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Population dynamics of free living, nitrogen fixing bacteria *Azospirillum* in Manakkudi mangrove ecosystem, India

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Abstract

Seasonal variations of population dynamics of free living nitrogen fixing bacteria, *Azospirillum* in relation to chemical parameters in Manakkudi mangrove eco system was assessed in root and rhizosphere soil samples of mangroves and mangrove associated plants. In rhizosphere soil and root samples, the counts of *Azospirillum* were recorded maximum in *Acrostichum aureum* as 8.63±0.92 x10^4 and 115.48±17.36 x 10^4 CFU g^{-1}, respectively. The counts of *Azospirillum* in non-rhizosphere soil varied from 0.01±0.001x 10^4 to 5.77±0.92x 10^4CFU g^{-1} and found maximum in February and minimum in March and September. *Azospirillum* counts in water samples were found maximum (2.24 x 10^4 CFU l^{-1}) in February. During seasonal variations maximum counts of *Azospirillum* were recorded during southwest monsoon season in *Avicennia officinalis* (1.40 x 10^4 CFU g^{-1}) followed by *Rhizophora mucronata* (1.07 x 10^4 CFU g^{-1}). The average maximum population density of *Azospirillum* counts was found during non monsoon season (9.73 x 10^4 CFU g^{-1}) and the average maximum population density of *Azospirillum* counts was found with the mangrove associated root samples (13.73 x 10^4 CFU g^{-1}). Of the selected isolates *Azospirillum lipoferum* (60%) was found to be predominant followed by *Azospirillum brasilense* (25%), *Azospirillum irakense* (5%), *Azospirillum halopraeferens* (5%) and *Azospirillum amazonense* (5%). Of the isolated species, *A. halopraeferens* exhibited better growth at 35 g l^{-1} NaCl. The level of Fe, Cu, Zn and Mn were varied from 0.91 to 15.93 ppm. The level of Mn (12.13 ppm) was found maximum during non-monsoon of rhizosphere soil sample. Highest rainfall (192.80 mm) and atmospheric temperature (25.10 °C) were recorded during south west monsoon and non monsoon seasons. The increased population density was greatly influenced by the pH (r=+0.686). The present finding provides enough information on the nitrogen flow through biological process in Manakkudi mangrove ecosystem which can be useful for the effective implementation of mangrove management plan.

Key words

Azospirillum, Ecosystem, Mangrove forest, Nutrient cycle

Introduction

Mangroves are highly productive tropical and subtropical tidal zone ecosystem which hosts a wide range of coastal and offshore marine organisms for protected breeding (Lacerda *et al.,* 1993; Ronnback, 1999; Primavera *et al.,* 2004; Maloy Kumar Sahu *et al.,* 2007). Nutrient cycles in the mangrove ecosystem suggest that, there is a close relationship between heterotophic microorganisms and macro fauna and flora. This recycling preserves most of the necessary nutrients for the natural sustainability of these ecosystems (Toledo *et al.,* 2001; Bano *et al.,* 1997; Vazquez *et al.,* 2000). Nitrogen is one of the most important limiting nutrients, which could affect the development of mangrove vegetation. Inorganic nitrogen in aquatic environments is in a variety of oxidation status ranging from nitrate, nitrite and ammonia (Ravikumar *et al.*, 2007; Vazquez *et al.,* 2000; Sarnaik *et al.,* 2006). Fixation of nitrogen in marine and terrestrial environment can be influenced by several microbial genuses of *Azospirillum, Azotobacter, Rhizobium, Clostridium* and *Klebsiella* (Sahoo and Dhal, 2009). Among these, *Azospirillum* are free living, nitrogen fixing heterotrophic bacteria (Swêdrzyñska and Sawicka, 2001), which can also mineralize nutrients from the soil, to sequester Fe, to survive in harsh environmental conditions, and to favour beneficial mycorrhizal-plant associations (Bashan *et al.,* 2004; Datta *et al.,* 2009). The increment or reduction of the *Azospirillum* sp. population may continuously affect the nitrogen status of the soil. The objective of the present study was to explore the population dynamics in relation to environmental factors of nitrogen fixing *Azospirillum* persistence in Manakkudi mangrove ecosystem.

