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Research Article

In Vitro Screening and Production of Plant Growth Promoting Substances by *Azospirillum* Isolates from Rhizoplane of Foxtail Millet [*Setaria italica* (L.) Beauv]

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ABSTRACT

Isolation and identification of Azopirillum spp. with enhanced potential to promote plant growth among the natural bacterial population associated with rhizosphere soil and roots of Foxtail millet collected from forty different locations in Raichur and Koppal districts of Northern Karnataka was carried out. Further in order to select the most efficient isolates as candidates for plant growth promotion forty Azospirillum isolates were screened for nitrogen fixation and other PGPR activity. Out of forty isolates, twenty three isolates were identified as A. brasilense and remaining seventeen as A. lipoferum as well as screened for their potential nitrogen fixation, IAA and siderophore production under sterile conditions. Among 40 isolates obtained 21 were able to fix nitrogen. Nitrogen fixation ranged from 21.41 mg (MARV-18) to 2.09 mg of N per g of malate used. Twenty one isolates produced indole-3-acetic acid (IAA)ranged from 1.14 to 18.44 µg (MARV-18) per 100 ml. Six efficient Azospirillum strains produced siderophore that ranged from 47.33 to 72.67 (MARV-18) per cent. These bacterial isolates revealed potential to increase crop productivity, pot crop experiments in climatic conditions of UAS, college of agriculture was done in order to formulate recommendations for their use as inoculants.

Key words: *Azospirillum, Foxtail millet, IAA, Nitrogen fixation, Siderophore.*

INTRODUCTION

In recent years, agricultural systems have changed to improve environmental quality and avoid environmental degradation. One of the most promising techniques to avoid environmental degradation is the use of bio-inoculants composed of diazotrophic bacteria as an alternative to use of nitrogen fertilizers[18,10]. It has been demonstrated that diazotrophic bacteria are not only able to fix atmospheric nitrogen but also to produce metabolites having agricultural interest, including phytohormones like indole-3-acetic acid (IAA) which is one of the most well-known and studied auxins, playing a governing role in plant growth[2].

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The ability to synthesize IAA is widespread among soil and plant-associated bacteria and it has been estimated that 80% of the rhizospheric bacteria have the ability to synthesize plant growth regulators. Pramod *et al*.[12] studied mechanisms of *Azospirillum* in plant growth promotion.

Isolation of microorganisms, screening for desirable characters and selection of efficient strains are important steps to optimize high crop yields and improve the sustainability of the ecosystem. Among plant-associated bacteria, *Azospirillum* spp. has been found as a promising bacterium with respect to its ability to produce plant hormones and fix nitrogen in association with more than 100 species of cereals and non cereals[2]. *Azospirilla* are Gram-negative free-living nitrogen-fixing rhizosphere bacteria. They display a versatile C- and N-metabolism, which makes them well adapted to establish in the competitive environment of the rhizosphere[14]. Such features confer on *Azospirillum* the capability to be not only a cereal growth promoter but also a general plant growth promoting bacterium. Apparently, inoculation of *Azospirillum* spp. reduced the use of chemical fertilizers, especially nitrogen by 20–50%, and provided superior results when organic fertilizers were incorporated[2]. Furthermore, they colonize niches that may have reduced oxygen tension, which is necessary for nitrogenase expression[7,3]. The objective of this study was to isolate and identify *Azopirillum* spp. with enhanced potential to promote plant growth among the natural bacterial population associated with rhizosphere soil and roots of Foxtail millet collected from forty different locations in Raichur and Koppal districts of Northern Karnataka.

MATERIAL AND METHODS

Investigations were carried out at the Department of Agricultural Microbiology, University of Agricultural sciences, Raichur for the isolation, characterization and screening of *Azospirillum* isolates from foxtail millet rhizoplane and their inoculation effect on growth and yield of foxtail millet.

Isolation of *Azospirillum* strains from foxtail millet root samples and their biochemical characterization.

