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RESEARCH ARTICLE

STUDIES ON DISTRIBUTION, NITROGENASE ACTIVITY, SEM IMAGING AND MOLECULAR IDENTIFICATION OF FREE LIVING DIAZOTROPHIC BACTERIAL ISOLATES OF FOREST ECOSYSTEM

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ABSTRACT

The present study was focused on distribution of microbial and diazotrophic *Azotobacter* and *Azospirillum* population in rhizosphere soil samples. Total microbial population viz, bacteria, fungi, actiomycetes and diazotrophic *Azotobacter* and *Azospirillum* were enumerated from five different rhizosphere soil samples and compared the distribution of diazotrophic *Azotobacter* and *Azospirillum* with other three microbial groups. The result of enumeration of total microbial population indicates the higher presence of bacterial population followed by *Azotobacter* and *Azospirillum* population in various rhizosphere soil samples. Based on the predominant growth, five strains of *Azotobacter* and five strains of *Azospirillum* (one isolate from each sample) were isolated and identified through morphological and biochemical characteristics. In order to asses the nitrogen fixing potential of *Azotobacter* and *Azospirillum* isolates, Acetylene Reductase Assay(ARA) was carried out using Gas Chromatography(GC).The results of GC-ARA reveals that *Azotobacter* strain-5 and *Azospirillum* strain-1 exhibit better nitrogen fixing potentials than other strains. All the ten diazotrophic bacterial strains were authenticated through SEM imaging and 16S rRNA phylogenetic tree analysis. Based on Neighbor joining algorithm using NCBI BLAST tool, all the ten diazotrophic bacterial strains were authenticated viz., Azotobacter *nigricans, A. beijerinckii, A. chroococcum, A. vinelandii, A. chroococcum, Azosprillum fermentarium, A. humicireducens, A. thiophilum, A. melinis, A. canadens*

INTRODUCTION

Agriculture in the 21st century faces multiple challenges and as consequences, it has to produce more food and fiber to feed a growing population (Hazell & Pachauri, 2006). According to United Nations Food and Agriculture Organization (FAO) the total demands for agricultural products will be 60 percent higher in 2030 than present time and more than 85% of this additional demand will come from developing countries. Therefore, the world has relied on increasing crop yields to supply an ever increasing demand for food. Further, the world cereal production increased significantly during last two decades and this dramatic increase in world grain production was the result of a 122 percent increase in crop yields. However, this trend of grain production cannot be maintained due to decreasing cultivable land for rapid urbanization. Therefore, vertical expansion for food production is necessary (Mia and Shamsuddin, 2010).

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In order to increase world food production in a sustainable manner, farmers have to use balanced fertilizer timely. One of the most important factors in the generation of high yields from modern rice cultivars is nitrogen fertilizer which is very costly and make the environment hazardous especially when use discriminately (Mia *et al.,* 2010). Due to this fact, agricultural sector are strongly depending on development of fertilizers with mineral nutrients and supply phytohormones for the growth, development and production of the plants. Replacement of chemical fertilizers with biofertilizers is an attractive goal for sustainable agriculture (Kifle *et al.,* 2016). Soil fertility can be restored effectively through adopting the concept of integrated soil fertility management (ISFM) encompassing a strategy for nutrient management-based on natural resource conservation, biological nitrogen fixation (BNF) and increased efficiency of the inputs (Vlek and Vielhauer, 1994). The process of BNF performed by symbiotic nitrogen-fixing bacteria and non symbiotic nitrogen-fixing bacteria provides high sustainability for ecosystems (Bomfeti *et al*., 2011). These microorganisms can promote plant growth not only by supplying nitrogen but also by other mechanisms,