Materials and Methods

Sampling site: Manakkudi estuary is unique and specific as it is the first estuary located on the south west coast of peninsular India (8°05' E latitude; 77°32' N longitude). It is also the second largest estuary in Kanyakumari district. Manakkudi estuary is a typical bar built estuary, which remains land locked during non-monsoon season (January, February, March, April, May and September). It opens during southeast monsoon (June to August) and northwest monsoon seasons (October to December). Mangrove plant species of *Rhizophora mucronata* and *Avicennia officinalis* are planted in the area, which has enhanced prawn breeding.

Isolation and identification of bacteria *Azospirillum* sp.: Studies were conducted during the months of October, 2001 to September, 2002 for a period of 1 year. Root and rhizosphere soil (soil directly associated with root sample) samples were collected from mangrove (*Avicennia officinalis*, *Rhizophora mucronata*) and mangrove associated plants (*Hymenachene acutigluma, Acrostichum aureum* and *Panicum repens*). Additionally soil and water samples of the mangrove region were also collected. About 1g of soil/ root and 1 ml of water samples were serially diluted with sterilized 50% aged seawater (seawater stored in dark condition for 30 days to remove phytoplankton and zooplankton contaminations) and were plated with the nitrogen free basal agar (Nfb) medium (Hi Media). After solidification, the plates were incubated in an inverted position for 9 days at 30°C. All the determinations were carried out in triplicates. *Azospirillum* colonies developed on the Nfb solid medium were counted and the total number of bacterial counts was expressed as colony forming unit (CFU) per ml or gram of sample. The different morphological colonies were picked up from the petriplates and re streaked in appropriate Nfb agar plates and stored in agar slants for identification (Holt *et al.,* 1994).

Determination of chemical parameters: The soil, water samples were subjected for the determination of various parameters *viz.,* pH, electrical conductivity, inorganic phosphate, nitrate, nitrite, particulate organic carbon, chloride, sulphate, potassium, calcium

