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Isolation, Characterization and Screening of *Azospirillum* from Tuberose Rhizoplane in Raichur

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A B S T R A C T

An experiment was carried out to isolate, characterize and screening of *Azospirillum* from tuberose rhizoplane. 40 bacterial strains were isolated showing characteristics of *Azospirillum*. Isolates were characterized based on utilization of glucose, biotin requirement, acid production in glucose peptone broth and denitrification test. Out of 40 isolates, 20 isolates required biotin for their growth and remaining 20 showed growth without biotin. 16 isolates showed positive for denitrification and they were tentatively identified as *Azospirillum brasilense*. All isolates produced acid and utilized glucose as carbon source. Isolates showed negative for denitrification test were screened through *In vitro* analysis.

Introduction

Azospirillum, a ubiquitous rhizosphere bacterium, representing the main group of microaerophilic free living/associative nitrogen fixing bacteria (Dobereiner and Day, 1976). They are isolated from the rhizosphere of many grasses and cereals all over world and their roles on plant growth and yield have been well established (Wani, 1990; James, 2000). Two species *viz.*, *Azospirillum brasilense* and *Azosprillium lipoferum* have been found in soil of a temperate zone (Coninck *et al.*, 1988) and even in the cold climate of Finland (Haathella *et al.*, 1983). According to Reynders and Vlassak (1979) *Azospirillum* occurs in about 90% of tropical soil and in about 60% of soils in the temperate zone. *A. brasilense* is attributed to have affinity with plants with photosynthesis type C3 (wheat and chilli), whereas *A. lipoferum* with plants of C4 type (Maize and Sorghum). N_2 fixation may be one of the minor mechanisms involved in plant growth promotion by *Azospirillum* (Michiels *et al.*, 1989; Bashan and Levanony, 1990). The success of *Azospirillum* inoculants in promoting plant growth will largely depend on its movement towards the host plant both in black soil and red soil in the rhizosphere (Bashan *et al.*, 1987 and Bashan and Holguin, 1994). The stimulatory effect exerted by

Azospirillum has been attributed to several mechanisms including secretion of phytohormones, biological nitrogen fixation, and enhancement of mineral uptake by plants (Okon and Itzigsohn, 1995; James, 2000).

Materials and Methods

Isolation of *Azospirillum*

The standard isolation procedure, as reported by Dobereiner and Day (1976), was followed for isolation of *Azospirillum* from tuberose root samples. Fresh root samples were cut into bits of 0.5cm length and then were washed thoroughly in running tap water and surface sterilized by dipping in 0.1% $HgCl_2$ 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 distilled water. The root bits were then placed at subsurface level in screw cap tubes containing sterilized Nitrogen-free semi solid malate medium (Okon *et al.,* 1977) under aseptic conditions.

The tubes were incubated at 37^{o}C for a period of 4-5 days and observed for growth of *Azospirillum* as subsurface white undulating pellicles. The isolates were purified by repeated sub culturing. A loopful of culture was streaked on malate agar plates containing 1 per cent NH_4Cl. After a week of incubation, typical small, white dense single colonies were picked and transferred to semisolid N-free malate medium in culture tubes. The isolates that formed characteristic subsurface white undulating pellicle in this medium were tentatively considered as *Azospirillum*.

Biochemical characterization

The biochemical tests *viz.,* utilization of glucose, biotin requirement, acid production in glucose peptone broth and denitrification tests were carried out for identification of the *Azospirillum* isolates. The isolates of *Azospirillum* were grown in nitrogen free malate medium (nfb) for 48 hrs at 28°C (±2) over an environmental shaker (100 rpm).

Utilization of glucose

Five ml of semi-solid nitrogen free glucose broth was dispensed into test tubes and autoclaved at 15lbs for 20 minutes. These test tubes were inoculated with 0.1ml of the standard inoculum of *Azospirillum* isolates. The tubes were incubated at 28°C (±2) for 3 days. Observations were recorded for the appearance of turbidity in the test tubes as indicated by the utilization of glucose.

Biotin requirement test

Two sets of test tubes containing 10ml of nitrogen free semi-solid medium were prepared, one with biotin (100mg/L) and another without biotin. The test tubes were sterilized at 121°C for 30 minutes and cooled to room temperature. These test tubes were inoculated with 0.1ml of the standard inoculum of *Azospirillum* isolates. The tubes were incubated at 28°C (±2) for 3 days. After three days of incubation, growth of *Azospirillum* isolates was observed in the tubes. In case where growth occurred in the medium without biotin, a second transfer was made to fresh medium without biotin and biotin requirement was confirmed.

