Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2019; 8(1): 114-118
Received: 24-11-2018
Accepted: 26-12-2018

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Morphological and biochemical characterization of *Azospirillum* isolates from rhizoplane of foxtail millet [*Setaria italica* (L.) Beauv.]

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Abstract
Azospirillum represents the best characterized genus of plant growth promoting rhizobacteria. Forty different strains of *Azospirillum* spp. were isolated from the different rhizoplane samples of foxtail millet grown in forty different locations in Raichur and Koppal districts of Northern Karnataka and were studied for their morphological and biochemical characters. Out of 40 isolates, 17 isolates were positive for biotin requirement, glucose utilization and acid production tests. 21 isolates were negative for denitrification test and all forty isolates were positive for nitrate reduction test. Out of forty isolates, twenty three isolates were identified as *A. brasilense* and remaining seventeen as *A. lipoferum*.

Keywords: *Azospirillum*, Foxtail millet, NFBTB

Introduction
Indiscriminate use of chemical fertilizers adversely affects soil microorganisms, fertility status of soil and environment. So, PGPRs are replacing agrochemicals for plant growth promotion economically, environmentally beneficial for lower production cost and for sustainable agriculture. Although nitrogen is available in abundance in gaseous form in the atmosphere unless it is reduced to ammonia it is not available to the plants. This process is popularly known as nitrogen fixation and is carried out by the prokaryotic microorganisms like *Azospirillum* which are capable of fixing atmospheric nitrogen. Experiments on sorghum, pearl millet and rice conducted at different places in India have indicated the positive response of the cultivars to *Azospirillum* inoculation (Subba Rao, 1981 and Rao *et al.*, 1983) [15, 13]. *Azospirillum* live in close association with plants in the rhizosphere. The plant stimulatory effect exerted by *Azospirillum* has been attributed to several mechanisms, including biological nitrogen fixation and production of plant growth promoting substances (Okon and Itzigsohn, 1995; Salomone *et al.*, 1996) [14]. Upon *Azospirillum* inoculation change in root morphology was observed, which has been described to the bacterial production of plant growth regulating substances (Umalia-Garcia *et al.*, 1980; Tien *et al*., 1979) [18, 17]. An increased number of lateral roots and root hairs enlarge the root surface available for nutrients. These results in higher nutrient uptake by inoculated root sand an improved water status of the plant, which in turn could be the main factor for enhancing plant growth (Fallik and Okon, 1996) [6]. Associative nitrogen fixing microorganisms *(Azospirillum*) developed elsewhere have not been very consistent in their performance everywhere, due to their poor adaptability to the changing soil and agro-climatic conditions. Thus there is a need to study the associative nitrogen fixing microorganism *Azospirillum* and develop region specific *Azospirillum* strains for the foxtail millet crop.

Material and methods
Isolation of *Azospirillum* strains from foxtail millet root samples
A total of 40 rhizosplane and foxtail millet root bit samples were collected from the foxtail millet fields located in the Raichur and Koppal districts of Northern Karnataka for isolation of *Azospirillum* strains. Isolation was done by enrichment culture technique as adopted by (Dobereiner and Day, 1976) [5] and (Baldani and Dobereiner, 1980) [3]. The fresh roots of foxtail millet were collected from the farmer's fields at different locations of Raichur and Koppal districts. The plants were uprooted carefully with root system intact and brought to the laboratory in sterile propylene bags. Roots were thoroughly washed in running tap water, cut into small bits of 1 cm length and surface sterilized by dipping in 0.1 per cent $HgCl_2$ solution for three minutes followed by dipping in 70 per cent alcohol for one minute.

The roots were finally washed in six to eight changes of sterile distilled water. The root bits were aseptically placed in tubes containing sterilized semisolid N free malate medium (Baldani and Dobereiner, 1980) [3]. The tubes were incubated at 30 ^{0}C for a period of one week and observed for growth of *Azospirillum* as subsurface white undulating pellicles. The repeated sub culturing was done to confirm the *Azospirillum* isolates.

