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Selection of efficient *Azospirillum* isolates from rhizosphere and root samples of paddy using acetylene reduction assay (ARA)

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Abstract
Azospirillum is a known diazotroph fixes atmospheric nitrogen through biological process by having the vital enzyme nitrogenase. Studying the nitrogen fixation is a effective and fruitful tool to know the efficiency of bacterium on nitrogen fixation.
In the present study about 20 isolates were isolated from 10 different locations of Namakkal district. Among the twenty isolates 10 isolates from rice rhizosphere soil and another ten isolates from rice root. All the twenty isolates were studies for n fixing efficiency through ARA assay, all the isolates recorded ARA activities, among the twenty different isolates, the isolates from root samples of rice recorded much significant values on ARA activity compared with the isolates from rice rhizosphere soil samples.

Keywords: *Azospirillum*, isolates, rhizosphere, root

Introduction
Azospirillum, known associative symbiotic diazotropic, microaerophillic, gram negative, curved rod shaped bacteria always associated with cereals, especially rice (*Oryza sativa*. L). *Azospirillum* fixes appreciable amount of nitrogen in the rice field ecosystem by having associative symbiosis with rice. These diazotrophic bacteria fixes 40-60kg of nitrogen per cropping season. Nitrogen is a vital element in agricultural production of various crop plants and all types of crops are responding positively to the nitrogenous fertilizers in terms of their growth and yield.
Rice crop requires 120 kg of nitrogen to produce their optimum growth and grain yield. In commercial cultivation the entire amount added in the form of synthetic (chemical) fertilizers,
These fertilizers causes damages to the physical, chemical and biological properties of soil, in addition the chemical residues enters in to the food chain and causes alteration in metabolism of living organisms are also possible. Hence in order to avoid nitrogen pollution an alternative nitrogen source must be essential. N fixing efficiency studies are fruitful tool to exploit potentiality of soil bacteria on N fixation .The efficient isolates of diazotrophs must be studied under lab and field condition.

Materials and Methods
Screening of *Azospirillum* for nitrogen fixing efficiency
Ten different *Azospirillum* isolates were screened for their nitrogen fixing efficiency by assaying nitrogenase activity using acetylene reduction assay (ARA) method and fixed nitrogen in culture medium.

Acetylene Reduction Assay
The acetylene reduction activity of the 10 rhizosphere isolates of *Azospirillum* and the standard strains were determined by following the procedure of Bergersen (1980). Semisolid nitrogen free malic acid medium was prepared and dispensed in 40 ml quantities in 130 ml glass bottles and sterilized. All the ten *Azospirillum* isolates and reference strain were individually inoculated to the media by adding 1.0 ml of log phase cells and incubated at 32◦C under static condition. After four days of growth, cotton plugs were replaced with sterilized rubber stoppers. The air inside the vials was withdrawn through a gastight syringe and 1.0 ml of pure acetylene gas was injected without disturbing cultures. Control vial were also maintained. The vials were incubated for 24h at room temperature. At the end of the incubation period, one ml of the gas sample was withdrawn from the vial and injected in to a gas chromatograph (chemito 3800 model), (with FID and 80 to 100 Poropak R column). The column temperature was maintained at 85◦C injector temperature at 100c and ionization temperature at 125◦C .The flow rate of carrier gas (nitrogen) was 30 ml S^{-1}, hydrogen 30 ml sec^{-1}, and oxygen 60 ml sec^{-1}.

The area of the ethylene peak was recorded for each culture. The acetylene reduction activity (ARA) was calculated using formula.

Peak length (mm) ×Attenuation × Range × 0.00446* ×

$$\text{ARA Assay} = \frac{\text{Volume of the gas inside the flask (ml)}}{\text{Incubation period (h)} \times \text{volume of gas sample injected in to gas Cromatograph (ml)}}$$

* This factor was arrived by injecting pure ethylene at different concentration under the experimental condition.

The protein contents of the culture were estimated by following standard procedure of Bradford (1976) and the ARA was expressed as n moles of C_2H_4 produced hr^{-1} mg^{-1} cell protein.

Screening for cell nitrogen content

The quantity of nitrogen fixed in vitro *Azospirillum* isolates of maize rhizosphere soil samples was estimated by to Humpries (1956) [3] method. Semisolid nitrogen free malic acid medium was prepared and dispensed in 100ml quantities in 250ml Erlenmeyer's' s flask. The medium was inoculated with one ml of each *Azospirillum* isolate and incubated at 32◦C for seven days. After seven days of incubation, for culture was mixed thoroughly with homogenizer.