such as production of siderophores, exopolysaccharides (EPS), and phytohormones; phosphate solubilization; and protection against phytopathogenic fungus (Mazinani *et al.,* 2014). Biofertilizers are also important components of integrated nutrients management system. Biofertilizers are the products containing living cells of different types of microorganisms which when, applied to seed, plant surface or soil, colonize the rhizosphere or the interior of the plant and promotes growth by converting nutritionally important elements (nitrogen, phosphorus) from unavailable to available form through biological process such as nitrogen fixation and solubilization of rock phosphates (Rokhzadi *et al.,* 2008). Beneficial microorganisms in biofertilizers accelerate and improve plant growth and also protect plants from pests and diseases (El-yazeid *et al.,* 2007). The role of soil microorganisms in sustainable development of agriculture has been reviewed by several scientists (Lee and Pankhurst, 1992; Wani *et al.,* 1995). Biofertilizer is consider as a valuable alternative for agricultural practices and are broadly classified as nitrogen fixers (N-fixer), potassium solubilizer (K-solubilizer) and phosphorus solubilizer (P- solubilizer) (Dar *et al.,* 2014).The major biofertilizers were used as *Rhizobium, Azotobacter, Azospirillum* and phosphate solubilizing bacteria (Subba Rao, 2001). Nitrogen-fixing bacteria (NFB) that function transform inert atmospheric N2 to organic compounds (Bakulin *et al.,* 2007). Biological nitrogen fixation is one way of converting elemental nitrogen into plant usable form (Gothwal *et al.,* 2007).

Among the various group of biofertilizer, *Azotobacter* (family Azotobacteriaceae) is a aerobic, free living, and heterotrophic organism present in neutral or alkaline soils and are capable converting nitrogen to ammonia, which in turn is taken up by the plants. *Azotobacter* exists in various species such as A. *chroococcum*, A. *vinelandii*, A. *beijerinckii*, A. *insignis* and A. *macrocytogenes* (Kamil *et al.,* 2008). The *Azotobacter* colonizing the roots not only remains on the root surface but also a sizable proportion of them penetrates into the root tissues and lives in harmony with the plants. *Azotobacter* do not, however, produce any visible nodules or out growth on root tissue but they synthesize biologically active growth promoting substances such as vitamins – B, indole acetic acid (IAA) and gibberellins (Dar *et al.,*2014). The other important free living diazotropic bacteria is *Azospirillum* (belong to the family Spirilaceae) which are heterotrophic and associative in nature and are highly beneficial for many crops such as cereals, millets, sugarcane, cotton, sunflower and other crops. Further, *Azospirillum* assimilates atmospheric nitrogen and also secretes phytohormones in the plant root regions, which in turn enhances the root growth. The geneus *Azospirillum* has many species such as *A.amazonense, A.halopraeferens, A.braisilense and A.lipoferumare* and are distributed worldwide (Arun, 2007). The aim of the present is focused on studies on distribution, nitrogenase activity, SEM imaging and molecular identification of diazotrophic bacterial isolates from rhizosphere soil of forest ecosystem.

MATERIAL AND METHODS

Study on Distribution of Total Microbial and Diazotrophic *Azotobacter* and *Azospirillum* Population in Rhizosphere Soil: Soil samples were collected from the rhizosphere zone in the Vattakanal Forest, Kodaikanal, Dindigul District, and Tamil Nadu at the depth of 0-15cm with latitude 10.2381^0N, Longitude of 77.4892^0E and Altitude of 2,133m above the sea level. The soil samples were kept in polythene bags and brought to the laboratory for further analysis. The total colony forming unit (CFU) of bacteria, fungi, actinomycetes, *Azotobacter* and *Azospirillum* population were enumerated using standard procedures (Ahmad *et al*., 2008). The percentage distribution of *Azotobacter* and *Azospirillum* were calculated using the following formula.

$$CFU = \frac{\text{Number of colonies}}{\text{Vol of Sample x Dilution factor}}$$

The ten predominant bacterial strains viz., *Azotobacter* (5 strains) and *Azospirillum* (5 strains) were isolated and identified through morphological and biochemical characteristics as described in Bergey's Manual of Determinative Systematic Bacteriology (Holt *et al*.,1984).