Characterization of *Azospirillum*

Morphological characters

The *Azospirillum* will form characteristic colonies like typical small white dense on nitrogen-free malate agar medium and also were tested for gram reaction as per the standard procedures given by Cappuccino and Sherman, 1992 [4].

Microscopic observation of *Azospirillum* isolates

The *Azospirillum* strains were studied for cell morphology, Gram reaction. The Gram staining was done using 24 hours old culture by the modified procedure of Hucker's (Rangaswami and Bagyaraj, 1996) [12]. The observations of Gram reactions and cell morphology were recorded.

Simple staining

The cell shape was observed by simple staining. A smear of each isolates were made, air dry and stained with crystal violet for 30 sec. Stained smear was then washed, air dried and observed under oil immersion compound microscope (Akhter *et al*., 2012) [1].

Gram staining

Smear of *Azospirillum* was prepared on a clean glass slide and treated with crystal violet solution for 1 min, the smear was gently rinsed off and iodine solution was applied for 1 min. This in turn was drained off and the smear will be decolorized using, Ethyl alcohol (95 %), safranin was used as a counter stain for 10 sec. Then slide was gently rinsed off with water and blotted off. The result was recorded as Gram positive and Gram negative (Akhter *et al*., 2012) [1].

Motility test

For determining the motility of the *Azospirillum*, motility medium was used. The tubes containing the medium supplemented with 2, 3, 5-Triphenyltetrazolium chloride were inoculated by stabbing with straight wire and incubated at 37 ^{0}C for 48 h. After incubation, the tubes were observed for the growth of *Azospirillum* (Akhter *et al*., 2012) [1].

Biochemical characterization of *Azospirillum* isolates

For the identification of *Azospirillum* isolates, biochemical tests *viz.* utilization of glucose, biotin requirement, acid production in glucose peptone broth and denitrification test were carried out.

Utilization of glucose

The utilization of glucose was tested by using 5ml of semi-solid nitrogen free glucose broth into test tubes and autoclaved. The medium in the test tubes was inoculated with 48 hrs old culture and test tubes were incubated at 37^{0}C. The test tubes were observed for the appearance of turbidity which indicated utilization of glucose.

Biotin requirement test

Azospirillum culture grown in nutrient broth (for 48 hrs) was centrifuged, washed twice with sterile phosphate buffer and re-suspended in water to a uniform density. A quantity of 0.1 ml of this suspension was used to incubate 5.0 ml volume of N free medium supplemented with biotin (0.0001g per liter) and inoculated for 48 hours at 37 ^{0}C. In case where slight growth occurred in medium without biotin, a second serial transfer was made to fresh medium without biotin for confirmation of the biotin requirement (Baladni and Dobereiner, 1980) [3].

Acid production in glucose peptone broth

Glucose peptone broth with Bromo Thymol Blue (5 ml) was taken into test tubes and autoclaved. Then overnight cultures of *Azospirillum* isolates were inoculated. The tubes were incubated at 37°C for three days. The change in color of BTB from green to yellow was recorded.

Test for denitrification

The denitrification test was carried out as described by Neyra *et al.* (1977) [9]. In the NFBTB medium NH_4NO_3 was added (5mM) and 5ml of the medium in the test tubes were inoculated with each isolates and incubated at 37^{0}C for 60 hours. Nitrite was determined on 0.1 ml aliquots from the culture by adding 2.0 ml of a 1:1 (v/v) solution of 0.02 % (1-naphthyl) ethylene diamine hydrochloride and 1.0% sulfanilamide in 1.5 m HCl. Final volume make up of 4.0 ml was done and tube were incubated for 15 minutes. The colour development of a purple red indicated the presence of nitrite. Accumulation of NO_2 was evidence of NO_3 reduction, while disappearance of NO_2 from the medium was evidence for NO_2 reduction.