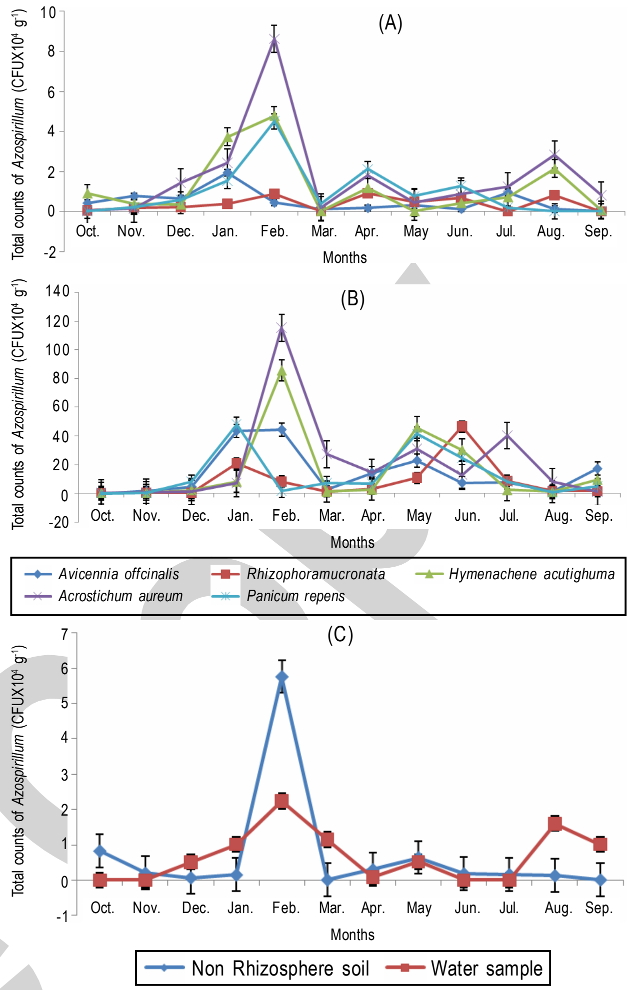

Fig. 1: Monthly variations of *Azospirillum* counts in various samples (A) Rhizosphere soil (B) Root, (C) Non-rhizosphere soil and water

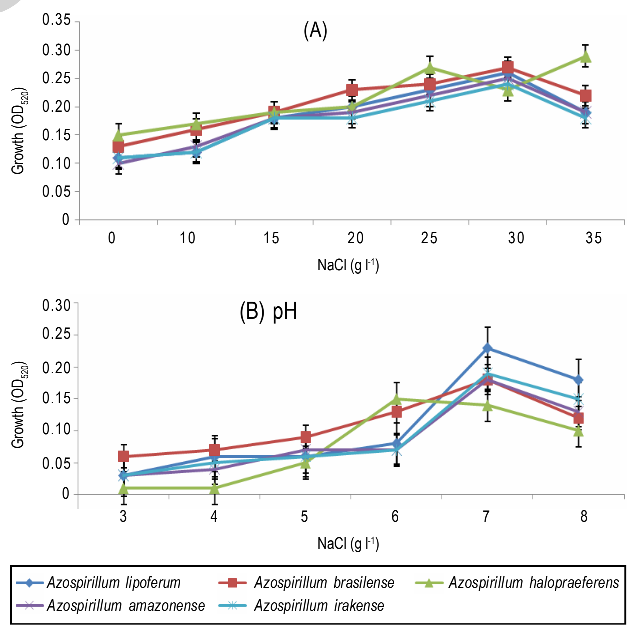

Fig. 2: Effect of (A) salinity and (B) pH on growth of *Azospirillum* species

and magnesium were calculated by using standard protocols (Ravikumar *et al.,* 2002; Young, 1997; Sanchez *et al.,* 1997). All the determinants were carried out in triplicates and the results were mentioned in ±SD values. The statistical analysis was carried out with the Statplus Proplus software.

Results and Discussion

The counts of *Azospirillum* varied from 0.01±0.02 x10^4 to 8.63±0.92 x10^4 CFU g^{-1} in rhizosphere soil and the bacterial counts were recorded maximum in *Acrostichum aureum* in February and counts were not observed in July and September in *Rhizophora mucronata* (Fig. 1A). In root samples, the counts of *Azospirillum* varied from 0.02±0.003x10^4 to 115.48±17.36 x 10^4 CFU g^{-1} and was found maximum in the month of February in *Acrostichum aureum* but counts were not recorded in October in *Panicum rupens* and *Avicennia officinalis* (Fig. 1B). The counts of *Azospirillum* in non-rhizosphere soil varied from 0.01±0.001 x 10^4 to 5.77±0.92 x 10^4 g^{-1} CFU and was found maximum in February and minimum in March and September.

Table - 1: Seasonal variation of *Azospirillum* counts in rhizosphere soil, root, non-rhizosphere soil and water samples

Sample types	Plant species monsoon	North east monsoon	Non monsoon	South west average	Total
Rhizosphere soil					
Mangroves	*A. officinalis*	0.61	0.50	1.40	0.69
	R. mucronata	0.16	0.44	1.07	
Mangrove associates	*H. acutigluma*	0.54	1.63	1.09	1.04
	A. aureum	0.55	2.38	0.85	
	P. repens	0.28	1.55	0.50	
Root					
Mangroves	*A. officinalis*	1.99	24.12	5.30	9.68
	R. mucronata	0.14	7.64	18.86	
Mangrove associates	*H. acutigluma*	0.98	25.56	11.20	13.73
	A. aureum	0.63	32.69	20.4	
	P. repens	2.71	18.31	11.18	
Non-rhizosphere soil		0.36	1.14	1.56	1.02
Water		0.17	1.00	0.53	0.56
Total average		0.76	9.73	6.16	