Studies on N_2 fixing potential by diazotrophic bacterial isolates using GC – ARA

Nitrogen fixing potential of all the ten selected free living diazotrophic isolates were determined through Acetylene Reductase Assay (ARA) using Gas Chromatography (Hardy *et al.,* 1968). All the ten selected diazotrophic isolates were incubated individually in glass bottles with the Burk's N free broth for *Azotobacter* strains and Rojo Congored broth for *Azospirillum* strains. All the reaction containers were incubated at 28˚C in Shaking Incubator with 120rev min^{-1} for 3 days. Acetylene gas (1ml v/v) injected in to all the culture bottles and incubated over night at 28˚C. Gaseous sample (1ml v/v) from each acetylene gas treated bottles were carefully collected and injected immediately in to Gas Chromatography (Perkin Elmer auto systems Gas Chromatography fitted with a propak T column and H- flame ionization detector having injected temperature of 110˚C; Column temperature of 75˚C and detector temperature of 120˚C). ARA activity was calculated using the following formula:

$$\text{ARA (Area n mol ml}^{-1}) = \frac{\text{Area count} \times \text{volume of flask} \times 0.0006}{\text{Vol of gas injected} \times \text{Hrs of incubation} \times \text{Vol of sample}}$$

SEM imaging analysis of five diazotrophic *Azotobacter* and five *Azospirillum* strains: All the ten selected diazotrophic bacteria strains viz., *Azotobacter* (5 strains) and *Azospirillum* (5 strains) were authenticated through nano scale microscopic imaging using SEM (Baldi *et al.,* 1990). A well grown bacterial sample from ten isolates (10 ml each) was collected and centrifuged at 10,000 rpm at 4ºC for 5 to 10 mins. The pellet containing bacterial cell sediment was washed thoroughly with sterilized Triple Distilled Water (TDW), immersed in glutaraldehyde (2.5% v/v, Fluka) for 2 hrs at room temperature and washed thoroughly with sterilized TDW. The pellet was then subjected to osmium tetraoxide staining (2% v/v, Fluka) for 1 hr and washed thoroughly with sterilized TDW. Followed by, the pellet was dehydrated by transferring it into a series of 25, 50, 70, 90 and 100% (v/v) of ethanol (Fluka) for 5 mins. The final dehydration in 100% ethanol was carried out for 10 min. The dehydrated pellet was then dried overnight in an oven and mounted on a glass slide 120 stab with a double- stick carbon tab. Followed by, coating with a thin layer of gold under vacuum to increase the electron

Table 1. Distribution of total microbial and diazotrophic *Azotobacter* and *Azospirillum* population in rhizosphere soil samples (values are mean of three replicates ± standard error)

Samples	Bacteria ($\times10^7$ CFU g^{-1}soil)	Fungi ($\times10^4$ CFU g^{-1}soil)	Actinomycetes ($\times10^3$ CFU g^{-1}soil)	*Azotobacter* ($\times10^4$CFU g^{-1}soil)	*Azospirillum* ($\times10^5$ CFU g^{-1} soil)
Soil Sample-1	3.3±0.01	5.2±0.01	2.9±0.01	5.10±0.02	4.63±0.01
Soil Sample-2	6.9± 0.02	5±0.01	2.4±0.02	8.15±0.01	4.87±0.01
Soil Sample-3	4.2±0.02	2.9±0.01	3.1±0.02	6.43±0.01	5.80±0.01
Soil Sample-4	7.1±0.03	4.1±0.02	1.9±0.01	8.17±0.01	5.47±0.01
Soil Sample-5	7.8±0.03	5.6±0.02	3.2±0.01	8.37±0.01	6.57±0.01

Table 2. Cultural and biochemical characteristics of five *Azotobacter* and five *Azospirillum* isolates

