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 isolatingendophytic bacteria, performingbiochemical and molecular characterization, and evaluating their ability to stimulate plant development through biochemical tests.
Place and Duration of Study: Department of Bioscience and Biotechnology, BanasthaliVidyapith, 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 Bargey's Manual 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 generation, 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 Genebank NCBI as*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 shows potential to promote plant growth.

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

1. INTRODUCTION

A crucial staple crop in arid and semi-arid areas, pearl millet (*Pennisetum glaucum* (L.) R. Br.) makes a substantial contribution to the world's 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., 2021). They frequently get 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

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identified from bean (Struz et al., 1997). Even while 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 (Watts et al., 2023).
It is well known that a dynamic microbial population inhabits the rhizosphere, the area where plant roots and soil meet, with endophytic bacteria playing a major role in maintaining the plant's general health. A thorough investigation of the endophytic bacteria linked to 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 applied to nutritional agar (NA) plates and incubated at 28±2°C for 3 days to check for and surface contamination.
To isolate bacterial endophytes, the above procedure was followed and then 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 placed over NA plates and incubated at 28± 2°Cand examined at periodic intervals. Bacterial colonies that were classified based on several visual observation and colony characteristics were noted (Don et al., 2005).Pure culture was established. For subsequent studies, the bacterial plates were kept at 4±1°C and glycerol stock was prepared for long term storage.

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2.3. Characterization of plant growth-promoting activities

2.3.1.Phosphate solubilization

To perform the phosphate solubilization test, Pikovskaya's agar was prepared and inoculated with isolated bacteria for 5-7 days at28±2°C.The plates were periodically checked for clear zones have formed around the bacterial growth (Karpagam et al., 2014).

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

To check the drought tolerant traits of bacterial isolates which were able to solubilize phosphate (as above 2.3.1), Nutrient agar media (NAM) was prepared with different PEG$_{6000}$ concentrations 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, which was then 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.

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2.3.3Ammonia production test

To conduct the ammonia production test, the suspension of bacterial isolate was added to 10 ml of peptone water, which was then incubated for 3–5 days at 28±2°C(Cappuccino etal., 2008). Nessler's reagent, 0.5ml, was added drop by drop from the side of the wall after the medium became turbid (Chaudharyetal., 2021).

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2.3.4. HCN test

To perform the HCN test, Glycine (4.4 g/ l) was added to the nutrient agar medium (Lorck, 2004; Manasa et al., 2017). The spreading plate technique was used to inoculate the bacterial culture onto the plate and after growth at 28±3°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 color.

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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, Ahmadabad, 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 could be grown in pure culture from the pearl millet roots. Among 14 bacterial isolates only 6 (BVCA 14, BVCA15, BVCA16, BVCA 17, BVCA18, BVCA19) showed phosphate solubilization capacityas revealed by formation of distinct clear zone surrounding bacterial disc (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 is consistent with research showing that chemicals such as PEG 6000 can cause osmotic stress, which can hinder the development of bacteria (Johnson et al., 2024).

It was observed that among the six isolates, BVCA-16 showedvisible growth in even higher PGG 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 etal., 2018). In the present study, positive results were obtained when the yellow medium was converted to brown showing that ammonia synthesis was occurring (Fig. 3a).

The ability of some bacteria to produce hydrogen cyanide (HCN), which is essential to their metabolic capacities, is a notable characteristic. With implications for both microbial identification and interactions with the environment, the bacteria isolate BVCA-16 was test for its capacity to produce hydrogen cyanide. BVCA-16 showed a positive result in the HCN test, demonstrating the existence of hydrogen cyanide generation (Fig. 3b). According to Bumunang et al., 2014, hydrogen cyanide can operate as an effective antibacterial agent and participate in allelopathy, which affects how bacteria and other organisms interact.

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The tests for biochemical charecterisation are represented in Fig. 3c-j. The presence of indole in the broth's reddish-pink color indicates that the organism canproduce 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). The diffusion of bacterial growth from the place of inoculation in a semisolid agar medium during the motility test indicated that the motility was positive. The capacity of the bacteria to use citrate as its only carbon source is shown by a positive citrate test, whichhelps identifyEnterobacteriaceae (MacFaddin et al., 2000). The use of citrate is confirmed by the observed alkaline pH change. 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 et al., 2022). The positive result of the nitrate test indicated that the bacteria were reducing nitrate to nitrite or nitrogen gas (MacFaddin et al., 2000). The H_2S test produced a negative result, indicating that the bacterial isolatedo not create 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 etal., 2021). The liquefaction of gelatin showed a favorable result for the gelatinase test. This implies that the bacterial isolate canproduce the enzyme gelatinase, which aids in the breakdown of gelatin into smaller peptides and amino acids.

Genomic DNA of the bacterial isolate BVCA-16 strain was obtained and PCR was conducted with the use of universal primers, the 16S rRNA and the PCR amplicon sent for sequencing.

