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# Isolation and Characterization of Native *Azospirillum* and Phosphorus Solubilizing Bacteria from Finger Millet Rhizosphere

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***Authors’ contributions***

*This work was carried out in collaboration among all authors. ‘Author A’ designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. ‘Author B’ and ‘Author C’ managed the analyses of the study. ‘Author C’ managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

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| **Background:** Finger millet is an important staple crop in different semi-arid and tropical regions of the world.In order to enhance finger millet yields and promote soil fertility, it is crucial to combine chemical fertilizers with biofertilizers such as *Azospirillum*, *Azotobacter,* and bacteria that solubilize phosphorus and potash in soil.  **Aim:** This study aims to characterize the *Azospirillum* and Phosphate-solubilizing bacteria (PSB) isolates from finger millet soil.  **Methodology**: A total of nineteen rhizospheric soil samples of finger millet were collected from villages in the Radhanagari, Karveer, Shahuwadi, and Panhala tehsils of Kolhapur district, Maharashtra, India. The samples were brought to the laboratory for the isolation of Azospirillum and phosphorus-solubilizing bacteria. Isolation was carried out on NFb and Pikovskaya’s medium for *Azospirillum* and PSB, respectively. Six isolates of *Azospirillum* and three isolates of PSB were obtained from these samples.  **Results:** Most of the *Azospirillum* isolates showed variability in cell morphology, i.e., rod and vibroid. Colonies of *Azospirillum* on semi-solid NFb medium showed a white sub-surface pellicle. In the biochemical analysis, all the isolates showed positive results for the catalase test and KOH test. Most of the PSB isolates showed variability in colony shape, i.e., circular and irregular. All three PSB isolates showed a smooth colony surface. In the biochemical analysis, all the isolates showed positive results for the methyl red test.  **Conclusion**: Significant morphological and biochemical diversity was found when Azospirillum and phosphate-solubilizing bacteria (PSB) isolates from finger millet soil were characterized. In terms of biochemistry, every PSB isolate tested positive in the methyl red test, indicating the possibility of acid generation. These results advance our knowledge of the microbial diversity in the rhizosphere of finger millet and could guide the creation of biofertilizers in the future. |

*Keywords: Azospirillum; phosphorus-solubilizing bacteria; morphological & biochemical analysis; finger millet; rhizosphere.*

## 1. INTRODUCTION

Finger millet is an important staple crop in different semi-arid and tropical regions of the world with excellent nutraceutical properties. It is a staple food in parts of Eastern and Central Africa and India. Around 4.5 million tons of finger millet are produced annually worldwide. Africa produces 2.5 million tons of finger millet annually, while India produces 1.2 million tons. Finger millet (‘*Eleusine coracana L*.’) [Family (Gramineae)] ranks 4th in importance among millets in the world after sorghum (*Sorghum bicolor*), pearl millet (*Pennisetum glaucum*), and foxtail millet (*Setaria italica*) (Upadhyaya *et al*., 2007; Maharanjan *et al*., 2019). Among the different states of India, it is widely grown in which Maharashtra is one. Finger millet is grown on an area of 1159.40 thousand hectares in India with a production of 1998.36 thousand tons (Misal et al.,2024). Maharashtra cultivated over an area of 0.70 lakh ha with a total production of 0.88 lakh tonnes and a total yield of 1251 kg per ha (Department of Agriculture and Farmers Welfare 2023-24). The technology of N-fixing bacteria inoculation is quite widespread (Balbinot et al., 2020). Biological N fixation is the primary mechanism by which *Azospirillum* bacteria increase plant growth and yield; besides, the efficiency of water and nutrients absorption is improved by facilitating the development of root system and making a higher soil volume available to plant roots (Bashan and Holguin, 1997; Reis *et al*., 2011). In order to enhance finger millet yields and promote soil fertility, it is crucial to combine chemical fertilizers with biofertilizers such as *Azospirillum*, *Azotobacter,* and bacteria that solubilize phosphorus and potash in soil (Misal et al.,2024). Phosphorus is widely called as “Bottleneck of world hunger” and an essential element with plays a vital role in plants’ growth and development (Angel et al., 2023). Rajakumar *et al*. (2014) isolated *Azospirillum* from soil samples were inoculated them in Nfb semisolid medium. After 48 hours, pellicles formed and were observed, and then moved within a day to the surface of the medium. Just 1 mm below the upper surface of the medium, thin, dense white, undulated pellicles were formed. The isolated strains formed a typical pellicle and showed spiral movement of cells under a microscope. This indicates the presence of *Azospirillum*. The isolated strains were occurred, white, small, dry, and often merged colonies on the Nfb agar plates. The use of phosphate fertilizers associated with inoculation with phosphate-solubilizing microorganisms has proved to be a technology that meets the precepts of ecological intensification of agriculture, both by reducing the use of synthetics in these environments and by adding beneficial microorganisms (Gomes et al., 2023). Sadiq *et al.* (2013) isolated bacterial strains using 10-fold serial dilutions. Serially diluted soil samples (up to 10-5) were spread on Pikovskaya’s agar and incubated at ±280C for 48 hrs. Single phosphorus-solubilizing bacterial colonies were streaked on fresh plates of Pikovskaya’s medium and incubated at ±280C for 48 h. Then, the appearance of halo zone was used for confirmation of the presence of phosphate-solubilizing bacteria (PSB). The use of PSB as inoculants increases P uptake by the plant and crop yield. The principal mechanism for mineral phosphate solubilization is the production of organic acids, and acid phosphatases play a major role in the mineralization of organic phosphorus in soil (Hilda Rodriguez, 1999). Therefore, the management of biofertilizers containing *Azospirillum* and phosphorus-solubilizing bacteria can help in regulating the nutrient use efficiency of chemical fertilizers, resulting in increased yield and productivity. By considering the nitrogen fixing ability, solubilization index, and biochemical test present investigation has been initiated to characterize the *Azospirillum* and PSB isolates from finger millet soil.