Values are average of three replicates

Table - 2: Seasonal variation of physico-chemical parameters in rhizosphere, non-rhizosphere and water samples

Parameters	Rhizosphere soil			Non-rhizosphere soil			Water		
	North east monsoon	Non-monsoon	South west monsoon	North east monsoon	Non-monsoon	South west monsoon	North east monsoon	Non-monsoon	South west monsoon
pH	5.46	6.22	4.41	6.67	5.91	7.6	7.93	7.5	7.00
EC (ds m^{-1})	2.41	5.06	6.26	1.23	3.07	2.77	6.73	8.56	7.43
N (µg g^{-1})	40.01	30.99	41.89	32.9	30.20	22.00	NT	NT	NT
HCO_3 (mil eq l^{-1})	NT	NT	NT	NT	NT	NT	2.83	5.9	3.4
Cl_2 (mil eq l^{-1})	NT	NT	NT	NT	NT	NT	72.23	36.92	21.83
SO_4 (mil eq l^{-1})	NT	NT	NT	NT	NT	NT	10.87	2.40	0.40
Ca (mil eq l^{-1})	NT	NT	NT	NT	NT	NT	10.00	6.47	5.33
Mg (mil eq l^{-1})	NT	NT	NT	NT	NT	NT	23.63	23.93	22.27
K (mil.eq.l^{-1})	NT	NT	NT	NT	NT	NT	4.90	2.92	2.96
NO_2 (mil eq l^{-1})	NT	NT	NT	NT	NT	NT	16.44	13.2	16.89
NO_3 (mil eq l^{-1})	NT	NT	NT	NT	NT	NT	0.496	0.25	0.67
P_2O_5 (µg g^{-1})	1.44	1.71	2.48	2.01	2.52	3.27	1.09	1.46	1.60
K_2O (µg g^{-1})	27.8	22.17	22.87	21.33	15.54	16.93	NT	NT	NT
Organic carbon (%)	1.14	1.59	2.19	1.17	1.37	0.97	0.85	0.55	0.90
Fe (ppm)	12.26	12.42	15.93	9.77	12.69	13.69	NT	NT	NT
Cu (ppm)	1.58	1.01	1.00	1.14	1.02	0.91	NT	NT	NT
Zn (ppm)	1.87	2.54	3.04	1.16	2.16	2.59	NT	NT	NT
Mn (ppm)	10.77	11.16	9.96	9.20	12.13	10.95	NT	NT	NT

EC = Electrical conductivity, N = Nitrogen, HCO_3 = Carbon source in water, Cl_2 = Chloride, SO_4 = Sulphate, Ca = Calcium, Mg = Magnesium K = Potassium, NO_3 = Nitrate, NO_2 = Nitrite, P_2O_5 = Inorganic phosphate, K_2O = Potassium oxide, Fe = Iron, Cu = Copper, Zn = Zinc, Mn = Manganese, NT = Not detected. Values are average of three replicates