Acid production in glucose peptone broth

Five ml of glucose peptone broth with Bromothymol Blue (BTB) was dispensed into test tubes and autoclaved at 121°C (15lbs) for 20 minutes. Overnight cultures of *Azospirillum* isolates were inoculated (0.1 ml) into the test tubes. The tubes were incubated at 37°C for three days. Colour change in BTB from green to yellow indicated acid production.

Denitrification test

Nitrogen free solid malate medium supplemented with 5M ammonium nitrate was prepared. The medium was dispensed in 5ml quantities in test tubes and sterilized at 121^{o}C (15lbs) for 30 minutes. The test tubes containing the medium was stab inoculated with the stock culture of *Azospirillum* isolates. The tubes were incubated at 28°C (±2) for three days. The tubes were observed for shredded agar block. Shredded agar block indicated that *Azospirillum* isolates were positive for denitrification test.

Results and Discussion

A total of forty isolates have been isolated from root bits of Tuberose grown in herbal garden, AC Raichur and tentatively confirmed as *Azospirillum* based on cell morphology i.e., spiral and twisted shape, characteristic rotating corkscrew type of motility, white dense colony morphology and formation of white pellicle in the subsurface of nitrogen free semisolid malate medium. Based on biotin requirement, of the total forty isolates characterized 20 isolates were found to be *A. brasilense* while remaining 20 isolates were unidentified. Total forty isolates characterized were coded serially from ATR-1 to ATR-40. Almost all isolates produced acid in glucose peptone broth and utilized glucose in the medium as a source of carbon (Table 1).

In vitro screening of *Azospirillum* isolates from root bits of Tuberose (Table 2)

In vitro N_2 fixation

Di-Nitrogen fixation is naturally the first major mechanism of action suggested for the enhancement of plant growth by *Azospirillum*. Total nitrogen fixation ranged from 12.7 mg of N/ g to 16.6 mg of N/g of malate utilized. Significantly highest nitrogen fixation was observed in the isolate ATR-06 (16.6 mg of N/g of malate). Significant higher N_2 production by ATR-06 may be due to well adoption to the surrounding environment and efficient utilization of nutrients present in the medium. Reports of nitrogen fixing efficiency of *Azospirillum* isolates isolated from grasses ranged from 3.4 mg to as high as 61.12 mg of nitrogen fixed per gram of carbon source consumed (Santosh swamy, 2006). Savalgi *et al.,* (2009) examined the *in vitro* N_2- fixation efficiency of *Azospirillum* isolates on N-free semi-solid malate medium and reported that N_2- fixed in the semi-solid medium ranged from 1.4 to 20.96 N mg /g of malate. Kanimozhi and Panneerselvam (2010) reported the maximum nitrogen fixation of 15.6 mg 'N'/g of malate in *A. brasilense* and minimum of 3.3 mg nitrogen/g of malate in *A. halopreferens* isolated from the soils of Thanjavur district.

In vitro IAA synthesis

The isolates were screened for IAA synthesis. IAA synthesized by *Azospirillum* isolates ranged from 6.50 µg/ml to 17.44 µg/ml of broth medium. Among all the isolates verified, the isolate ATR-32 synthesized significantly higher amount of IAA (17.44 µg/ml of broth medium) may be the isolate was more potential and well-adjusted to the medium. Gadagi (2000) observed 1.12 to 38.12 µg 100 ml^{-1} IAA production.

Ruíz-Sáncheza *et al.,* (2011) examined the response of rice plants to inoculation with *A. brasilense*. Result showed that *A. brasilense* was able to enhance ascorbate content on rice plants.

A total of forty *Azospirillum* isolates were isolated from root bits of Tuberose grown in Raichur and were characterized based on morphological and biochemical characteristics.

Table.1 Biochemical characterization of *Azospirillum* isolates isolated from rhizosphere soil of Tuberose grown at New Herbal garden, UAS Raichur