## 2. MATERIALS AND METHODS

## 2.1 Collection of Soil Samples

A total of nineteen rhizospheric soil samples of finger millet were collected from villages of Radhanagari, Karveer, Shahuwadi, and Panhala tehsil of Kolhapur district of Maharashtra and brought to the laboratory for isolation of *Azospirillum* and phosphorus-solubilizing bacteria. The rhizospheric soil and root samples were kept in sterile plastic bags after labelling and tagging with precise GPS coordinates (latitude, longitude and altitude). These samples were preserved in a refrigerator at 4°C for further use. Isolation was carried out on NFB medium from collected soil and root samples. Isolation of PSB was carried out by the serial dilution pour plate technique method on Pikovskaya's Agar medium for Phosphorus-solubilizing bacteria from collected rhizospheric soil samples of Karveer, Shahuwadi, Radhanagari, and Panhala tehsils of Kolhapur District. After that, the morphological and biochemical characteristics of obtained colonizes in both the medium were compared with those defined in Bergey’s manual (Krieg *et al*., 1994) to confirm them as *Azospirillum* and PSB isolates. The isolates with similar characteristics of *Azospirillum* and PSB were streaked on another medium plate and were purified by subsequent streaking after each growth till all the colonies in the petri plates appeared similar in morphology and characters. Then, morphological and biochemical tests were carried out for both *Azospirillum* and PSB isolates.

## 3. RESULTS AND DISCUSSION

A total of nineteen rhizospheric soil samples of finger millet were collected from different villages of Kolhapur district in the year 2023-24. Isolation of *Azospirillum* and phosphorus-solubilizing bacteria was done on NFB and Pikovskaya's medium respectively. During the investigation, six *Azosprillum* isolates were obtained, and three isolates of phosphorus-solubilizing bacteria were obtained. (Table 1)

## 3.1 Identification of *Azospirillum*

The identification of six *Azospirillum* isolates was done by using microscopic observations, morphological and biochemical characters, and six isolates were identified as *Azospirillum*. On NFB medium, the cultures of *Azospirillum* have been revived when required. Semi-solid and solid Nfb medium were used to study the morphological characters, such as Gram reaction, colour of colony, and cell morphology of six *Azospirillum* isolates were examined. All isolates of *Azospirillum* were Gram-negative. Isolates *Azospirillum-*1, *Azospirillum*-2, *Azospirillum*-5, and *Azospirillum*-6 were rod-shaped, and isolates *Azospirillum-*3 & *Azospirillum*-4 were vibroid in shape. All *Azospirillum* isolates showed white sub-surface pellicle on semi-solid medium. *Azospirillum-*1, *Azospirillum*-4, and *Azospirillum*-6 was showed smooth, raised, dense colonies on solid Nfb medium, and *Azospirillum*-2, *Azospirillum*-3, and *Azospirillum*-5 showed smooth, flat, dense colonies on solid Nfb medium. The present results are in correspondence with the findings of Cappuccino & Sherman (1992), Cassán *et al.* (2015), Yao Lin *et al*. (2015), Muthukumar *et al.,* (2021) who had also found vibrioid, slightly curved rods in shape, typical small white dense colonies of *Azospirillum*.