Nitrate reduction

Azospirillum isolates were inoculated in to the pre-sterilized nitrate broth and kept it for incubation at 37 ^{0}C for 48 h. In a porcelain dish, three drops of nitrate test reagent and one drop of sulphuric acid was mixed with one drop of culture. Formation of blue color was taken as positive result for the test.

Results and Discussion

Isolation and identification of *Azospirillum*

Isolation of *Azospirillum* was made from rhizoplane of foxtail millet root samples obtained from Raichur and Koppal districts. Forty *Azospirillum* isolates were obtained by adopting enrichment culture technique.

Rajyalakshmi *et al*. (2007) [8] isolated *Azospirillum* from rhizosphere of 30, 45, 60 and 75 days old foxtail millet. Attitalla *et al*., (2010) [2] Occurrence and microbiological characteristics of *Azospirillum* strains associated with leguminous and non-leguminous plants in Al jabal Al Akhdar eco-Region, Libya).

Morphological characteristics

All the isolates were studied for their morphological characteristics and results are presented in Table 1. The colony morphology of isolates on N-free malate medium was small to medium, pale white dense, spindle and transparent pale shiny white in colour. All the isolates formed subsurface pellicles in NFBTB medium and turned olive green colour of Bromo Thymal Blue (BTB) to brilliant blue including the reference strain. The cell shape of all the isolates was spiral; all the isolates were Gram negative and had cork screw movement when observed under microscope. These results are similar to reference strain and to the genus *Azospirillum* as

described by Tarrand *et al.* (1978) [16] and Krieg and Dobereiner (1984) [7].

Formation of pellicle in NFBTB indicates the isolates seem to be *Azospirillum* spp. (Okon *et al.* 1977) [11]. The pellicle formation may be considered as one of the criteria for identification because other genera like *Bacillus* and *Herbaspirillum* are also known to form pellicle (Krieg and Dobereiner 1984) [7].

The morphological characteristics of the isolates in the present study were appeared similar to the description of *Azospirillum* spp. given by Krieg and Dobereiner (1984) [7] and Tarrand *et al.* (1978) [16].

Biochemical characteristics

All the isolates were tested for biochemical characteristics. The data pertaining to the biochemical characters are presented in Table 2.

Biotin requirement

Biotin free medium favored the growth of *Azospirillum brasilense, Azospirillum lipoferum* required biotin for growth. Out of 40 isolates, 17 isolates utilized the biotin, whereas 23 isolates did not utilized the Biotin.

Glucose utilization

Azospirillum brasilense, Azospirillum lipoferum were characterized by its in ability to utilize glucose as a carbon source for its growth and nitrogen fixation. Out of 40, seventeen isolates utilized glucose in the medium, whereas remaining twenty-three failed to utilize glucose in the medium.

Production of acid from glucose peptone broth

Among the 40 isolates, 17 produced acid in glucose peptone broth and remaining 23 isolates failed to produce acid.

Denitrification test

Out of 40 isolates of *Azospirillum* tested for denitrification test, twenty-one isolates were found to be negative whereas, nineteen isolates showed positive for the test. For further studies, only the isolates which showed negative for denitrification test were retained.

Nitrate reduction test

All the 40 isolates were tested for nitrate reduction and all 40 isolates showed positive result.

Characterization of isolates was done according to Bergey's manual of systematic Bacteriology. Accordingly, out of 40 isolates, 17 were tentatively identified as *A. lipoferum* and remaining 23 as *A. brasilense.* These characterizations of species of *Azospirillum* were in similarity with the description of Tarrand *et al.* (1978) [16].

Table 1: Morphological characteristics of native *Azospirillum* strains isolated from roots of foxtail millet.

