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# Isolation and identification of *Azospirillum* isolates from different Onion fields

**Rajesh C, Kumaresan G and Sivasakthivelan P**

**Abstract**
Samples of rhizosphere soil, non-rhizosphere soil and roots of rice plants were collected from the particular locations of the Trichy District. Twenty different colonies of *Azospirillum* species. Were isolated from the different samples from which ten isolates were finally selected for detailed study on the basis of their better growth in Nfb semi-solid medium. The selected isolates were T-1, T-2, T-3, T-4, T-5, T-6, T-7, T-8, T-9 and T-10. The selected isolates were thoroughly characterized on the basis of their response to a number of morphological and physiological tests. Forming the confirmation to the identification based on the characteristics of these isolates Bergey's Manual of Determinative Bacteriology was followed. In this investigation, six isolates were selected T-3, T-4, T-6, T-7, T-8 and T-10 were identified as *A. lipoferum*; three isolates T-1, T-2 and T -5 were identified as *A. brasilense* and one isolate T-9 was identified as *A. halopraeferns*. Variations were found in all of the isolates in colonial, morphological and physiological characteristics and ability of fermenting various carbohydrates. All of the selected isolates developed characteristics scarlet colonies on RC medium.

**Keywords:** *Azospirillum*, isolates, isolation, inoculation, identification, bio-fertilizer, Trichy

## Introduction
Plants and different microorganisms in the rhizosphere have a well-researched connection that is often described as mutualistic and helpful (Kiersi and Denison, 2008) [17]. According to Bashan *et al*. (2004) [28], *Azospirillum* is thought to be the most significant rhizobacterial genus for enhancing crop production or plant development globally under a range of soil and environmental circumstances. Because of their adaptable C and N metabolism, azospirilla can survive in the rhizosphere (Hartmann and Zimmer 1994) [29]. Microaerophilic bacteria that fix nitrogen on their own, *Azospirillum* species are widely known to be associated with cereals and grasses (Boddey and Dobereiner, 1988; Baldani *et al*., 1997; Peng *et al*., 2006) [5, 2, 24].
Utilizing biological methods to boost crop yields is typically less costly, risk-free, and accessible to all nations. By using biological nitrogen fixation (BNF) technology, urea-N usage can be reduced, soil organic matter can be prevented from being depleted, and environmental pollution can be significantly decreased. According to Saikia and Borah (2007) [27], the application of biofertilizers can simultaneously boost crop output by 10–20% and reduce chemical fertilizer consumption by 20–50%.
The rhizosphere, the region around a plant's roots, has a low oxygen content and an abundance of substrates that bacteria can use. Meanwhile, the plant gains from the bacteria's provision of fixed nitrogen and substances that promote plant growth (Madigan *et al*., 2014) [21]. Relationships between rhizobacteria and plants indicate that during the course of evolution, a significant and long-lasting degree of adaptation has emerged between the two types of organisms (Nehl and Knox, 2006) [22]. It can create an associative symbiotic relationship in the roots of many different plants, including onions. It has been discovered that *Azospirillum* inoculation significantly boosts the growth and productivity of a variety of crops, including onions. The yield response to *Azospirillum* inoculants used for bacterization was nearly equal to what could be obtained by applying 15-20 kg N/ha. This organism is very adaptable to a variety of environmental circumstances. Various BNF systems are used on a restricted basis in

## Materials and Methods

### Collection of samples

For the purpose of collecting samples, specific onion fields in the Tamil Nadu districts of Trichy were chosen. Twenty villages in the Trichy District served as the settings. A non-rhizosphere soil sample was taken six feet away from each plant, while rhizosphere soils were taken from the plant's rhizosphere areas at a depth of two to three centimeters. To get a root sample, the plants were uprooted, and the soil that was affixed to the roots was extracted. Each sample was collected and transported to the lab in a separate polythene bag. The specimens were refrigerated for preservation.

