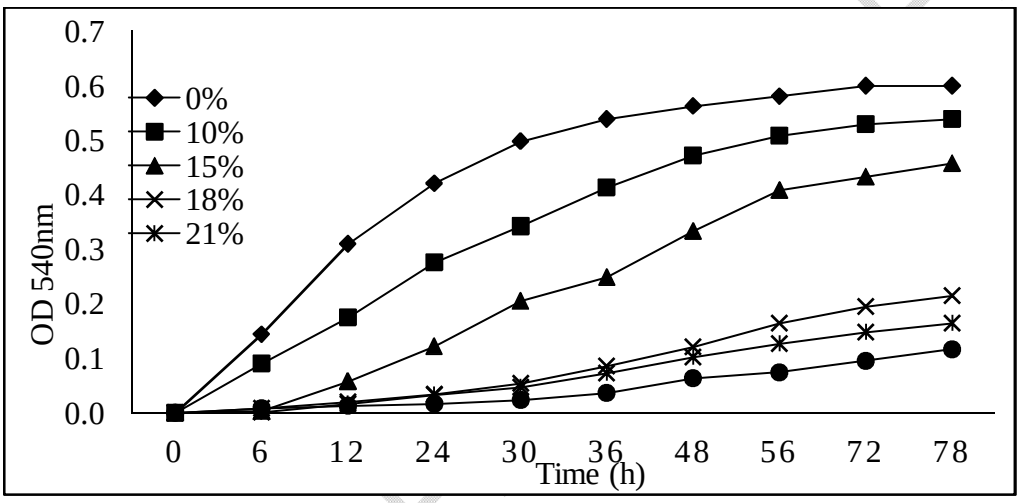

Figure 2. The strain BVCA-16 growth curve at 0% - 24% polyethylene glycol (PEG; PEG_{6000}).

Non-inoculatedInoculated Non-inoculated Inoculated

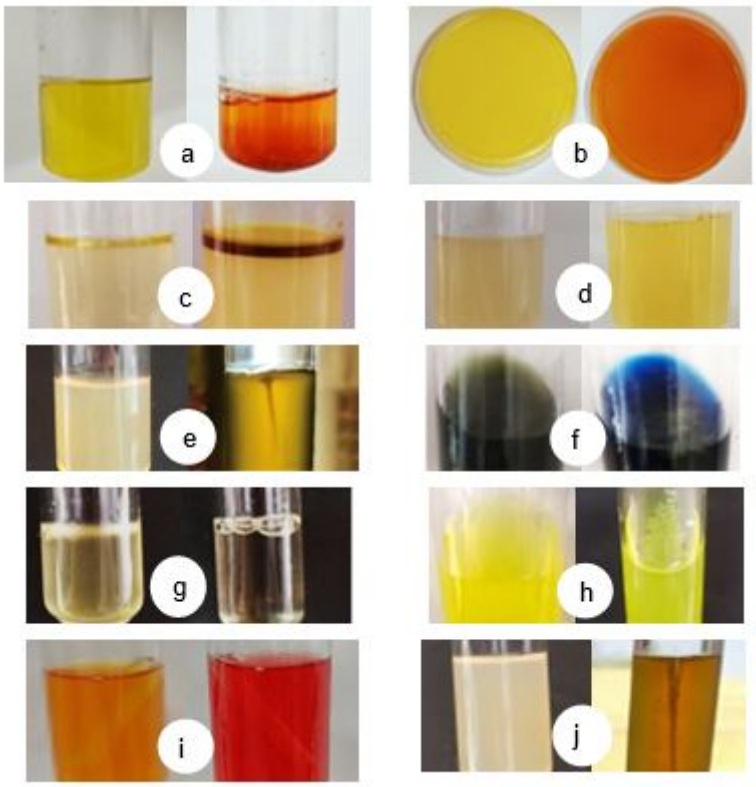

Figure 3. Biochemical analysis of a selected bacterial strain (BVCA-16): a) ammonia production, b) HCN production, c) Indole test, d)MR-VP test, e) motility test, f) citrate test, g) catalase test, h) urea test, i) Nitrate test j) H_2S test by selected bacterial strains.

4. CONCLUSION

In the Pearl millet variety MSH-1970, the endophytic bacteria *Pseudomonas* sp. strain BVCA-16 was identified and it exhibited potential in promoting plant growth. Initially 14 colonies of endophytic bacteria were isolated from the pearl millet variety MSH-1970 on Nutrient agar medium. One of the colonies, BVCA-16 could solubilize phosphate, and generate hydrogen cyanide, and ammonia. Using 16f-16r and 63f-1244r primers, a molecular analysis of BVCA16 revealed 99.92% similarity with *Pseudomonas* sp. AUS22.The sequence was submitted to Genebank NCBI as *Pseudomonas* sp. strain BVCA-16, accession number OR350932.

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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