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Morphological and biochemical study of *Azospirillum* and phosphate solubilizing bacterial isolates

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Abstract
The rhizosphere is a dynamic region governed by complex interactions between plants and the organisms that are in close association with the root. *Azospirillum* is one of the versatile non-symbiotic, free living diazotrophic bacteria which appears to have a world-wide distribution and occurs in large number in the rhizosphere soil of a variety of grasses and cereals. There are various types of soil microbes which can solubilise the fixed form of P and make it available to plants. Twenty root and rhizospheric soil samples were collected from different tehsils of Kolhapur District. Total seven *Azospirillum* and eight Phosphate solubilizing bacteria (PSB) isolates were obtained. All *Azospirillum* and PSB isolates were identified on the basis of morphological, microscopic features and different biochemical tests. Biochemical tests were studied *viz*. methyl red test, catalase test, starch hydrolyse test, gelatine hydrolysis test, gas production, H2S production, indol test, and nitrate reductase test. Most of isolates showed positive while some showed negative test. All the isolates of *Azospirillum* were gram negative and had white subsurface pellicle on semi solid NFB media. All the isolates were rod shaped except the isolate 5 which was vibroid shaped. All the eight Phosphate solubilizing bacterial isolates had white, smooth colonies. Some showed irregular colonies whereas some showed circular colonies. All isolates were gram negative.

Keywords: Morphological and biochemical, *Azospirillum*, phosphate solubilizing bacterial

Introduction
The rhizosphere is a dynamic region governed by complex interactions between plants and the organisms that are in close association with the root. *Azospirillum* is a gram-negative, microaerophilic, non-fermentative, nitrogen-fixing bacterium belonging to the Rhodospirillaceae family. It is aerobic, but many of them can also function as microaerobic diazotrophs, meaning they can survive in low-oxygen environment. It may fix 20-40 kg of nitrogen per hectare per year. *Azospirillum* is mainly studied under associative symbiotic nitrogen fixers because of its association with different grasses. Currently, 17 species of *Azospirillum* have been reported and among them, *Azospirillum lipoferum* and *Azospirillum brasilense* are mainly studied, and described (Kour *et al*., 2020) [9]. These species have been isolated from the soil as well as from the aerial parts of plants having nitrogen- fixing abilities. Apart from nitrogen fixation, these microorganisms also produce indole acetic acid (IAA), cytokinins, and gibberellins. It promotes root propagation by secreting growth hormones (Van *et al*. 1997) [18]. The use of Phosphate solubilizing bacteria as inoculants increases phosphate uptake by plants while also increasing crop production. The formation of organic acid is the primary mechanism for mineral phosphate solubilization and enzyme phosphatases play an important role in the mineralization of organic phosphorus in soil (Richardson and Simpson, 2011) [14].

Biochemical tests are the most important methods for microbial identification by differentiating them on the basis of biochemical activities.

Identification and characterization of microorganisms is a key part of the microbial management. This technique is useful to identify bacteria or other unknown microorganisms in the bacterial population. The aim of this study is to revive *Azotobacter* and PSB isolates of microbial repository of Dept. of Agril. Microbiology, College of Agriculture, Raipur, characterize them and through systematic screening select the best performing *Azotobacter* and PSB isolates for their further use in crop production.

Materials and Methods

Roots and rhizospheric soil samples were taken from sorghum-growing areas of Shirol, Gadhinglaj, Karveer and Hatknangle tehsils of Kolhapur district. The rhizospheric soil of the sorghum crop as well as the plant roots were collected and the soil was carefully cleaned from the plant roots before being stored in the refrigerator at 4°C for further use. For the isolation of *Azospirillum*, root samples were used whereas the isolation of Phosphate solubilizing bacteria were carried out by serial dilution pour plate technique on Pikovskaya's media (PVK).

Isolation

Fresh root samples were cut into 0.5 cm lengths, washed well in running tap water and surface sterilized by dipping in 0.1 percent $HgCl_2$ solution for three minutes, followed by one minute in 70 percent alcohol. Finally, six to eight changes of distilled water were used to cleanse the roots. Under aseptic conditions, the root bits were inserted subsurface in screw cap tubes containing sterilized Nitrogen-free semisolid malic acid media.

Serial dilution and pour plate technique

One gram of well mixed soil sample was added in 9ml of distilled water blank. Tenfold serial dilutions were prepared up to 10-7 dilution. One ml aliquot was transferred from 10-4 to 10-6 in sterilized petriplates under aseptic conditions. After that each petriplate with aliquot was filled with sterilized Pikovskaya's Agar medium (15-20 ml) and mixed gently. After solidification of the medium, plates were incubated at 28±2 °C for 4 to 5 days.

