Characterization of *Azospirillum* and Phosphorus solubilizing bacteria

ABSTRACT

Nineteen soil and root samples were collected from finger millet field. 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. Most of the *Azospirillum* isolates showed variability in cell morphology i.e., rod and vibroid. Colonies of *Azospirillum* on semi-solid NFb medium showed white sub-surface pellicle. In the biochemical analysis all the isolates showed positive results for 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 smooth colony surface. In the biochemical analysis all the isolates showed positive results for methyl red test.

Keywords: *Azospirillum*, Phosphorus solubilizing bacteria, Morphological & biochemical analysis.

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). Maharashtra cultivated over an area of 0.70 lakh ha with total production of 0.88 lakh tones and total yield 1251 kg per ha

(Department of Agriculture and Farmers Welfare 2023-24). 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 higher soil volume available to plant root (Bashan and Holguin, 1997; Reis *et al*., 2011). Rajakumar *et al*., (2014) isolated *Azospirillum* from soil samples, were inoculated in Nfb semisolid medium. After 48 hours, pellicles formed and 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 typical pellicle and showed spiral movement of cells under microscope. This indicates the presence of *Azospirillum*. The isolated strains were occurred, white, small, dry and often merged colonies on the Nfb agar plates. 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 $\pm 28^0$C for 48 hrs. Single phosphorus solubilizing bacterial colonies were streaked on fresh plates of Pikovskaya's medium and incubated at $\pm 28^0$C for 48 h. Then, the appearance of halo zone was used for confirmation of the presence of 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.

MATERIALS AND METHODS

Collection of soil samples

Total nineteen rhizospheric soil samples of finger millet was 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 refrigerator at 4°C temperature for further use. Isolation was caried out on Nfb medium from collected soil and root samples. Isolation of PSB was carried out by 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 characters of *Azospirillum* and PSB were streaked on another medium plates and were purified by subsequent streaking after each growth till all the colonies in petri plates appeared similar in morphology and characters. Then morphological and biochemical test were carried out for both *Azospirillum* and PSB isolates.

RESULTS AND DISCUSSION

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

Identification of *Azospirillum*

The identification of six *Azospirillum* isolates was done by using microscopic observations, morphological and biochemical characters, six isolates were identified as *Azosprillum*. 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, *Azospirillum*-6 were rod shaped and isolates *Azospirillum*-3 & *Azospirillum*-4 were vibroid in shape. All *Azospirillum* isolates were showed white sub surface pellicle on semi solid medium. *Azospirillum*-1, *Azospirillum*-4, *Azospirillum*-6 was showed smooth, raised, dense colonies on solid Nfb medium and *Azospirillum*-2, *Azospirillum*-3, *Azospirillum*-5 was 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 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 methyl red test except isolate *Azospirillum*-2. All *Azospirillum* isolates were positive for indole test except *Azospirillum*-5. The present results revealed similarities with Akhter *et al*., (2012), Hossain *et al*., (2015), Sulaiman *et al*., (2019), M. Gayathri (2021), Gandhimaniyan *et al.,* (2020).

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 4^0C in 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 PSB isolates were gram negative. All PSB isolates were showed smooth surface and white colony colour. Isolate PSB-1 had irregular shape and isolates PSB-2 and PSB-3 showed 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 obtained isolates. The isolates PSB-1 and PSB-2 were positive for catalase test and negative for PSB-3. Both isolates PSB-2 and PSB-3 were positive for Starch hydrolysis test and negative for PSB 1. Oxidase test was positive for isolates PSB-1 and PSB-3 and negative for PSB-2. Gelatin liquefaction test was positive for isolates PSB-1 and PSB-2 and negative for PSB-3. Nitrate reductase test was positive for isolates PSB-2 and PSB-3 and negative for PSB-1. All isolates were positive for methyl red test. Isolates PSB-1 and PSB-3 were positive for indole test and negative for PSB-2. These results were similar with the findings of Damor and Goswami (2016), Bashir *et al*., (2019), Saisree C. (2017).

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

CONCLUSIONS

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, biochemical characterization.

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