The *Azospirillum* strains from foxtail millet samples were isolated by following the enrichment culture technique as adopted by[4] and[1]. For the identification of *Azospirillum* isolates, biochemical tests *viz.* utilization of glucose, biotin requirement, and acid production in glucose peptone broth and denitrification test were carried out. Out of 40 isolates, 17 isolates were tentatively identified as *Azospirillum lipoferum* and 23 were identified as *Azospirillum brasilense*. Out of 40 isolates, 17 isolates utilized the biotin, whereas 23 isolates did not utilized the Biotin, out of 40, seventeen isolates utilized glucose in the medium, 17 produced acid in glucose peptone broth, twenty-one isolates were found to be negative, these 21 isolates were used for further screening and *in vitro* tests.

***In vitro* nitrogen fixation by *Azospirillum* isolates**

The nitrogen fixation by each *Azospirillum* isolates was estimated according to the method described by Humphries[6]. The N free semi solid malate medium supplied with L-glutamic acid was used in this study. A quantity of 100 ml of the above medium was dispensed to a 250 ml capacity conical flask and autoclaved. The *Azospirillum* isolates grown for 24 hrs separately in NFB broth were used to inoculate at 2 ml/100 ml of the medium. Five ml of the homogenized culture was collected and digested with 5 ml concentration H_2SO_4 and 200 mg catalytic mixture (K_2SO_4 : $CuSO_4$, selenium) (100:10:1 ratio) till the contents become clear. After cooling, the volume was made up to 25 ml with distilled water. Then aliquot 5 ml was transferred to micro kjeldhal distill unit. An aliquot of 10 ml of 40 per cent sodium hydroxide was added and steam distilled. Ammonia evolved was collected and back titrated.

Estimation of growth promoting substances

Indole Acetic Acid (IAA)

Production of IAA by *Azospirillum* isolates was determined by the method described by Tien *et al*.[16]. N-free malate broth containing

0.005 M L-tryptophan (100 ml) was prepared. After seven days of incubation the cultures were refrigerated (4 ^{o}C), centrifuged at 6000 rpm and the supernatant obtained was used for estimation of IAA.

IAA present in the methanol extract was determined using the method explained by Gorden and Paleg[5]. From a standard curve prepared with known concentrations of IAA, the quantity of IAA in the culture filtrate was determined and expressed as µg per 100 ml of the medium.

Screening the isolates for siderophores production

In order to screen the production of siderophores, the *Azospirillum* isolates were grown in Chrome Azurol Sulfonate (CAS) agar medium. The inoculated agar plates were incubated at 37 ^{o}C for 24 h. The observation was made for the change of medium color from blue to reddish yellow to determine the siderophores production. Siderophores assay was carried out based on the CAS shuttle assay of Payne[11].

RESULTS AND DISCUSSION

***In vitro* screening of *Azospirillum* isolates for their nitrogen fixing ability**

Selected *Azospirillum* isolates were used to screen under *in vitro* conditions, for their nitrogen fixing ability, IAA and siderophore producing capacity along with reference strain.

The amount of nitrogen fixed by the isolates was expressed as mg of nitrogen fixed per gram of malate utilized. The results pertaining to nitrogen fixation are presented in Table 1. Among the 21 isolates of *Azospirillum*, significantly higher amount of nitrogen (21.41 mg of N per g of malate used) was fixed by MARV-18. The lowest amount of nitrogen was fixed by MARV-23 (2.09 mg of N per g of malate utilized).

These results are in accordance with the findings of Sawalgi *et al*.[13] who examined the *in vitro* N fixation efficiency of *Azospirillum* isolates on NFBTB and reported that nitrogen fixed ranged from 1.4 to 20.96 mg g^{-1} of malate. Kanimozhi *et al*.[8] studies on isolation and nitrogen fixation ability of *Azospirillum* spp. isolated from Thanjavur district revealed that among 30 isolates tested, only 28 isolates were able to fix nitrogen. Further among 28, only 10 isolates were able to produce the highest amount of nitrogen (from 11.0 to 15.06 mg 'N'/kg).