Morphological Characteristics

Morphological characteristics such as gram staining, cell shape, colony colour, colony shape, 3% KOH test were studied. Gram staining was done as per standard procedure described by Sagar Aryal (2018) [15].

KOH (Potassium Hydroxide) test

A week-old colony's loop complete culture of bacteria was mixed with a drop of 3 percent aqueous KOH solution on a clean glass slide, then swirled in a rapid circular motion for 5-10 seconds with a needle. The needle was lifted a few millimetres above the glass slide and watched for the formation of viscid material stands to confirm the gram reaction. When a needle was used to pick up a thread-like slime, gram negative bacteria were found (Sunslow *et al*., 1982)**.**

Biochemical characteristics

Biochemical tests were studied *viz*. methyl red test, catalase test, starch hydrolyse test, gelatine hydrolysis test, gas production, H2S production, indol test, nitrate reductase test. The biochemical characterization was done as per the procedures outlined by Cappuccino and Sharman (1987) [4].

Methyl red (MR) test

Fresh isolates of *Azospirillum* were inoculated in test tubes containing MR-VP broth under aseptic conditions and were incubated at 28±2 °C for 48 hours. 5 ml of methyl red solution was added to each after the incubation period. The formation of red colour in the broth indicated the positive result and the formation of yellow colour indicated negative result for the test.

Catalase test

Catalase activity of the isolates were determined by placing the 24 hour old cultures on a clean labelled glass slide. A drop of 3% H_2O_2 was added on the samples on the glass slide. The development of gas bubbles indicated the positive catalase test.

Gelatin hydrolysis test

The gelatine medium stabs were prepared and the fresh grown cultures of *Azospirillum* were inoculated on the stabs under aseptic conditions. The stabs were then incubated for 48 hours at 28 °C.

After incubation of 2 days, the gelatine dip tubes were placed in refrigerator at 4 °C for about 20 minutes. The culture was then examined whether the medium was solid or liquid. The liquefaction of the medium indicated positive test.

Nitrate reductase test

The isolates of *Azospirillum* were inoculated to the test tubes containing nitrate broth under aseptic conditions. The test tubes were incubated at 28±2 °C for 4 days. After incubation added 6-8 drops of nitrate reagent A (Sulfanillic acid) and 6-8 drops of nitrate reagent B (naphthylamine). Observed for the red colour development. Red colour development indicates the positive test.

Starch hydrolysis test

The isolates were inoculated on the sterilized petriplates containing starch agar media and incubated at 28±2 °C for 4 days. After the incubation for 4 days, flooded the surface of plates with iodine solution with a dropper for 30 seconds. Examined for the clear zone around the line of bacterial growth. Formation of clear halo zone around the bacterial colony indicates the positive test.

Indol test

Growing the organism in test tube containing 4 ml of tryptophan broth. Incubated the test tubes for four to five days at room temperature (28±2 °C). After incubation added 0.5 ml of Kovac's reagent to broth culture. Observed for the formation of pink to red coloured reagent layer on top of medium within seconds of adding the reagent. Red coloured ring formation indicates positive test.

Results and Discussion

Twenty root and soil samples of sorghum growing fields were collected from different fields of Kolhapur District in year 2021-22. Seven isolates of *Azospirillum* and eight isolates of Phosphate solubilizing bacteria were obtained from twenty root and soil samples respectively from Kolhapur District.

Morphological characteristics of *Azospirillum* isolates

NFB medium were used to study morphological characteristics such as cell morphology, growth on semi solid and solid NFB medium, gram reaction, colour of colony and KOH test etc. The results found that all the isolates of *Azospirillum* were gram negative. *Azospirillum* Isolates 1, 2, 3, 4 and 7 were rod shaped while the isolates 5 & 6were vibroid in shape. All isolates were positive for KOH test. All isolates showed white sub-surface pellicle in semi solid NFB medium. Isolates 1, 3, 5 & 7 showed smoot, raised, dense colonies on solid agar medium while isolate 2, 4 and 6 showed smooth, flat, dense colonies on NFB solid agar medium. (Table 1)

The results are in support with the scientists Narayan *et al.*, (2018) [12] who reported that the *Azospirillum* isolates were gram negative, vibroid, rod shaped and colonies were white, smooth, flat, dense or thin. It forms white subsurface pellicle on semi solid NFB medium. Vijayalakshmi and Mahadeva, (2019) [19] noticed that the colonies of *Azospirillum* were white, dense, pale, shiny on NFB solid medium and forms white subsurface pellicle in NFB semi solid medium.