EZTaxon and NCBI BLAST tools were used to examine the partial sequence BVCA-16. The sequence, exhibited >99% similarity to other strains on BLAST analysis at NCBI portal (Table 1) and was submitted to the NCBI database as*Pseudomonas sp.* (BVCA-16) with the accession code OR350932.

Endophytes, in addition to phyllosphere microorganisms, contribute to maintaining plant health. It is common to find endophytes, which are mostly made up of bacteria and fungus, in all parts of the plant, including the roots, stems, leaves, fruits, and seeds. *Kosakoniacowanii, Bacillus subtilis, B. tequilensis, B. velezensisPantoeastewartii, Paenibacillusdendritiformis and Pseudomonas aeruginosa* were among the seed endophytic bacteria that are reported from pearl millet seeds (Kumar et al., 2021). *P. aeruginosa* and other diazotrophic endophytic bacteria are associated with pearl millet (Gupta et al., 2022). The soil microbiome and the phyllospheric and endophytic elements of the millet-associated microbiome are often engaged in nutrient cycling, absorption, pathogen defence, and plant growth promotion; as a result, they provide a great deal of promise for use in creating microbial formulations that will increase agricultural productivity, yield and growth.

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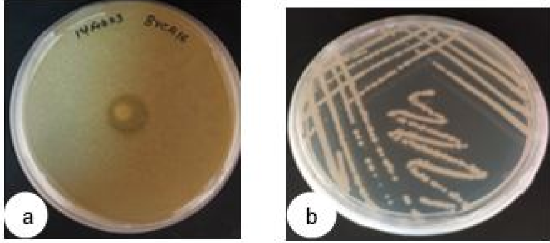

Figure 1.a) Clear zone around bacterial culture (BVCA-16) in phosphate solubilizing test, b)bacterial isolate BVCA-16 grown in NA medium.

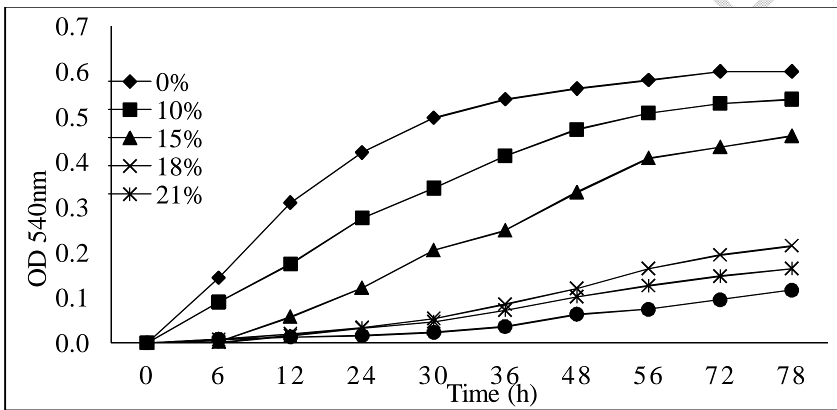

Figure 2. The strain BVCA-16 growth curve at 0% - 24% polyethylene glycol (PEG; PEG_{6000}).

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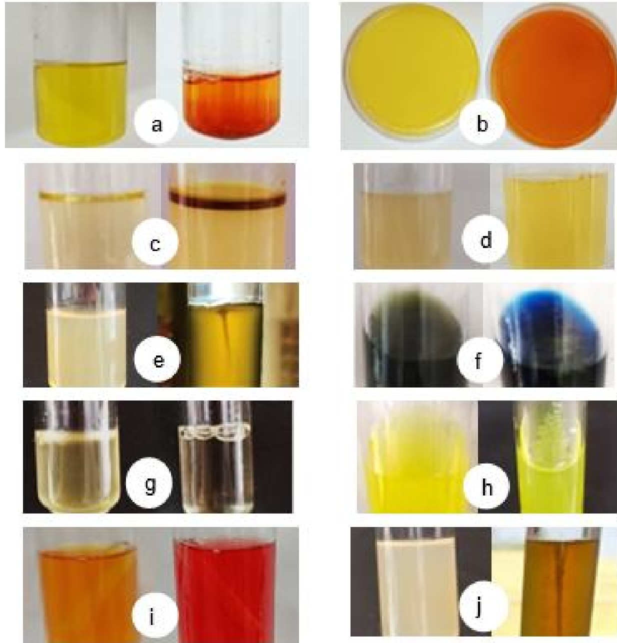

Figure 3. Biochemical analysis of a selected bacterial strain (BVCA-16): a) ammonia production, b) HCN production, c) Indole test, d)MR-VP test, e) motility test, f) citrate test, g) catalase test, h) urea test, i) Nitrate test j) H_2S test by selected bacterial strains.

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4. CONCLUSION

In the Pearl millet variety MSH-1970, the endophytic bacteria *Pseudomonas* sp. strain BVCA-16 was identified and it shows potential to promote plant growth.