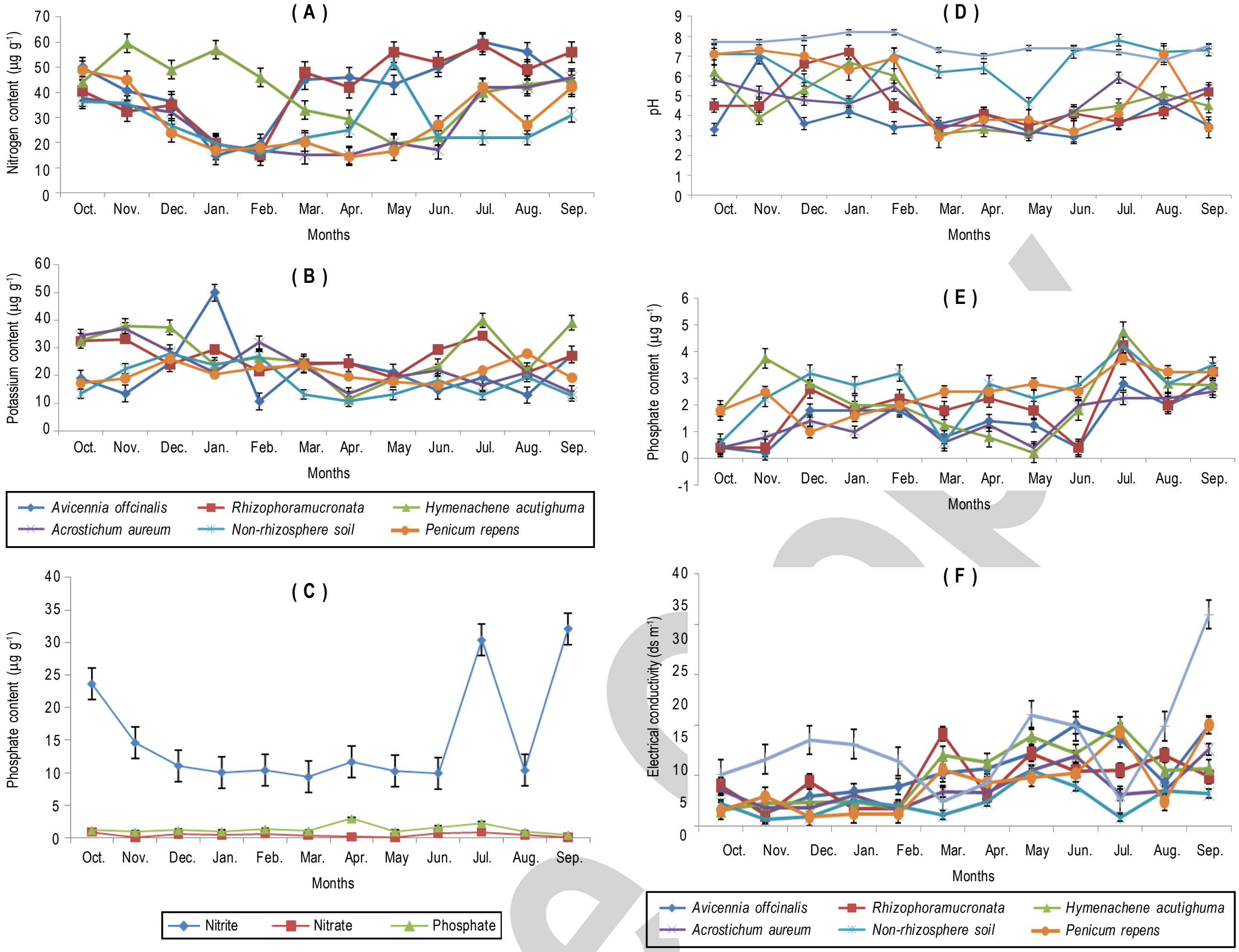

Fig. 3: Monthly variations of chemical constituents, pH and EC in various samples, (A) Rhizosphere and non-rhizosphere soil, (B) Water, (C) Rhizosphere and non-rhizosphere soil, (D) Rhizosphere, non-rhizosphere soil and water, (E) Rhizosphere and non-rhizophere soil, (F) Rhizosphere, non-rhizosphere soil and water. Values are mean of three replicates ±SD

Azospirillum counts in water samples were found maximum (2.24±0.91 x 10^4 CFU l^{-1}) in February and the counts were not recorded in October, November, 2001 and June, July, 2002 (Fig. 1C).