Table 1: Morphological characterization of Azospirillum isolates

Sr. No	*Azospirillum* Isolates	Morphology of cell	Colonies on semi solid NFB medium	Colony on solid NFB medium	Stain colour	Gram reaction	Colour of colony	KOH test
1	Isolate 1	Rod	White sub- surface pellicle	Smoot, raised, dense	Pink	- Ve	White	+ Ve
2	Isolate 2	Rod	White sub- surface pellicle	Smoot, flat, dense	Pink	- Ve	White	+ Ve
3	Isolate 3	Rod	White sub- surface pellicle	Smooth, raised, dense	Pink	- Ve	White	+ Ve
4	Isolate 4	Rod	White sub-surface pellicle	Smooth, flat, dense	Pink	- Ve	White	+ Ve
5	Isolate 5	Vibroid	White sub- surface pellicle	Smoot, raised, dense	Pink	- Ve	White	+ Ve
6	Isolate 6	Vibroid	White sub- surface pellicle	Smoot, flat, dense	Pink	- Ve	White	+ Ve
7	Isolate 7	Rod	White sub- surface pellicle	Smooth, raised, Dense	Pink	- Ve	White	+ Ve

Biochemical characterization of *Azospirillum* isolates

Various biochemical tests of *Azospirillum* isolates were conducted. From Table 2, the results revealed that all isolates of *Azospirillum* were positive for catalase test. All isolates of *Azospirillum* except Isolate 6were positive for methyl red test. *Azospirillum* Isolates 1, 2, 4, 5 and 7were positive for starch hydrolysis test however, isolates *Azospirillum* Isolates 3& 6were negative for starch hydrolysis test. All the isolates except the isolate 7were positive for gelatine hydrolyse test. *Azospirillum* Isolates 1, 2, 3, 5 and 7were positive for nitrate reductase test however, isolates 4and 6were negative for nitrate reductase test. All isolates except the Isolate 6 were positive for indol test. Table 2

The results of present studies are in accordance with the researchers Ilyas *et al.*, (2012) [7], found that all isolated strains of *Azospirillum* showed positive results for catalase test and gelatin hydrolysis test. Usha and Kanimozhi, (2011) [17], had identified ten strains of *Azospirillum* based on their biochemical characteristics, out of ten isolates, eight isolates showed starch hydrolase positive test, nine isolates showed indol positive test, nine isolates showed methyl red positive test while, eight isolates showed positive results for catalase test. Vijayalakshmiand Mahadeva Swamy, (2019) [19] also isolated forty *Azospirillum* strains out of these, all strains showed positive results for nitrate reductase test.

Table 2: Biochemical characterization of *Azospirillum* isolates

Sr. no.	*Azospirillum* Isolates	Methyl red test	Catalase test	Starch hydrolase test	Gelatine hydrolase test	Nitrate reductase Test	Indol test
1	Isolate 1	+	+	+	+	+	+
2	Isolate 2	+	+	+	+	+	+
3	Isolate 3	+	+	-	+	+	+
4	Isolate 4	+	+	+	+	-	+
5	Isolate 5	+	+	+	+	+	+
6	Isolate 6	-	+	-	+	-	-
7	Isolate 7	+	+	+	-	+	+

Legends: (+) Positive test, (-) Negative test

Morphological characteristics of Phosphate solubilizing bacterial isolates

Based on the formation of halo zones around the colonies and morphological characteristics *viz.* colony shape, colony colour, surface, Gram reaction etc. of all isolates were examined on PVK medium and identified as PSB.

From the results it is observed that most of PSB isolates were gram negative and rod shaped. Results are tabulated Table 4. PSB isolates 1, 4 & 8 had irregular colony shape whereas PSB isolates 2, 3, 5, 6 and 7 had circular shape. All PSB isolates had smooth surface and white colony colour. (Table 4)

Table 4: Morphological characterization of Phosphate solubilizing bacterial Isolates

Sr. No.	PS Bisolates	Colony shape	Colony colour	Gram reaction	Stain colour	Surface
1	Isolate 1	Irregular	White	- Ve	Pink	Smooth
2	Isolate 2	Circular	White	- Ve	Pink	Smooth
3	Isolate 3	Circular	White	- Ve	Pink	Smooth
4	Isolate 4	Irregular	White	- Ve	Pink	Smooth
5	Isolate 5	Circular	White	- Ve	Pink	Smooth
6	Isolate 6	Circular	White	- Ve	Pink	Smooth
7	Isolate 7	Circular	White	- Ve	Pink	Smooth
8	Isolate 8	Irregular	White	- Ve	Pink	Smooth

The results of the present investigation are in confirmation with the findings of scientists, Uddin *et al*. (2016) [16] studied the morphological characteristics of PSB isolates i.e. colony morphology, colony shape, gram reaction etc.