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REFERENCES

1. Aneja, K. R. (2007). Experiments in microbiology, plant pathology, and biotechnology. New Age International, India.
2. Bumunang, E.W., &Babalola, O.O. (2014). Characterization of rhizobacteria from field-grown genetically modified (GM) and non-GM maize. *Brazilian Archives of Biology and Technology*, *57*, 1-8.
3. Cappucino, J.G., & Sherman, N. (2005). Microbiology a Laboratory Manual 7thEd. *Aurora*.
4. Chaudhary, T., Gera, R., & Shukla, P. (2021). Deciphering the potential of Rhizobium pusense MB-17a, a plant growth-promoting root endophyte, and functional annotation of the genes involved in the metabolic pathway. *Frontiers in Bioengineering and Biotechnology, 8*, 617034.
5. Chaudhary, P., Agri, U., Chaudhary, A., Kumar, A., & Kumar, G. (2022). Endophytes and their potential in biotic stress management and crop production. *Frontiers in Microbiology*, *13*, 933017.
6. Zheng, T., Li, J., Ji, Y., Zhang, W., Fang, Y., Xin, F., ... & Jiang, M. (2020). Progress and prospects of bio electrochemical systems: electron transfer and its applications in the microbial metabolism. *Frontiers in Bioengineering and Biotechnology*, *8*, 10.
7. Das, S.R., Haque, M.A., Akbor, M.A., Abdullah-Al-Mamun, M., Debnath, G.C., Hossain, M.S., ... & Cho, K.M. (2022). Organophosphorus insecticides mineralizing endophytic and rhizospheric soil bacterial consortium influence eggplant growth promotion. *Archives of Microbiology*, *204*(3), 199.
8. Del Carmen Orozco-Mosqueda, M., Fadiji, A. E., Babalola, O.O., Glick, B.R., & Santoyo, G. (2022). Rhizobiome engineering: Unveiling complex rhizosphere interactions to enhance plant growth and health. *Microbiological Research*, *263*, 127137.
9. Chandra, S., & Chakraborty, D. (2023). Isolation and characterization of plant growth promoting bacteria from root nodules of *Cicerarientinum* L. *International Journal of Plant & Soil Science*, *35*(14), 404-409.
10. Gupta, G., Paul, S., Singh, S., Pietramellara, G., Pathan, S. I., Danish, S., ... & Jha, P. N. (2022). Exploring functional diversity and community structure of diazotrophic endophytic bacteria associated with *Pennisetum glaucum* growing under field in a semi-arid region. *Land*, *11*(7), 991.
11. JF, M. (2000). Biochemical tests for identification of medical bacteria. Lippinccot, Williams & Williams, Baltimore.
12. Johnson, E.R., & Patel, K.N. (2021). Hydrogen Sulfide Test: A Rapid and Reliable Method for Detecting Hydrogen Sulfide-Producing Bacteria. *Journal of Microbiology Methods*, 87(3), 197-201.
13. Kabiraz, M.P., Majumdar, P.R., Mahmud, M.C., Bhowmik, S., & Ali, A. (2023). Conventional and advanced detection techniques of foodborne pathogens: A comprehensive review. *Heliyon*, *9*(4).
14. Kumar, K., Verma, A., Pal, G., Anubha, White, J.F., & Verma, S.K. (2021). Seed endophytic bacteria of pearl millet (*Pennisetum glaucum L.*) promote seedling development and defend against a fungal phytopathogen. *Frontiers in Microbiology*, *12*, 774293.
15. Lorck, H. (1948). Production of hydrocyanic acid by bacteria. *PhysiologiaPlantarum*, *1*(2).

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UNDER PEER REVIEW

16. Numan, M., Serba, D.D., &Ligaba-Osena, A. (2021). Alternative strategies for multi-stress tolerance and yield improvement in millets. *Genes*, *12*(5), 739.
17. Pikovskaya, R. (1948). Mobilization of phosphorus in soil in connection with vital activity of some microbial species. *Microbiology* 17: 362–370.
18. Devi, R., & Thakur, R. (2018). Screening and identification of bacteria for plant growth promoting traits from termite mound soil. *Journal of Pharmacognosy and Phytochemistry*, *7*(2), 1681-1686.
19. Srivastava, S., Singh, V., Kumar, V., Verma, P.C., Srivastava, R., Basu, V., ... & Rawat, A.K. (2008). Identification of regulatory elements in 16S rRNA gene of Acinetobacter species isolated from water sample. *Bioinformation*, *3*(4), 173.
20. Sturz, A.V., Christie, B.R., Matheson, B.G., & Nowak, J. (1997). Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. *Biology and Fertility of Soils*, *25*, 13-19.
21. Watts, D., Palombo, E. A., Jaimes Castillo, A., &Zaferanloo, B. (2023). Endophytes in agriculture: potential to improve yields and tolerances of agricultural crops. *Microorganisms*, *11*(5), 1276.
22. Weisburg, W.G., Barns, S.M., Pelletier, D.A., & Lane, D.J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology*, *173*(2), 697-703.
23. Wheelis, M. (2007). *Principles of modern microbiology*. Jones & Bartlett Publishers.

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