Seasonal variations of *Azospirillum* counts in rhizosphere soil varied from 0.16 x10^4 to 2.38 x 10^4 CFU g^{-1}, maximum counts of *Azospirillum* were recorded during southwest monsoon season in *Avicennia officinalis* (1.40 x 10^4 CFU g^{-1}) and in *Rhizophora mucronata* (1.07 x 10^4 CFU g^{-1}). Maximum counts in non-monsoonal months were recorded in *Hymenachene acutigluma* (1.63 x 10^4 CFU g^{-1}), *Acrostichum aureum* (2.38 x 10^4 CFU g^{-1}) and *Panicum repens* (1.55 x 10^4 CFU g^{-1}). *Azospirillum* counts in root samples varied from 0.14 x 10^4 to 32.69 x 10^4 CFU g^{-1}. Maximum counts of *Azospirillum* were recorded during non-monsoon season in *Acrostichum aureum* (32.69 x 10^4 CFU g^{-1}) followed by *Hymenachene acutigluma* (25.56 x 10^4 CFU g^{-1}). Whereas maximum counts of *Azospirllum* were recorded during non-monsoon in water (1.00 x10^4 CFU ml^{-1}) and non rhizosphere (1.14 x 10^4 CFU g^{-1}) samples. Among the plant species *Acrostichum aureum* harboured maximum counts in both rhizosphere soil and root samples than the other plants (Table 1). The average maximum population density of *Azospirillum* counts was found during non monsoon season (9.73 x 10^4 CFU g^{-1}) and the average maximum population density of *Azospirillum* counts was found with the mangrove associated root samples (13.73 x 10^4 CFU g^{-1}). Out of seventy isolates of *Azospirillum* sp. five different species were identified among them, *Azospirillum lipoferum* (60%) was found to be predominant than the other species *viz., Azospirillum brasilense* (25%), *Azospirillum irakense* (5%), *Azospirillum halopraeferens* (5%) and *Azospirillum amazonense* (5%). Effect of salinity and pH on growth of *Azospirillum* sp. revealed that, all the bacterial species showed maximum growth at 30 g l^{-1} NaCl, but *A. halopraeferens* exhibited better growth at 35 g l^{-1} NaCl (Fig. 2A) and the 5 bacterial strains prefer to grow at the pH of 7.0 (Fig. 2B). The level of physico chemical parameters in the collected samples were also carried out by the present study in every month for one year [Fig. 3(a-f) and Fig. 4(a-f)]. To find out the seasonal variations in the physico chemical parameters, the data were proved corresponding to the season and are represented in Table 2. The level of pH was found maximum (7.93) in water samples during north east monsoon season. The highest level of nitrogen (41.89