Morphologically the colonies of isolates were found irregular, circular, round shaped, small to large size, slightly raised elevation, smooth surface, viscous consistency and whitish in colour on Picovskaya's medium. Mustamu *et al*. (2021) [11] found the circular, irregular, flat elevated, smooth edged, white and yellow coloured colonies of PSB isolates obtained from biogas sludge.

Biochemical Characteristics of Phosphate solubilizing bacterial Isolates

All the PSB isolates were biochemically tested. The isolate 1, 4, 5, 7 and 8 were positive for methyl red test however, isolate 2, 3 and 6were negative for methyl red test. All isolates except Isolate 6and 8were positive for catalase test. Isolates 1, 2, 4, 5and 7were positive for starch hydrolysis test however, isolate 3, 6and 8were negative for starch hydrolysis test. Isolates 2, 3, 5, 7and 8were positive for gelatine hydrolyse test however, Isolates 1, Isolate 4and 6were negative for gelatine hydrolyse test. Isolates 1, 3, 4, 6, 7and 8were positive for nitrate reductase test while Isolates 2and 5were negative for nitrate reductase test. Isolates Isolate 1, 3, and 5 were positive for indol test while Isolates 2, 4, 6, 7and 8 were negative for indol test. Results are tabulated in Table 5

Table 5: Biochemical Characteristics of Phosphate solubilizing bacterial Isolates

Sr. No.	Isolates of PSB	Methyl red test	Catalase test		Starch hydrolase test	Gelatine hydrolase test	Nitrate reductase test	Indol test
1	Isolate 1	+		+	+	-	+	+
2	Isolate 2	-		+	+	+	-	-
3	Isolate 3	-		+	-	+	+	+
4	Isolate 4	+		+	+	-	+	-
5	Isolate 5	+		+	+	+	-	+
6	Isolate 6	-		-	-	-	+	-
7	Isolate 7	+		+	+	+	+	-
8	Isolate 8	+	-		-	+	+	-

Legends: (+) Positive test, (-) Negative test

These results are in accordance with Bashir *et al.*, (2019) [3] Seven PSB isolates were biochemically characterized, out of 7 Isolates, 5 Isolates were positive for catalase test, 5 isolates were positive for methyl red test, 4 isolates were positive for starch hydrolysis test, 3 isolates were positive for gelatine hydrolysis test. Damor and Goswami (2016) [4], isolated four PSB isolates, all were positive for starch hydrolysis test, 3 isolates were positive for catalase test, 1 isolate were positive for nitrate reductase test.

Screening of Phosphate solubilizing bacteria

The PSB isolates were observed for formation of clear halo zones around the colonies on the Pikovskaya's medium supplemented with tricalcium phosphate. The solubilizing index (SI) was calculated from the diameter of halo zone and diameter of colony. The PSB Isolate 7showed maximum zone of solubilization with solubilization index of 4.14 followed by isolate 6 (3.85). The Isolate 3(1.76) showed lowest solubilizing index as shown in Table 6.

Table 6: Phosphate Solubilizing index (SI) showed by the Phosphate solubilizing bacteria

Sr. No.	PS Isolates	SI index of P solubilization
1	Isolate 1	3.25
2	Isolate 2	2.64
3	Isolate 3	1.76
4	Isolate 4	3.56
5	Isolate 5	2.40
6	Isolate 6	3.85
7	Isolate 7	4.14
8	Isolate 8	1.96

Similar finding were reported by Animesh, (2012) [2], investigated that the isolates showed varying levels of halo zones on Pikovskaya's agar medium in plate assay. The strain OS07 produced the biggest clear zone (PSI = 4.7 mm) in agar medium while the strain OS08 produced smallest clear zone (PSI = 1.1 mm) in agar medium. Alam *et al.*, (2002) [1], and Murkhi *et al.*, (2014) [10], who observed the solubilizing index of the isolates from rice rhizosphere ranged from 1.16-1.18. Nagalakshmi and Karpagam, (2014) [12] observed the zone of solubilization index of eight PSB isolates ranged from 1.13-3.0.

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