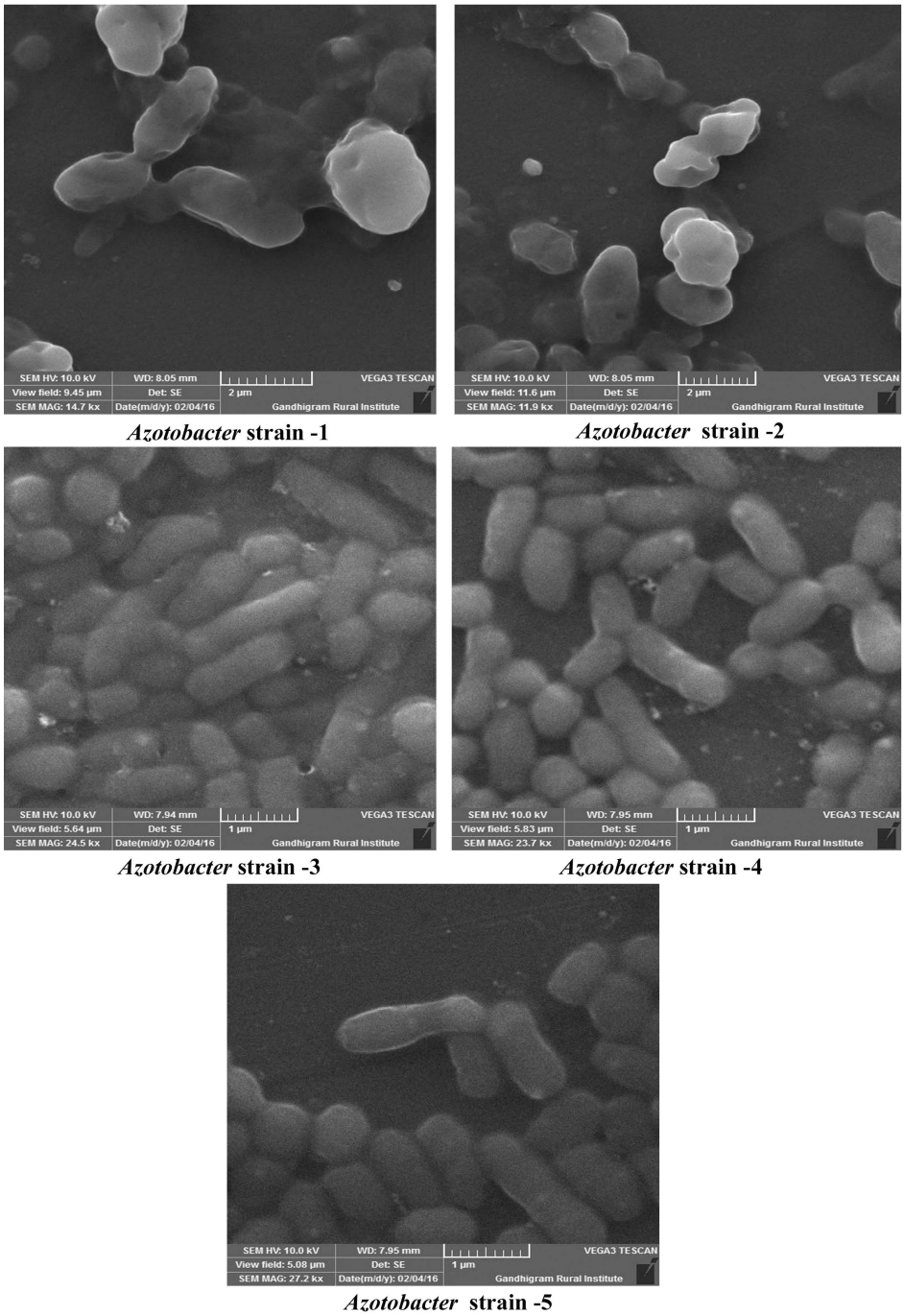
Characteristics	Characterization of ten diazotrophic bacterial isolates									
	Five *Azotobacter* isolates					Five *Azospirillum* isolates				
	Azotobactr strain 1	*Azotobactr* strain 2	*Azotobactr* strain 3	*Azotobactr* strain 4	*Azotobactr* strain 5	*Azosirillm* strain 1	*Azosirillm* strain 2	*Azosirillm* strain 3	*Azosirillm* strain 4	*Azosirillm* strain 5
Gram staining	-	-	-	-	-	-	-	-	-	-
Shape	Blunt Rod	Blunt Rod	Blunt Rod	Blunt Rod	Blunt Rod	Vibrioid	Vibroid	Rod	Rod	Rod
Indole production	-	-	-	-	-	-	-	-	-	-
Methyl red reaction	+	-	-	-	-	+	-	+	-	-
Vogues proskauer test	-	-	-	-	-	+	+	-	-	+
Nitrate reduction	-	-	+	+	+	-	+	+	+	+
Oxidase reaction	+	+	+	+	+	+	+	+	+	+
Catalase reaction	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis	-	-	+	+	-	-	-	-	+	+
Citrate utilization test	-	-	-	-	-	-	-	-	-	-
Urease production	+	-	-	-	+	-	-	-	-	+
Gelatin hydrolysis	-	-	+	+	-	-	+	-	+	-