Five ml of culture was withdrawn and digested with 5 ml of concentrates sulphuric acid and 5g of digestion catalyst (K_2SO_4 and $CuSO_4$ in 10: 1 ration) in tectaor digestion system, until the content became colour less. After sufficient cooling, the aliquot was transferred to the Microkjeldahl distillation unit. Ten ml of 40 per cent sodium hydroxide was added and steam distilled. Ammonia evolved was collected in 20ml of two per cent boric containing two drops of double indicator (83.3mg bromocresol green +16.6 mg methyl red indicator dissolved in 10 ml of 95 per cent ethanol) back titrated against $N/50H_2SO_4$. Using the titre value, the nitrogen contents was calculated and expressed in mg per 5 ml of broth culture.

$$\text{Total nitrogen content} = \frac{\text{Titre value} \times 0.00028^* \times 1000/1}{^*N/50\ H_2SO_4 = 0.00028 \text{ g of N. N*mole / plant / h}}$$

Table 1: screening of *Azospirillum* isolates from root sample on nitrogenise activity

Name of the isolate	Nitrogenase activity*	Cell nitrogen content**
ARSS-1	234.60	32.00
ARSS-2	349.20	38.60
ARSS-3	220.10	29.00
ARSS-4	220.30	27.00
ARSS-5	350.26	38.00
ARSS-6	376.31	42.30
ARSS-7	362.36	41.10
ARSS-8	249.20	33.66
ARSS-9	325.30	35.00
ARSS-10	349.20	36.40
ATCC 29145	355.60	39.30
SEd	4.20	1.03
CD(P=0.05)	9.82	2.98

- * n moles of C_2H_4 produced mg^{-1} of cell protein h^{-1}
- ** mg g^{-1} cell weight
- * *ATCC 29145 – Azospirillum brasilense* reference strain

Table 2: Screening of *Azospirillum* isolates from rhizosphere soil sample on nitrogenise activity

Name of the isolate	Nitrogenase activity*	Cell nitrogen content**
ARSS-1	232.60	32.00
ARSS-2	260.00	34.00
ARSS-3	178.46	25.00
ARSS-4	152.92	23.00
ARSS-5	270.00	34.00
ARSS-6	230.00	30.00
ARSS-7	228.00	26.00
ARSS-8	210.00	20.00
ARSS-9	219.00	22.00
ARSS-10	226.00	24.00
ATCC 29145	230.00	29.00
SEd	4.20	1.03
CD(P=0.05)	9.82	2.98

Result and Discussion

In the present research an attempt was made to study the `N' fixing efficiency of isolated *Azospirillum* from rice field in lab condition.

In the present research about 20 different isolates of *Azospirillum* were isolated from the rhizosphere soil and root samples of rice from Namakkal District, in which 10 isolates from rhizosphere soil sample of rice and another ten isolates were obtained from root samples of rice. All the isolates were designated as ARSS-1 to ARSS-10 and ARRS-1 to ARRS-10 respectively for the isolates Of *Azospirillum* from rhizosphere soil and samples. *Azospirillum* was isolated from flooded condition Mlchiels et al., 1989 [5]. All the *Azospirillum* isolates were screened and studied for the `N' fixing efficiency and among the twenty different isolates; the isolates from root samples showed and recorded maximum value for `N'fixation compared with the other isolates from rhizosphere soil samples. In `N' fixation the isolate ARRS-6 from west valasu village of Namakkal District recorded much significant values on `N' fixation compared with other isolates. Further studies are needed to study the efficiency of *Azospirillum* isolate ARRS-6 on field condition.

The nitrogen fixing ability of isolates were determined by acetylene reduction assay. The nitrogenase activity is expressed in n moles of C_2H_4 produced mg^{-1} of cell protein h^{-1} and values recorded in ARA activity were present in Table 1.All the ten isolates of *Azospirillum* were tested for ARA activity and all isolates showed ARA positive trend. The overall nitrogenase activity were varied between 220.10 n moles of C_2 H_4 Produced mg^{-1} of cell protein h^{-1} to 376.31 n moles of C_2 H_4 produced mg^{-1} of cell protein h^{-1}.The highest amount of nitrogenase activity was observed in ARRS-6 which was recorded 376.31 n moles of C_2 H_4 Produced mg^{-1} of cell protein h^{-1}.The bacterial isolates ARRS-7 with 362.36 n moles of C_2 H_4 Produced mg^{-1} of protein h^{-1}.The reference strain ATCC 29145 also recorded appreciable amount of nitrogen fixation and values were 355.60 n moles of C_2 H_4 Produced mg^{-1} of cell protein h^{-1}. The cell nitrogen content was observed in mg^{-1} cell weight. The highest nitrogen content was recorded by ARRS-6 (42.30mg g^{-1} cell weight).These findings are similar to the findings of kucey and kloppstech1993.

The isolates from rhizosphere soil sample were also recorded appreciable values for `N' fixation. The values were ranged 220.10 to 376.31 n moles of C_2 H_4 $mg^{-1}$of cell protein h^{-1}.These research findings are in accordance with the findings of Dobereiner et al., 1988

Conclusion

The results of the present research clearly paved a way for selecting an efficient stain for nitrogen fixation. As per the findings while selecting a *Azospirillum* for nitrogen fixation it is quite essential to select isolate from roots of rice for maximum `N' fixation.

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