S. No.	Isolate code	Colony morphology on N-free malate medium	Pellicle formation	Cell shape	Motility	Gram reaction
1	MARV-1	White dense small	Present	Spiral	Cork screw	-ve
2	MARV-2	Pale white shiny and small	Present	Spiral	Cork screw	-ve
3	MARV-3	Pale shiny white medium	Present	Spiral	Cork screw	-ve
4	MARV-4	White dense medium	Present	Spiral	Cork screw	-ve
5	MARV-5	Spindle and pale green colour	Present	Spiral	Cork screw	-ve
6	MARV-6	White dense and small	Present	Spiral	Cork screw	-ve
7	MARV-7	Spindle and transparent	Present	Spiral	Cork screw	-ve
8	MARV-8	Pale shiny white medium	Present	Spiral	Cork screw	-ve
9	MARV-9	Medium wrinkled	Present	Spiral	Cork screw	-ve
10	MARV-10	White dense small	Present	Spiral	Cork screw	-ve
11	MARV-11	Spindle transparent	Present	Spiral	Cork screw	-ve
12	MARV-12	Spindle transparent	Present	Spiral	Cork screw	-ve
13	MARV-13	White dense and medium	Present	Spiral	Cork screw	-ve
14	MARV-14	Pale shiny white medium	Present	Spiral	Cork screw	-ve
15	MARV-15	Medium and wrinkled	Present	Spiral	Cork screw	-ve
16	MARV-16	White dense and medium	Present	Spiral	Cork screw	-ve
17	MARV-17	Flat white colonies elevated borders	Present	Spiral	Cork screw	-ve
18	MARV-18	Flat white colonies elevated borders	Present	Spiral	Cork screw	-ve
19	MARV-19	Spindle and transparent	Present	Spiral	Cork screw	-ve
20	MARV-20	White dense medium	Present	Spiral	Cork screw	-ve
21	MARV-21	Pale shiny white medium	Present	Spiral	Cork screw	-ve

Table 1: Contd…..

S. No.	Isolate code	Colony morphology on N-free malate medium	Pellicle formation	Cell shape	Motility	Gram reaction
22	MARV-22	White dense and medium	Present	Spiral	Cork screw	-ve
23	MARV-23	Spindle and transparent	Present	Spiral	Cork screw	-ve
24	MARV-24	White dense small	Present	Spiral	Cork screw	-ve
25	MARV-25	Pale shiny white medium	Present	Spiral	Cork screw	-ve
26	MARV-26	Medium and wrinkled	Present	Spiral	Cork screw	-ve
27	MARV-27	White and dense small	Present	Spiral	Cork screw	-ve
28	MARV-28	Pale white shiny and small	Present	Spiral	Cork screw	-ve
29	MARV-29	White dense medium	Present	Spiral	Cork screw	-ve
30	MARV-30	Medium and wrinkled	Present	Spiral	Cork screw	-ve
31	MARV-31	White dense small	Present	Spiral	Cork screw	-ve
32	MARV-32	Pale shiny white medium	Present	Spiral	Cork screw	-ve
33	MARV-33	Pale shiny white medium	Present	Spiral	Cork screw	-ve

34	MARV-34	Medium and wrinkled	Present	Spiral	Cork screw	-ve
35	MARV-35	Medium and wrinkled	Present	Spiral	Cork screw	-ve
36	MARV-36	Medium and wrinkled	Present	Spiral	Cork screw	-ve
37	MARV-37	Pale shiny white medium	Present	Spiral	Cork screw	-ve
38	MARV-38	White dense small	Present	Spiral	Cork screw	-ve
39	MARV-39	Spindle and transparent	Present	Spiral	Cork screw	-ve
40	MARV-40	White dense medium	Present	Spiral	Cork screw	-ve
41	Ref strain	Pale shiny white medium	Present	Spiral	Cork screw	-ve

Table 2: Biochemical characteristics of native *Azospirillum* isolate from rhizoplane of foxtail millet