Table 3. Acetylene Reductase Activity of ten diazotrophic strains including ethylene control

S.No	Strain	GC Area (mV*sec)	ARA (nmol ml-1)
1	*Azotobacter* strain-1	17256.25	34.51
2	*Azotobacter* strain -2	19359.38	38.71
3	*Azotobacter* strain -3	18235.98	36.47
4	*Azotobacter* strain -4	14254.24	28.50
5	*Azotobacter* strain -5	27256.21	54.51
6	*Azospirillum* strain -1	21226.08	42.45
7	*Azospirillum* strain-2	19487.57	38.97
8	*Azospirillum* strain-3	15352.58	30.70
9	*Azospirillum* strain -4	8965.72	17.92
10	*Azospirillum* strain -5	20777.31	41.55
11	Ethylene standard	3534.79	7.06

conduction and to improve the quality of the micrographs. SEM imaging study was performed using a 20 kV with Scanning Electron Microscopy (Tescan Vega 3).

Molecular sequencing of 16S rRNA and phylogenetic tree analysis for two potential strains: Bacterial Genomic DNA from all the ten diazotrophic isolates was isolated using the Insta Gene TM Matrix Genomic DNA isolation kit (Chen *et al*., 2006). The bacterial gene fragment was amplified with 16S rRNA Universal primers using Applied Biosystem Thermal Cycler. Then, PCR product was sequenced using the 518F/800R sequencing primers. The forward primer 518F includes 20 unique sequences (CCAGCAGCCGCG GTAATACG) while a reverse primer 800R includes 18 unique sequences (TACCAGGGTATCTAATCC). Sequencing reactions were performed using a ABI PRISM® BigDyeTM Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems). The 16S rRNA sequence was subjected for blast using NCBI online tool. The bacterial 16S rRNA gene sequence was carried out through phylogeny analysis of the closely related blast sequence with multiple sequence alignment using MUSCLE 3.7 software (Edgar, 2004). The resulting aligned sequences were cured using the program Gblocks 0.91b, which eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana, 2007). Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as substitution model. PhyML was shown to be at least as accurate as other existing phylogeny programs using simulated data while being one order of magnitude faster.