Sl. No.	Isolate code	Glucose utilization	Denitrification test	Acid production	Biotin requirement	Tentative identification
1.	ATR - 1	+	+ ve	++	- ve	*Azospirillum brasilense*
2.	ATR - 2	++	- ve	++	- ve	*Azospirillum brasilense*
3.	ATR - 3	+++	- ve	+	+ ve	Unidentified
4.	ATR - 4	+++	- ve	+++	+ ve	Unidentified
5.	ATR - 5	+	+ ve	++	- ve	*Azospirillum brasilense*
6.	ATR - 6	+++	- ve	++	+ ve	Unidentified
7.	ATR - 7	++	+ ve	+	+ ve	Unidentified
8.	ATR - 8	++	- ve	+	- ve	*Azospirillum brasilense*
9.	ATR - 9	++	+ ve	++	- ve	*Azospirillum brasilense*
10.	ATR -10	+++	+ ve	+	+ ve	Unidentified
11.	ATR - 11	+++	+ ve	+++	- ve	*Azospirillum brasilense*
12.	ATR - 12	++	- ve	++	- ve	*Azospirillum brasilense*
13.	ATR - 13	+	- ve	++	+ ve	Unidentified
14.	ATR - 14	+	- ve	+	- ve	*Azospirillum brasilense*
15.	ATR - 15	+	- ve	+	+ ve	Unidentified
16.	ATR - 16	++	- ve	+++	- ve	*Azospirillum brasilense*
17.	ATR - 17	++	- ve	++	+ ve	Unidentified
18.	ATR - 18	+	+ ve	++	- ve	*Azospirillum brasilense*
19.	ATR - 19	+++	- ve	++	+ ve	Unidentified
20.	ATR - 20	++	+ ve	++	- ve	*Azospirillum brasilense*
21.	ATR - 21	++	+ ve	++	+ ve	Unidentified
22.	ATR - 22	+	- ve	++	- ve	*Azospirillum brasilense*
23.	ATR - 23	++	- ve	+	- ve	*Azospirillum brasilense*
24.	ATR - 24	+	- ve	+	- ve	*Azospirillum brasilense*
25.	ATR - 25	+	- ve	++	+ ve	Unidentified
26.	ATR - 26	++	+ ve	+	- ve	*Azospirillum brasilense*
27.	ATR - 27	+	- ve	++	+ ve	Unidentified
28.	ATR - 28	+	+ ve	++	- ve	*Azospirillum brasilense*
29.	ATR - 29	+	+ ve	+	+ ve	Unidentified
30.	ATR - 30	++	- ve	+++	+ ve	Unidentified
31.	ATR - 31	+	- ve	++	- ve	*Azospirillum brasilense*
32.	ATR - 32	+++	- ve	++	+ ve	Unidentified
33.	ATR - 33	++	+ ve	++	- ve	*Azospirillum brasilense*
34.	ATR - 34	+	- ve	+	+ ve	Unidentified
35.	ATR - 35	+	- ve	+++	- ve	*Azospirillum brasilense*
36.	ATR - 36	+++	- ve	++	+ ve	Unidentified
37.	ATR - 37	++	+ ve	+	+ ve	Unidentified
38.	ATR - 38	++	+ ve	+	+ ve	Unidentified
39.	ATR - 39	+++	- ve	++	+ ve	Unidentified
40.	ATR - 40	++	+ ve	++	- ve	*Azospirillum brasilense*

Table.2 *In vitro* screening of *Azospirillum* isolates through Nitrogen fixation and IAA production

Sl. No.	Isolate code	IAA (µg/ml of medium)	Nitrogen fixed (mg/g of malate)
1.	Control	0.00	0.06
2.	ATR-2	11.60	14.0
3.	ATR-3	8.36	12.8
4.	ATR-4	13.40	13.1
5.	ATR-6	14.18	16.6
6.	ATR-8	13.08	14.2
7.	ATR-12	10.30	12.8
8.	ATR-13	9.60	13.9
9.	ATR-14	9.70	14.0
10.	ATR-15	6.50	13.3
11.	ATR-16	11.76	12.7
12.	ATR-17	12.60	13.0
13.	ATR-19	14.06	15.8
14.	ATR-22	10.30	13.4
15.	ATR-23	12.04	13.4
16.	ATR-24	8.48	13.7
17.	ATR-25	11.70	14.5
18.	ATR-27	7.80	14.2
19.	ATR-30	10.84	13.7
20.	ATR-31	14.02	14.0
21.	ATR-32	17.44	16.2
22.	ATR-34	11.44	15.1
23.	ATR-35	13.42	12.7
24.	ATR-36	16.64	15.4
25.	ATR-39	14.88	15.3
26.	Reference strain (ACD-15)	13.99	14.3

Twenty four isolates were negative for denitrification test and these isolates were used for further screening (Isolates which are positive for denitrification were not used for further studies, as denitrification is a harmful process for agriculture with the loss of available nitrogen). The isolate ATR-6 was found to fix significantly higher nitrogen under *in vitro* conditions. All the isolates could produce IAA but significantly highest IAA was synthesized by isolate ATR-32. Isolates *viz.*, ATR-06 and ATR-32 shall be used as bio-inoculants for bio fertilizer production.

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