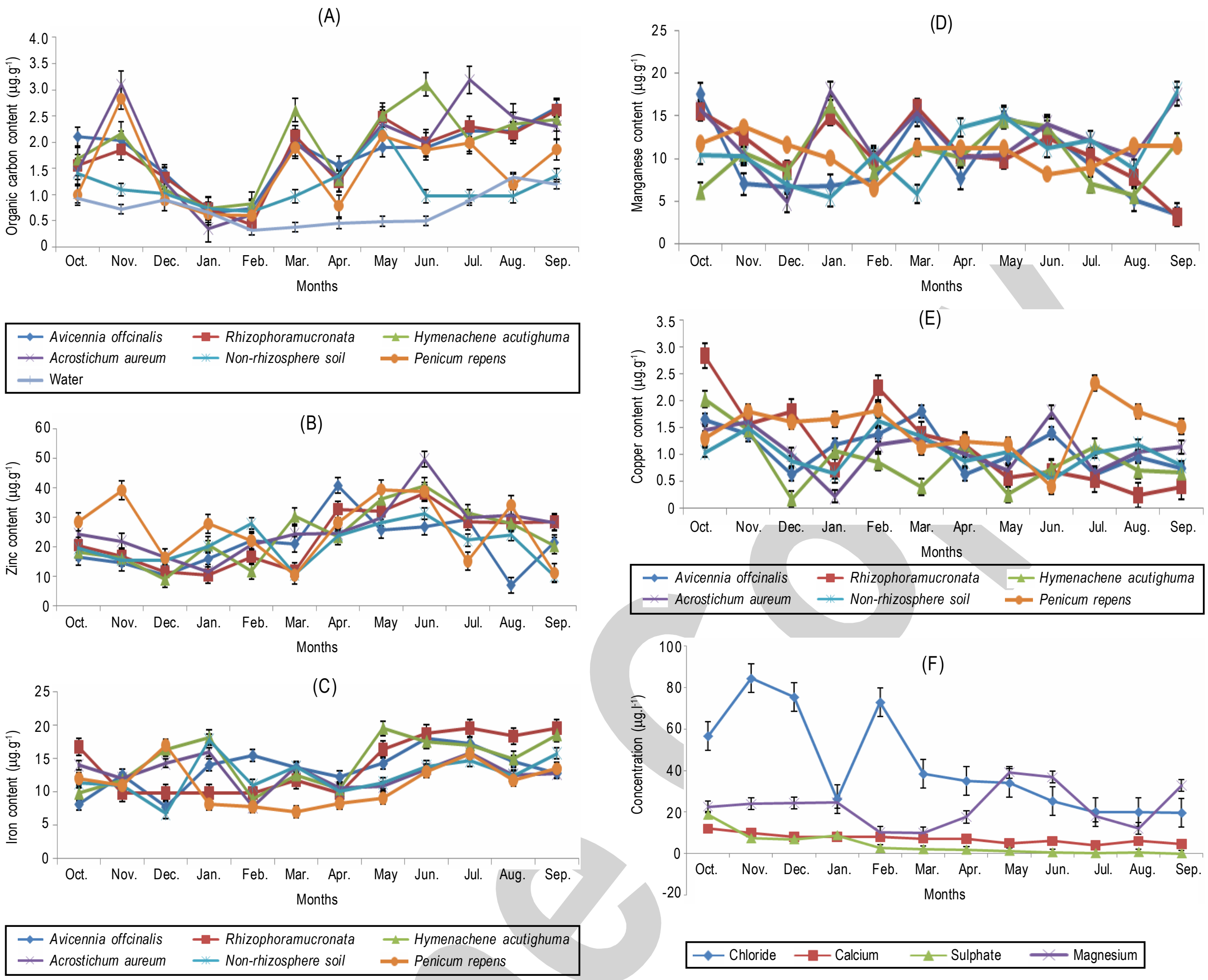

Fig. 4: Monthly variations of chemical constituents in various samples, (A) Rhizosphere and non-rhizosphere soil and water in rhizosphere and non-rhizosphere soil, (B) Rhizosphere and non-rhizosphere soil, (C) Rhizosphere and non-rhizosphere soil, (D) Rhizosphere, non-rhizosphere soil and water, (E) Rhizosphere and non-rhizophere soil, (F) Water. Values are mean of three replicates ±SD