***Azotobacter* strain -1** ***Azotobacter* strain -2**

***Azotobacter* strain -3** ***Azotobacter* strain -4**

***Azotobacter* strain -5**

Figure 3: SEM micrograph of five Azotobacter isolates

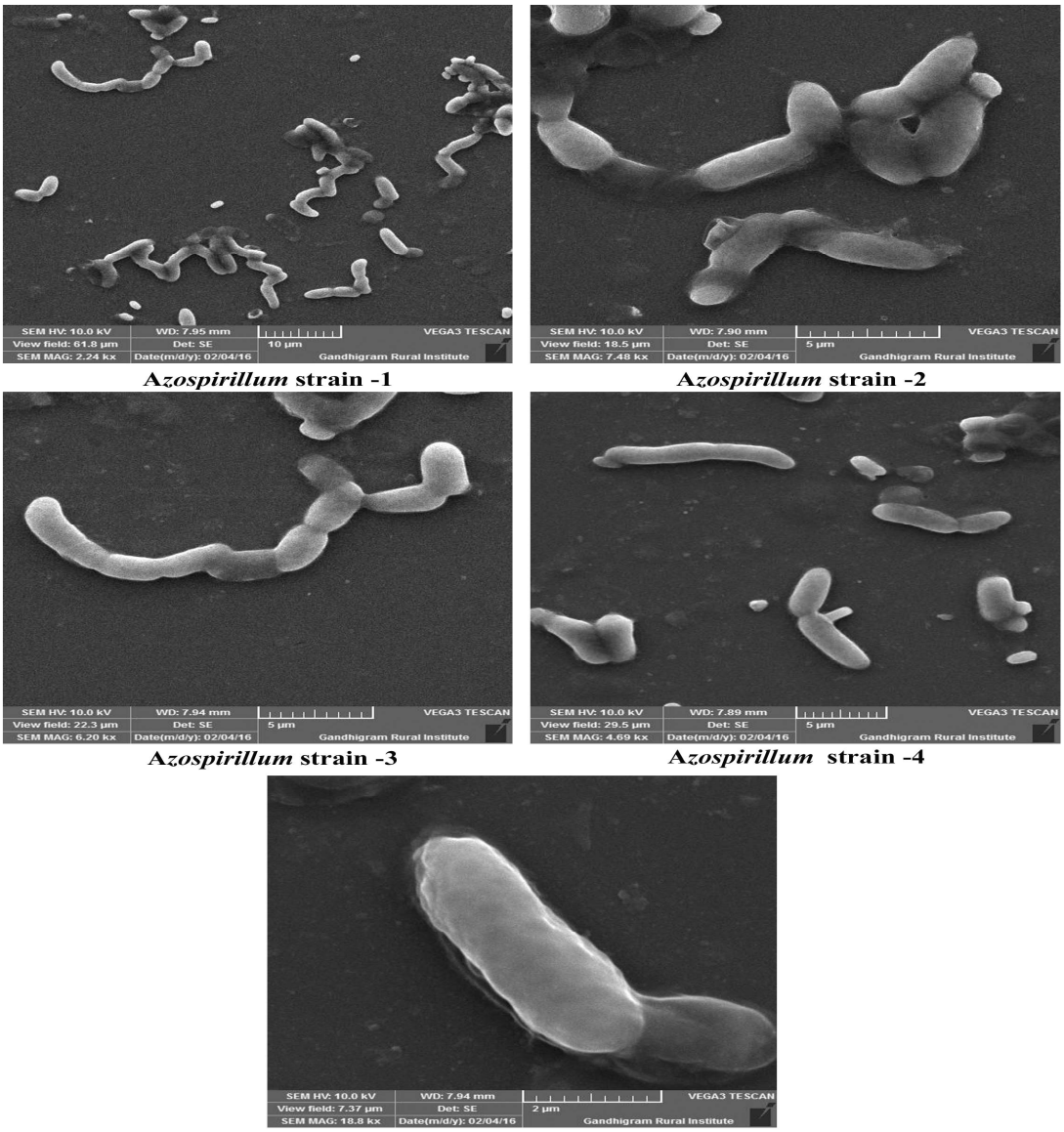


Figure 4: SEM micrograph of five Azospirillum isolates

The program Tree Dyn 198.3 (Dereeper *et al*., 2008) was used for tree rendering of all the ten potential diazotrophic strains.

RESULTS AND DISCUSSION

Agriculture ecosystem will be severely affected by frequent application of mineral fertilizer. The negative environmental impacts of chemical fertilizers and their rising costs, enhances the application of plant growth promoting biofertilizer which is valuable in the sustainable agricultural practices (El-Metwaly, 1998, Abdalla, 2001, Adam *et al*., 2002). Therefore, the present study was focused on "studies on distribution, nitrogenase activity, SEM imaging and molecular identification of free living diazotrophic bacterial isolates of forest ecosystem". In the present study, enumerated total colony froming units of bacteria, fungi and actinomycetes, *Azotobacter* and *Azospirillum* from rhizosphere soil and the results are presented in Table 1. Comparatively, the free living diazotrophs like *Azotobacter* strains and *Azospirillum* strains were distributed significantly among the total microbialpopulation.

This results are in close conformity with the findings of Watanabe and Baraqui (1979) who have revealed that the nitrogen fixing bacteria present in greater number in root of wet land rice. The results of the present study was also supported by the findings of Mukhopadhyay *et al.,* (1996) and Stoltzfus *et al.,(*1997).