### Preparation of samples for inoculation

by gently washing the roots under a stream of water to remove any soil that was adhered to them. The roots were thoroughly cleaned with sterile distilled water multiple times until they were free of any remaining soil sticking to them. Sterile scissors were used to cut the roots into tiny pieces. For this aim, one gram of each root sample was employed. After two minutes in 70% alcohol, the roots were repeatedly cleaned with sterile distilled water. These root samples were serially diluted after being macerated in sterile mortars.

### *Azospirillum* isolates

Using a sterile pipette, 0.1 ml of each sample suspension was added to Nfb semi-solid medium in screw-capped tubes. The tubes were then incubated for 72 hours at 37 °C. Following incubation, *Azospirillum* emerged in the tubes and formed a thin, dense, white pellicle that was visible a few millimeters below the medium's surface (Dobereiner, 1980) [10].

Gram negative, vibroid, and motile cells were detected under a microscope in the pellicles. Krieg (1981) [19] states that new semi-solid Nfb-medium was filled with a loopful of the pellicle that had formed in tubes, and the tubes were screw-capped and incubated at 37 °C. Three times, at 72-hour intervals, the white sub-surface pellicle that had developed in the fresh medium was placed into the fresh semi-solid Nfb-medium to be examined under a microscope for the presence of gram-negative, curved, motile cells. Next, a loopful of the pellicle was spread onto Nfb-medium plates that had 20 milligrams of yeast extract per liter, and it was then solidified using 1.5% agar. For a week, the plates were incubated at 37 °C. Tiny, arid, somewhat convex, and Rugose colonies were moved to solid malate medium slants that had 0.1% ammonium chloride in them. To obtain pure colonies, cultures in the slants were streaked on malate agar medium plates containing 0.1% NH4Cl. The pure colonies were transferred to the identical medium and kept intact. In the end, the isolates with good growth only and relatively quick growth were chosen for additional research.

### Morphological study of the *Azospirillum* isolates

The morphological features of the chosen isolates were examined using solid Nfb-medium, N-plus-malate agar medium, BMS agar medium (Dobereiner and Baldani, 1979a, b) [7, 8], Rojo-Congo red (RC) medium (pH 7.0) (Rodrignuez-Caceres, 1982) [26], LG medium (pH 6.8) (Dobereiner, 1980) [10], and MPSS medium. For this, gram staining was also employed.

### Biochemical characterization of the *Azospirillum* isolates

Tests on the organisms' physiological activities included those for oxygenase, catalase, urease, nitrate reduction, biotin requirement, starch hydrolysis, utilization of glucose or sucrose as the only carbon source for N2-dependent growth, acidification of peptone-based glucose medium test (Krieg and Dobereiner, 1984) [18], carbohydrate fermentation, citrate utilization, denitrification, ammonification, gelatin-liquification test, and motility. Visual observation was used in each case to record the isolates' growth. In accordance with Bergey's Manual of Determinative Bacteriology (1994), all of the chosen isolates were identified.

**Table 1:** Sources from which selected isolates were isolated

| Selected isolates | Sources | Locations |
|---|---|---|
| T-1 | Root | P.K. Agaram |
| T-2 | Root | M.R. Palayam |
| T-3 | Root | Sanamangalam |
| T-4 | Root | Edhumalai |
| T-5 | Root | Thirupattur |
| T-6 | Root | Padalur |
| T-7 | Root | Siruganur |
| T-8 | Root | Konalai |
| T-9 | Root | Perakambi |
| T-10 | Root | Vazhaiyur |

**Fig 1:** White colonies of *Azospirillum*

**Table 2:** Morphological and cultural characterization of the new isolates of *Azospirillum*

| Sl. No. | *Azospirillum* isolates | Gram Reaction | Capsule | Microcyst Formation | Solid agar media | Semi-solid media | Color of colony |
|---|---|---|---|---|---|---|---|
| 1. | T-1 | Negative | Present | + | Smooth, Raised, Dense | White sub-surface pellicle | White |
| 2. | T-2 | Negative | Present | + | Smooth, Raised, Dense | White sub-surface pellicle | White |
| 3. | T-3 | Negative | Present | + | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 4. | T-4 | Negative | Present | + | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 5. | T-5 | Negative | Present | + | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 6. | T-6 | Negative | Present | + | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 7. | T-7 | Negative | Present | + | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 8. | T-8 | Negative | Present | + | Smooth, Flat, Dense | White sub-surface pellicle | White |
| 9. | T-9 | Negative | Present | + | Smooth, Raised, Dense | White sub-surface pellicle | White |
| 10. | T-10 | Negative | Present | + | Smooth, Raised, Dense | White sub-surface pellicle | White |