The findings of the biochemical characterization of all the obtained isolates of *Azospirillum* are presented in Table 4. The results revealed that all *Azospirillum* isolates were positive for the catalase test. Starch hydrolysis and oxidase test were positive for isolates *Azospirillum*-2, *Azospirillum*-3, *Azospirillum*-5 & *Azospirillum*-6 and negative for *Azospirillum*-1 & *Azospirillum*-4. *Azospirillum*- 1 isolate positive for gelatin hydrolysis, and all remaining isolates were negative. Nitrate reductase test was positive for *Azospirillum*-1, *Azospirillum*-3, *Azospirillum*-4, and *Azospirillum*-6 and negative for *Azospirillum*-2 & *Azospirillum*-5 isolates. KOH test for all *Azospirillum* isolates were positive. All isolates of *Azospirillum* were positive for the methyl red test except isolate *Azospirillum*2. All *Azospirillum* isolates were positive for indole test except *Azospirillum*-5. The presen results revealed similarities with Akhter *et al*., (2012), Hossain *et al*., (2015), Sulaiman *et al*., (2019), M. Gayathri (2021), Gandhimaniyan *et al.,* (2020).

**Table 1. Collection of soil samples of finger millet from Kolhapur District.**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Name of Tehsil** | **Name of Village** | **No. of Sample** | **Obtained isolates of *Azospirillum*** | **Obtained isolates of PSB** | **Latitude** | **Longitude** | **Altitude** |
| Karveer | Ispurli | 1 |  |  | 16.5001 | 74.0973 | 681m |
|  | Ispurli | 1 |  | PSB-2 | 16.5018 | 74.0954 | 717m |
|  | Yevati | 1 |  |  | 16.4672 | 74.0869 | 794m |
|  | Shelewadi | 1 | ***Azosprillum*-1** |  | 16.4805 | 74.0912 | 765m |
|  | Mhalunge | 1 |  |  | 16.5849 | 74.0824 | 564m |
|  | Nigave | 1 |  |  | 16.5761 | 74.0822 | 598m |
| Shahuwadi | Jadhavwadi | 1 | ***Azosprillum*-2** |  | 16.5506 | 74.0951 | 566m |
|  | Yelur | 1 |  | PSB-1 | 16.5015 | 74.1168 | 568m |
| Radhanagari | Dherewadi | 1 |  |  | 16.5608 | 74.1797 | 596m |
|  | Dherewadi | 1 |  | PSB-3 | 16.3354 | 74.1051 | 574m |
|  | Chakareshwarwadi | 1 | ***Azosprillum*-3** |  | 16.5398 | 74.1488 | 569m |
|  | Baradwadi | 1 |  |  | 16.4938 | 74.1301 | 584m |
|  | Sonali | 1 | ***Azosprillum*-4** |  | 16.5426 | 74.1490 | 568m |
|  | Mhalsawade | 1 |  |  | 16.5180 | 74.1445 | 563m |
|  | Rashiwade | 1 | ***Azospirillum*-6** |  | 16.9259 | 73.9112 | 618m |
|  | Thipkurli | 1 |  |  | 16.9340 | 73.9195 | 614m |
| Panhala | Pisatri | 1 |  |  | 16.7206 | 73.9336 | 545m |
|  | Kisrul | 1 | ***Azospirillum*-5** |  | 16.7297 | 73.9642 | 552m |
|  | Kaljawade | 1 |  |  | 16.7147 | 73.9484 | 568m |

**Table 2. Morphological characteristics of *Azospirillum* isolates**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Sr. No.** | **Isolates** | **Morphology of cell** | **Colonies on semi-solid Nfb medium** | **Colonies on solid Nfb medium** | **Gram reaction** | **Colour of colony** |
| 1. | *Azospirillum*-1 | Rod | White sub-surface pellicle | Smooth, raised, dense | -ve | Greenish blue |
| 2. | *Azospirillum*-2 | Rod | White sub-surface pellicle | Smooth, flat, dense | -ve | Greenish blue |
| 3. | *Azospirillum*-3 | Vibroid | White sub-surface pellicle | Smooth, flat, dense | -ve | Greenish blue |
| 4. | *Azospirillum*-4 | Vibroid | White sub-surface pellicle | Smooth, raised, dense | -ve | Blue |
| 5. | *Azospirillum*-5 | Rod | White sub-surface pellicle | Smooth, flat, dense | -ve | Blue |
| 6. | *Azospirillum*-6 | Rod | White sub-surface pellicle | Smooth, raised, dense | -ve | Blue |