Production of growth promoting substances

Apart from nitrogen fixation, *Azospirillum* is also known to produce growth promoting substances *viz.*, Indole Acetic Acid (IAA) and siderophore which in turn affect the root growth and other plant growth parameters

Indole acetic acid

Twenty one *Azospirillum* isolates along with a reference strain were tested for production of indole acetic acid under *in vitro* condition. The detailed results are depicted in the Table 2. The IAA production of selected *Azospirillum* isolates ranged from 1.14 to 18.44 µg per 100 ml. Among the *Azospirillum* isolates examined, the highest production of IAA was observed with strain MARV-18, which produced 18.44 µg per 100 ml. These results are in accordance with work of Pramod *et al*.[12] who studied mechanisms of *Azospirillum* in Plant Growth Promotion**;** *Azospirillum* is one of the successful inoculants for di-nitrogen fixation and plant growth promoter in non-legume crops.

Siderophore production

Six efficient strains of *Azospirillum* along with a reference strain were tested for production of siderophore under *in vitro* condition. The detailed results are depicted in the Table 3. The siderophore production of selected *Azospirillum* isolates ranged from 47.33 to 72.67 per cent. Among the *Azospirillum* isolates examined, the highest production of Siderophore was observed with strain MARV-18, which produced 72.67 per cent. Earlier workers *viz.*, Tortora *et al*.[17] observed that the both rhizosphere and endophytic strains of *A. brasilense* reached to a maximum yield of siderophore production on the fourth and fifth day of incubation respectively, but after that, when cells reached a high cellular density, Siderophore production stopped and the yield remained constant.

Table 1: *In vitro* N_2-fixation of *Azospirillum* isolates

Sl. No.	Isolate code	N_2 fixed (mg/g of malate)
1	**MARV-2**	**18.61**
2	MARV-4	18.00
3	MARV-6	17.91
4	MARV-8	19.11
5	MARV-9	6.94
6	MARV-11	7.80
7	MARV-13	19.02
8	MARV-16	7.95
9	**MARV-17**	**20.32**
10	**MARV-18**	**21.41**
11	MARV-20	9.74
12	MARV-23	2.09
13	**MARV-25**	**18.85**
14	MARV-26	10.19
15	MARV-27	4.25
16	MARV-29	6.60
17	**MARV-30**	**17.88**
18	**MARV-33**	**18.63**
19	MARV-34	15.53
20	MARV-38	4.48
21	MARV-39	7.95
22	**Reference strain**	**18.96**
S.Em±		0.05
C.D.at 1%		0.21

Values are average of three replications

Table 2: IAA production potential of *Azospirillum* isolates under *in vitro* condition

Sl. No.	Isolate code	IAA (µg/50ml of medium)
1	**MARV-2**	**16.80**
2	MARV-4	13.65
3	MARV-6	16.80
4	MARV-8	13.06
5	MARV-9	6.20
6	MARV-11	1.80
7	MARV-13	17.58
8	MARV-16	7.58
9	**MARV-17**	**17.84**
10	**MARV-18**	**18.44**
11	MARV-20	5.55
12	MARV-23	2.92
13	**MARV-25**	**17.00**
14	MARV-26	2.98
15	MARV-27	1.14
16	MARV-29	9.35
17	**MARV-30**	**15.53**
18	**MARV-33**	**16.94**
19	MARV-34	14.81
20	MARV-38	13.65
21	MARV-39	13.06
22	**Reference strain**	**17.40**
S.Em±		0.06
C.D.at 1%		0.24

Values are average of three replications

Table 3: Siderophore production of *Azospirillum* isolates under *in vitro* condition

Sl. No.	Isolate code	Siderophore production (%)
1	MARV-2	53.79
2	MARV-17	69.80
3	MARV-18	72.67
4	MARV-25	58.47
5	MARV-30	47.33
6	MARV-33	57.07
7	Reference strain	65.86
	S.Em±	0.05
	C.D.at 1%	0.24

Values are average of three replication

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