**Table 3:** Identification of the selected isolates

| Selected isolates | Identified species | Selected isolates | Identified species |
|---|---|---|---|
| T-1 | *Azospirillum brasilense* | T-6 | *Azospirillum lipoferum* |
| T-2 | *Azospirillum brasilense* | T-7 | *Azospirillum lipoferum* |
| T-3 | *Azospirillum lipoferum* | T-8 | *Azospirillum lipoferum* |
| T-4 | *Azospirillum lipoferum* | T-9 | *Azospirillum halopraeferens* |
| T-5 | *Azospirillum brasilense* | T-10 | *Azospirillum lipoferum* |

## Results and Discussion

Twenty distinct colonies were isolated from soil samples containing roots and rhizosphere for this investigation. In primary selection, 14 colonies out of 20 were selected, with the remaining colonies being eliminated. Ten isolates were ultimately chosen for additional research from these 14 primary selection colonies because they were able to grow more quickly and well in Nfb semi-solid medium in screw-capped test tubes but not in Nfb agar medium in plates. T-1, T-2, T-3, T-4, T-5, T-6, T-7, T-8, T-9, and T-10 were the isolates that were chosen.

Microorganisms are frequently found in onion fields. According to Idress *et al*. (2010) [16], rice plants primarily receive their nitrogen from biological nitrogen fixation by microorganisms, which reduces the need for chemical fertilizers, particularly nitrogen, by 20% to 50%. Although nutrient limitation is a significant stress factor in soil, the isolation of *Azospirillum spp.* from bulk soil does not always indicate that the cells are physiologically active in situ. Therefore, until a host plant becomes available, bacteria must survive in their vegetative or cystic forms, where they cannot grow or only grow temporarily. Globally, it has been confirmed that *Azospirillum spp.* can be isolated from bulk soil, albeit typically in smaller quantities than from rhizosphere soil (Bashan, 1999) [4]. The investigation examined the *Azospirillum* species found in rice plant roots, rhizospheres, and onion fields. In a good area of Trichy District for growing onions, root samples, rhizosphere soil samples, and non-rhizosphere soil samples were collected from various locations (Table 1).

For the purpose of separating distinct populations of nitrogen-fixing bacteria, the type of carbon source utilized in N-free media is crucial. *Azospirillum* can be isolated more easily when malate is used as the carbon source in the semi-solid nitrogen-free medium (Okon *et al*., 1977) [23]. On the other hand, selective media have been described (Caceres, 1982; Bashan and Levanony, 1985) [6, 3] for the purpose of isolating species that belong to *Azospirillum.* The selected isolates in this study were characterized using the medium reported by Caceres. According to Dobereiner (1980) [10], the development of a white, dense, and undulating fine pellicle a few mm below the medium's surface in the screw-capped tubes (Table 2) was used as the criterion of *Azospirillum* 's presence in the culture tube. He stated that, with practice, the researcher would be able to identify the pellicle that *Azospirillum* forms in semi-solid Nfb medium in screw-capped tubes. Finally, ten *Azospirillum* isolates were chosen for in-depth investigation.

The chosen isolates' colonies exhibited traits akin to those of *Azospirillum* (Krieg and Dobereiner, 1984) [18]. The appearance of colonies of *Azospirillum lipoferum* and *brasilense* on bromothymol blue agar medium is similar (Dobereiner, 1992) [11]. According to Dobereiner (1992) [11], *Azospirillum* colonies on potato agar initially appear smooth and grayish before changing to a pinkish hue. According to Dobereiner *et al*. (1976) [9], the colonies of *Azospirillum* on bromothymol blue malate agar medium containing 50 mg of yeast extract per liter were dry, small, raised, round, or irregular, and white or pinkish after one week of incubation. According to Lakshmi-Kumari *et al*. (1980) [20], *Azospirillum* colonies were thin, dry, slightly convex, and rugose.