**Table 3. Morphological characteristics of Phosphorus solubilizing bacterial isolates**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Sr. no.** | **Isolates** | **Colony shape** | **Colony colour** | **Gram reaction** | **Surface** |
| 1. | PSB-1 | Irregular | White | -ve | Smooth |
| 2. | PSB-2 | Circular | White | -ve | Smooth |
| 3. | PSB-3 | Circular | White | -ve | Smooth |

**Table 4. Biochemical characterization of *Azospirillum* isolates**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Sr. No.** | **Biochemical tests** | ***Azospirillum-*1** | ***Azospirillum*-2** | ***Azospirillum*-3** | ***Azospirillum-*4** | ***Azospirillum-*5** | ***Azospirillum-*6** |
| 1. | Catalase test | +ve | +ve | +ve | +ve | +ve | +ve |
| 2. | Starch hydrolysis | -ve | +ve | +ve | -ve | +ve | +ve |
| 3. | Oxidase test | -ve | +ve | +ve | -ve | +ve | +ve |
| 4. | Gelatin liquefaction | +ve | -ve | -ve | -ve | -ve | -ve |
| 5. | Nitrate reduction | +ve | -ve | +ve | +ve | -ve | +ve |
| 6. | KOH test | +ve | +ve | +ve | +ve | +ve | +ve |
| 7. | Methyl red | +ve | -ve | +ve | +ve | +ve | +ve |
| 8. | Indole test | +ve | +ve | +ve | +ve | -ve | +ve |

**Table 5. Biochemical characterization of Phosphorus solubilizing bacterial isolates**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Sr. no.** | **Biochemical tests** | **PSB-1** | **PSB-2** | **PSB-3** |
| 1. | Catalase test | +ve | +ve | -ve |
| 2. | Starch hydrolysis | -ve | +ve | +ve |
| 3. | Oxidase test | +ve | -ve | +ve |
| 4. | Gelatin liquefaction | +ve | +ve | -ve |
| 5. | Nitrate reduction | -ve | +ve | +ve |
| 7. | Methyl red | +ve | +ve | +ve |
| 8. | Indole test | +ve | -ve | +ve |

## 3.2 Identification of Phosphorus Solubilizing Bacterial Isolates

The identification of PSB isolates was done by using microscopic observations, morphological, and biochemical characters. Three isolates were identified as Phosphorus Solubilizing Bacteria. The obtained PSB isolates were purified and maintained on Pikovskaya’s medium by frequent subculturing and stored at 4 °C in a refrigerator. On medium Pikovskaya’s, the cultures of PSB have been revived when required. The morphological characters of PSB isolates in Table 3 revealed that, all isolates showed all isolates were gram-negative. All PSB isolates showed a smooth surface and white colony colour. Isolate PSB-1 had an irregular shape, and isolates PSB-2 and PSB-3 showed a circular shape. The present investigation shows similarity with the findings of the scientists Uddin *et al*., (2016), Anbuselvi *et al*., (2015).

The different biochemical tests were performed for the obtained isolates. The isolates PSB-1 and PSB-2 were positive for the catalase test and negative for PSB-3. Both isolates PSB-2 and PSB-3 were positive for the Starch hydrolysis test and negative for PSB-1. Oxidase test was positive for isolates PSB-1 and PSB-3 and negative for PSB-2. The gelatin liquefaction test was positive for isolates PSB-1 and PSB-2 and negative for PSB-3. The nitrate reductase test was positive for isolates PSB-2 and PSB-3 and negative for PSB-1. All isolates were positive for the methyl red test. Isolates PSB-1 and PSB-3 were positive for the indole test and negative for PSB-2. These results were similar to the findings of Damor and Goswami (2016), Bashir *et al*., (2019), Saisree C. (2017).

## 4. CONCLUSION

Significant morphological and biochemical diversity was found when Azospirillum and phosphate-solubilizing bacteria (PSB) isolates from finger millet soil were characterized. All the native isolates of *Azospirillum* and Phosphorus-solubilizing bacteria isolated from the rhizospheric soil of finger millet cultivated in different villages of Kolhapur district were identified on the basis of morphological characteristics, microscopic observations, and biochemical characterization. All three PSB isolates showed a smooth colony surface. In the biochemical analysis, all the isolates showed positive results for the methyl red test. The results advance our knowledge of the microbial diversity in the rhizosphere of finger millet and could guide the creation of biofertilizers in the future.

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Competing interests

Authors have declared that no competing interests exist.

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