µg g^{-1}) was recorded in rhizosphere soil during southwest monsoon. The highest level of phosphate (3.27 µg g^{-1}) was recorded during south west monsoon in rhizosphere soil sample. The highest level of potassium (27.82 µg g^{-1}) was recorded during northeast monsoon in rhizosphere soil sample. The percentage of carbon (2.19%) was found maximum during southwest monsoon in rhizosphere soil sample. The level of Fe, Cu, Zn and Mn were varied from 0.91 ppm to 15.93 ppm however rhizosphere soil showed highest level of (15.93 ppm) of Fe and Zn (3.04 ppm) during southwest monsoon season. The level of Mn (12.13 ppm) was found maximum during non monsoon of rhizosphere soil sample. Highest rainfall (192.80 mm) and atmospheric temperature (25.10 °C) were recorded during south west monsoon and non monsoon seasons respectively (Table 2). Biological nitrogen fixation is estimated to contribute 180x10^6 metric tons/year globally (Postgate, 1998) of which 80% comes from symbiotic associations and the rest from free-living or associative systems (Gina Holguin and Yoav Bashan, 1996). The genus diazotrophic *Azospirillum* can fix nitrogen under microaerophilic conditions, and are frequently associated with root and rhizosphere of plants. Mangrove plants are ecologically important as its prevent soil erosion and also reduce the tidal speed like Tsunami. But these plants are fast disappearing due to reduction in bacterial counts and important mineral nutrients (Kathiresan and Bingham, 2001), hence the present study has proved out to investigate nitrogen fixing *Azospirillum* counts in Manakkudi mangrove ecosystem which were found to be the higher in during non-monsoon season in all the samples analyzed except rhizosphere soil. This might be due to the large amount of dissolved and particulate nutrient introduced into the study area during northeast and southwest monsoon season through land run-off and a major portion of these nutrients would have gradually settled on the bottom sediments thereby accounting for the higher nutrient content during the non-monsoon season which would have stimulated bacterial action (Ravikumar *et al.,* 2002). The total counts of *Azospirillum* were found higher in root samples than the soil samples. This may be due to the quantity and quality of root derived carbon which supports the biomass expansion.

As roots grow through the soil, it releases photosynthetically generated carbon in to the soil in a variety of soluble and insoluble form, the totality of which is referred to as rhizodeposition (Barassi *et al.,* 2007). Among the plant species, the fern species, *Acrostichum aureum* harbors maximum counts than the other plants. This may be due to the thick sparsely branched spongy stilt like roots providing more surface area for colonization of microbes, which is a specific root characteristic of a fern. Moreover, high fluctuations of *Azospirillum* in the rhizosphere soil of mangrove plants might be due to the level of leakiness of acidic principles and also the secretions from the microorganisms (Peng *et al.,* 2006). Among the isolated *Azospirillum* species, *Azospirillum brasilense* is the most dominant group in Manakkudi mangrove but, *A. lipoferum* is the most dominant one in the Pichavaram mangrove ecosystem (Ravikumar *et al.,* 2002) and *A. amazonense* was reported to be the predominant form in the Amazon region (Barassi *et al.,* 2007). All the five species of *Azospirilla* prefer to grow at a wide range of salinity and different pH regimes. Moreover, *A. halopraeferens* and *A. irakense,* could extend their growth upto 35 g l^{-1} of NaCl, which confirms their halotolerant property.

The environmental parameters are observed high during either northeast monsoon or southwest monsoon seasons. This might be due to high nutrient input from terrestrial run-off (Jeffries *et al.,* 2003), sewage discharge (Thompson *et al.,* 2002) and ground water seepage (Barassi *et al.,* 2007). Besides that, assimilation of dissolved nutrients by photosynthetic organisms and other microbes during mineralization and oxidation-reduction aided by physical processes such as diffusion, advection and circulation controlled the distribution of inorganic and organic nutrients as well as their interaction in the marine environment (Ravikumar *et al.,* 2002). Statistical analysis reveals that all the physico-chemical parameters did not directly influence the *Azospirillum* counts in all the samples analyzed, however pH alone has influenced the distribution of *Azospirillum* in the root of *Hymenachene acutigluma* (r = +0.686) and found a positive correlation, whereas, seasonal influence was noticed in the *Azospirillum* population in the present study. It is concluded that the occurrence, distribution and dominance of *Azospirillum* counts did not vary with the season, but varied with space and time, Moreover, the present finding provides enough information for the conservation and management of mangroves in Manakkudi mangrove ecosystem which can be useful for the mangrove managers for the effective mangrove management.

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