Their undulate margins and granular, wavy surface frequently gave them a fried-egg-like appearance. It is uncommon to find a pink *Azospirillum* colony on BMS (potato agar) and can only occur under extremely selective growth conditions. Congo red (37.5 mg/l) is present in this medium, which is the Rojo Congo red medium. The isolates that were chosen for this investigation formed distinct scarlet colonies and exhibited several traits listed below, as documented by Caceres (1982) [6]. It has become clear to us during this investigation that the growth conditions have an impact on the colony characteristics. These bacteria don't absorb Congo red, and their colonies are round, convex, translucent, smooth, and have an entire margin. This study demonstrated that impurities could be removed from the *Azospirillum* pellicle in the Nfb semi-solid medium by using RC medium, even after multiple transfers to the same medium. It was discovered that several *Azospirillum* isolates needed minimal amounts of yeast extract in order to grow in mineral medium (Dobereiner *et al*., 1976) [9]. For the isolates T-2, T-6, and T-8 to grow well in Nfb semi-solid medium, very little yeast extract was needed. Over time, scientific reports on Azospinillum's distinguishing characteristics have also changed. According to Balandreau (1983) [1], definitive traits at the *Azospirillum* species level are not always very clear.

Six of the ten isolates that were chosen for this investigation turned out to be *Azospirillum lipoferum*. The isolates T-3, T-4, T-6, T-7, T-8, and T-10 needed biotin to grow and could only use glucose as their only carbon source. Their growth was dependent on nitrogen. These six isolates used an acidified glucose medium based on peptone. The other three isolates, T-1, T-2, and T-5, did not require biotin to grow and were unable to use glucose as their only carbon source for nitrogen-dependent growth. *Azospirillum brasilense* was identified as the cause of these three isolates. According to Reinhold *et al*. (1987) [32], isolate T-9, the last one chosen, was able to use sucrose as the only carbon source and was unable to acidify a glucose medium based on peptone. *A. halopraeferens* was determined to be the isolate T-9.

## Conclusion

*Azospirillum* may be applied in agriculture as a biofertilizer. *Azospirillum* is one of the rhizobacteria that promote growth and is well-known for being an extremely active nitrogen fixer in both laboratory and natural soil environments, resulting in quick growth, improved plant health, and increased yield. This study shows that *Azospirillum* is abundant in roots and can be easily isolated from the rhizosphere and roots of onion plants. For isolation, NFB semi-solid media are essential. In the Trichy District,

*Azospirillum lipoferum* is more prevalent than *Azospirillum brasilense*. In order to use these isolates as a biological approach, more research is required.