S. No.	Isolate code	Biotin requirement	Glucose utilization	Acid production	Denitrification test	Nitrate reduction test	Tentative isolate
1	MARV-1	-	-	-	+	+	*Azospirillum brasilense*
2	MARV-2	+	++	++	-	+	*Azospirillum lipoferum*
3	MARV-3	-	-	-	+	+	*Azospirillum brasilense*
4	MARV-4	-	-	-	-	+	*Azospirillum brasilense*
5	MARV-5	+	+++	+++	+	+	*Azospirillum lipoferum*
6	MARV-6	-	-	-	-	+	*Azospirillum brasilense*
7	MARV-7	-	-	-	+	+	*Azospirillum brasilense*
8	MARV-8	+	+	+	-	+	*Azospirillum lipoferum*
9	MARV-9	-	-	-	-	+	*Azospirillum brasilense*
10	MARV-10	-	-	-	+	+	*Azospirillum brasilense*
11	MARV-11	-	-	-	-	+	*Azospirillum brasilense*
12	MARV-12	-	-	-	+	+	*Azospirillum brasilense*
13	MARV-13	+	+++	++	-	+	*Azospirillum lipoferum*
14	MARV-14	-	-	-	+	+	*Azospirillum brasilense*
15	MARV-15	+	++	++	+	+	*Azospirillum lipoferum*
16	MARV-16	-	-	-	-	+	*Azospirillum brasilense*
17	MARV-17	-	-	-	-	+	*Azospirillum brasilense*
18	MARV-18	+	+++	+	-	+	*Azospirillum lipoferum*
19	MARV-19	+	+++	++	+	+	*Azospirillum lipoferum*
20	MARV-20	-	-	-	-	+	*Azospirillum brasilense*
21	MARV-21	+	++	++	+	+	*Azospirillum lipoferum*

Table 2: Contd…..

S. No.	Isolate code	Biotin requirement	Glucose utilization	Acid production	Denitrification test	Nitrate reduction test	Tentative isolate
22	MARV-22	-	-	-	+	+	*Azospirillum brasilense*
23	MARV-23	-	-	-	-	+	*Azospirillum brasilense*
24	MARV-24	+	+	+	+	+	*Azospirillum lipoferum*
25	MARV-25	+	+++	+	-	+	*Azospirillum lipoferum*
26	MARV-26	-	-	-	-	+	*Azospirillum brasilense*
27	MARV-27	+	++	+++	-	+	*Azospirillum lipoferum*
28	MARV-28	-	-	-	+	+	*Azospirillum brasilense*
29	MARV-29	+	++	+	-	+	*Azospirillum lipoferum*
30	MARV-30	-	-	-	-	+	*Azospirillum brasilense*
31	MARV-31	-	-	-	+	+	*Azospirillum brasilense*
32	MARV-32	+	+++	++	+	+	*Azospirillum lipoferum*
33	MARV-33	+	++	+	-	+	*Azospirillum lipoferum*
34	MARV-34	-	-	-	-	+	*Azospirillum brasilense*
35	MARV-35	-	-	-	+	+	*Azospirillum brasilense*
36	MARV-36	+	+++	+	+	+	*Azospirillum lipoferum*
37	MARV-37	-	-	-	+	+	*Azospirillum brasilense*
38	MARV-38	+	+	+++	-	+	*Azospirillum lipoferum*
39	MARV-39	-	-	-	-	+	*Azospirillum brasilense*
40	MARV-40	+	+	++	+	+	*Azospirillum lipoferum*
41	Ref strain	+	++	++	-	+	*Azospirillum lipoferum*

–ve : Gram negative, **+++ : ** Good growth, **++ : ** Moderate growth, **+ : ** Less growth, **- :** No growth

Conclusion

Nitrogen is available in abundance in gaseous form in the atmosphere but it is unavailable to plants unless it is reduced to ammonia. The process of reduction of atmospheric nitrogen to ammonia is carried out by prokaryotic microorganisms. *Azospirillum* has potential use as biofertilizers in agriculture. Totally 40 samples were used for enumeration of *Azospirillum*. Based on morphological and biochemical characteristics, 40 *Azospirillum* isolates were obtained. Out of 40 isolates of *Azospirillum*, 17 were tentatively identified as *A. lipoferum* and remaining 23 as *A. brasilense.* In the present investigation 21 isolates were found to be negative for denitrification.

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