Isolation and identification of diazotrophic *Azotobacter* and *Azospirillum:* Ten diazotrophic bacterial strains viz., five strains of *Azotobacter* and five strains of *Azospirillum* were isolated based on their predominant growth in the respective selective medium. All the strains were identified according to the Bergey's manual of determinative bacteriology (Holt *et al*., 1994) considering the characteristics of shape, staining and various biochemical characteristics as shown in Table 2.

Studies on N2 fixing potential by diazotrophic bacterial isolates using GC – ARA: Nitrogen fixing potential of the ten selected diazotrophic bacterial strains (five strains of *Azotobacter* and five strains of *Azospirillum*) were determined through Acetylene Reductase Assay (ARA) using Gas

Chromatography and the results are recorded in Table 3. Out of ten bacterial strained screened for nitrogenase activity, only two strains such as *Azotobacter* strain 5 & *Azospirillum* strain 1 exhibited highest nitrogenase activity with 54.51 nmol ml^{-1} and 42.45 nmol ml^{-1} respectively (Table 3). Similar results were reported by Andrade *et al.,* (1997) who have screened 25 bacterial isolates and 11strains were found efficient in nitrogen fixing potential. Rózycki *et al.,* (1999) also showed similar results of nitrogenase activity by some of diazotrophic isolates belonging to the genera *Pseudomonas* and *Bacillus*. Naher *et al*., (2009) also reported on nineteen bacterial strain were evaluated for nitrogen fixation activity.

SEM imaging analysis of five Diazotrophic *Azotobacter* and *Azospirillum* strains: All the five diazotrophic strains of *Azotobacter* and five diazotrophic strains of *Azospirillum* were identified through SEM imaging and the results are presented in Figure 3 and 4. In this study, all the ten selected bacterial strains (Five *Azotobacter* and Five *Azospirillum*) were subjected for SEM analysis and the SEM images of all the five *Azotobacter* and five *Azospirillum* strains are shown in Figure 3 and 4 . The SEM micrograph clearly evident that, all the five *Azotobacter* and five *Azospirillum* strains are morphologically similar with bacterial genus *Azotobacter* and *Azospirillum* strain respectively (Naher *et al*., 2013).

Table 4. NCBI detailes of ten diazotrophic bacterial isolates of rhizosphere soil samples

S.No	NCBI Accession Number	Strain Name	Source
1.	KY856972	*Azotobacter nigricans*	Forest Soil Sample
2.	KY856973	*A. eijerinckii*	Forest Soil Sample
3.	KY856974	*A. hroococcum*	Forest Soil Sample
4.	KY856975	*A.vinelandii*	Forest Soil Sample
5.	KY856976	*A. chroococcum*	Forest Soil Sample
6.	KY856962	*Azospirillum fermentarium*	Forest Soil Sample
7.	KY856963	*A.humicireducens*	Forest Soil Sample
8.	KY856964	*A.thiophillum*	Forest Soil Sample
9.	KY856965	*A.melinis*	Forest Soil Sample
10.	KY856966	*A.canadens*	Forest Soil Sample

Molecular Characterization on Selected Isolates: The genomic DNA of all the ten isolates were sequenced and the similarity quarry was achieved with known database available online and successfully submitted to NCBI USA and obtained accession number for all the ten isolates (Table 4). In support to this study, Wolf *et al*.,(2001) have analysed the 16S rRNA sequence of diazotrophic bacterial strain AJK-3 which showed 99% similarity with *S. rhizophila* type. Naveed, *et al*., (2014) revealed that diazotrophic strains QAU-63 and QAU-68 had 97% similarity with belong to genes *Bacillus.* Similarly, Bhromsiri *et al.,* (2010) stated that 16S rRNA sequence of diazotrophic strains KR-4, KR-23, KR-5 and KR-6 are 99% similar with *Pseudomonas putida*, *Spingomonas azotifigens*, *Stenotrophomonas maltophila* and *Herbispirillum* respectively.

Conclusion

Based on the findings of the present study, it was concluded that the diazotrophic *Azotobacter* and diazotrophic *Azospirillium* strain could be effectively used as potential Nitrogen fixing biofertilizer for improved crop production towards sustainable agriculture.

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