## References


1. Balandreau J, Microbiology of the association, Can. J Microbiol. 1983;29:851-859.
2. Baldani JI, Caruso L, Baldani VLD, Goi SR, Döbereiner J. Recent advances in BNF with; c1997.
3. Bashan Y, Levanony H. An improved selection technique and medium for the isolation and enumeration of *Azospirillum brasilense*, Can. J Microbiol. 1985;3(10):947-952.
4. Bashan Y. Interaction of *Azospirillum spp*. in soils, Biol. Fartil. Soils. 1999;29:246-256.
5. Boddey RM, Döbereiner J. Nitrogen fixation associated with grasses and cereals: recent; c1988.
6. Caceres ERA. Improved medium for isolation of *Azospirillum* spp., Appl. Environ. Microbiol. 1982;44(4):990-991.
7. Dobereiner J, Baldani VLD. Ann. Acad. Brasil. Decienc, Cited from Dobereiner J and; c1979a.
8. Dobereiner J, Baldani VLD, Selective infection of maize roots by streptomycin resistant; c1979b.
9. Dobereiner J, Day JM. Associative symbiosis in tropical grasses: Characterization of microorganisms and dinitrogen fixing sites, In: Newton WE and Nyman CJ(ed.), Proceedings of the first international symposium on nitrogen fixation, Wahington State University Press, Pullman. 1976;2:518-538.
10. Dobereiner J. Forage grasses and grain crops, In: Methods of evaluating biological nitrogen-fixation, Bergersen, F. J. (ed.), John Wiley and Sons, New York; c1980. p. 541-555.
11. Dobereiner J. The genera *Azospirillum* and *Herbaspirillum. In*: The Prokaryotes (2nd ed.): A handbook on the biology of bacteria: eco physiology, isolation, identification, applications, Blows A, Truper HG, Dworkin M, Harder W and Schleifer KH (ed.), Vol. 3, Springer-verlag, New York Inc; c1992. p. 2236-2253.
12. Enumeration of *Azospirillum brasilense*, Can. J Microbiol. 1985;31(10):947-952.
13. Environ, Microbiol; c1982. p. 990-991.
14. Wiley and Sons, New York; c1980. p. 541-555.
15. Gerhardt P. (ed.), American Soc. Microbiol. Washington; c1981. p. 112-142.
16. Idress H, Attitalla M, Abobakar, Alhasin A, Muftah, Nasib. Occurrence and microbiological characteristics of *Azospirillum* strains associated with leguminous and non-leguminous plants in Al Akdar eco-region, Libya, American-Eurasian J Agric. & Environ. Sci. 2010;8(6):617-625.
17. Kiersi ET, Denison RF. Sanctions, cooperation, and the stability plant-rhizosphere; c2008.
18. Krieg NR, Dobereiner J. Genus *Azospirillum In:* Krieg NR, (ed.), Bergey's manual of systematic bacteriology, Williams and Wilkins, Baltimore. 1984;1:94-104.
19. Krieg NR, Enrichment and isolation, In: Manual of Methods for general bacteriology; c1981.
20. Lakshmi-kumari M, Lakshmi V, Nalimi PA, Subba Rao NS. Reactions of *Azospirillum* to certain dyes and their usefulness in enumeration of the organism, Curr. Sci. 1980;49(11):438-439.
21. Madigan MT, Martinko JM, Bender KS, Buckley DH, Stahl DA. Brock Biology of Manoharachary C, Singh J. (Eds.), Microbial Activity in the Rhizosphere, Springer-. Microorganisms, 14th Ed. Benjamin Cummings, San Francisco. Mutualisms. Annu Rev Ecol Evol Sys. 2014;39(1):215-236.
22. Nehl DB, Knox OGG. Significance of bacteria in the rhizosphere. In: Mukerji KG, non-legume plants. Soil Biol Bioch. 2006;29(5):911-922.
23. Okon Y, Albrecht SL, Burris RH, Methods for growing *Spirillum lipoferum* and for counting it in; c1977.
24. Peng G, Wang H, Zhang G, Hou W, Liu Y, Wang ET, *et al*. *Azospirillum melinis sp*. pure culture and in association with plants, Appl. Environ. Microbiol. 2006;33:85-87.
25. Reinhold B, Hurek T, Fendrik I, Pot B, Gillis M, Kersters K, *et al*. *Azospirillum halopraeferens* sp. nov, a nitrogen-fixing organism associated with roots of Kallar grass (*Leptochloa fusca*) (L.) Kunth.), Int. J Syst. Bacteriol. 1987;37:43-51.
26. Rodriguez-Caceres, Enrique A, Improved Medium for Isolation of *Azospirillum* spp, Appl. Environ, Microbiol; c1982. p. 990-991.
27. Saikia J, Borah P. *Azospirillum* Biofertilizer in Sweet Potato: Growth, Yield and Economics, Verlag, Heidelberg; c2007 pp. 89-120.
28. Rodriguez H, Gonzalez T, Goire I, Bashan Y. Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium Azospirillum spp. Naturwissenschaften. 2004 Nov;91:552-5.
29. Hartmann A, Zimmer W. Physiology of Azospirillum. Azospirillum/plant associations. 1994;1:15-39.