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Molecular characterization and stress tolerance level of nitrogen-fixing *Azotobacter* strain isolated from *Oryza sativa*

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Abstract

Characterization of four isolates obtained from *Oryza* sativa nodules grown under a stress environment was performed. Isolates were screened for their ability to tolerate different abiotic stresses; extreme temperature (50 ◦ C), salinity (1–5% (w/v) NaCl), and pH (4–12). The genomic analysis of 16S rRNA showed that isolates were phylogenetically related to *Azotobacter* spp. All the isolates can tolerate NaCl up to 3% and be able to grow between 30 and 40 ◦C with a pH tolerance of between 6 -10 indicating that the isolates were alkali and NaCl-tolerant. The tested isolates effectively utilize mono and disaccharides as carbon sources. Out of four, *Azotobacter vinelandii* (AR-3) showed the highest nitrogenase fixing ability. The plant growth-promoting characterization of all isolates revealed their effectiveness to solubilize inorganic phosphate (78-288 µg mL−1), and synthesizing indole acetic acid (IAA) (46–70 µg m.). The present finding revealed that treatment *A. vinelandii* (AR-3) are highly efficient to improve the growth and yield of rice crop, therefore the amplification of its nitrogen fixing gene can be explored as rice biofertilizer to enhance yield and N2-fixation for the resource-poor farmers of Kuje Area Council.

Keywords: Biofertilizer; Inorganic phosphate; Amplification; Salinity; Abiotic.

1. Introduction

Rice known as *Oryza sativa* is the most commonly consumed staple food for most of the world's human population. Nigeria is the largest producer of rice in the West Africa region (Zelda and Tamuno-Ina, 2022). However, of the nearly 7 million metric tons of rice consumed annually in Nigeria, only about 3.8 million metric tons is produced domestically (FAO, 2022). Rice is a high-yielding crop, but the current average yield is 10 to 15% yield is lower than its potential (Nwaobiala, 2016). There are many reasons for this yield gap, i.e., nutrient deficiencies, environmental stresses (biotic or abiotic), and management strategies. Abiotic stresses have been identified as the major causes of this low yield. Soil salinity is one of the several ecological stresses that is associated with huge crop losses globally (Ghulam *et al*., 2019). Salinity is one of the most brutal abiotic stresses limiting rice (*Oryza sativa L.*) productivity. The nitrogen-fixing bacteria played a vital role in crop production and improving soil health owing to their ability to fix atmospheric nitrogen, and proved to be environmentally friendly by minimizing pollution problems associated with the application of chemical fertilizers even under extreme conditions. Rice production depends largely on nitrogen (N) fertilizer application because most rice soils of the world are N-deficient (Sajid *et al*., 2017). As chemical fertilizers degrade soil and the environment, Biological Nitrogen Fixation (BNF) by *Azotobacter spp*. on the other hand has the potential to supplement 19–47 % of total N requirement, i.e., 0.4–0.9 t/ha (7– 20 %) in rice (James *et al*., 2011).

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Azotobacter spp. represents the main group of heterotrophic free-living nitrogen-fixing bacteria associated with plants and improves the productivity of other non-leguminous crops (Hadija *et al*., 2021). Nevertheless, rice being a monocot, associative N_2-fixing microbe like *Azotobacter* would be the key component for in situ nitrogen fortification (Swapna *et al*., 2018). Besides nitrogen fixation, the *Azotobacter spp*. produce different growth hormones (IAA and other auxins, gibberellins, and cytokinins), vitamins, siderophores, and antifungal compounds to fight against many plant pathogens (Dragana *et al*., 2015).

2. Material and methods

2.1. Isolation of Azotobacter spp.

The *Azotobacter* strains were isolated from root nodules of Rice (*Oryza sativa L*.). Host plants were sampled from swampy soils of different farmers' fields in Kuje Area Council, Nigeria (latitude 8.8821° N and longitude 7.2275° E). *Azotobacter spp*. were isolated from 100 µl extract of 10^{-4} dilution fraction of 1 g crushed nodules from the rice roots serially diluted with 9 ml of sterile distilled water and inoculated on Nitrogen free Jensen's medium. It was then incubated for 3–7 days at 28 ◦C as described by Akhter *et al.* (2012). Pure bacterial colonies were obtained by repeatedly streaking the individual colony on Nutrient Agar and preserved in an Agar slant at –80^{0}C for further use. A total of four *Azotobacter spp*. were obtained.

2.2. Screening of Isolates for Abiotic Stress Tolerance

Abiotic stress tolerance of isolates was carried out following the protocol of Rabia *et* al., (2020). The ability of the isolates to grow at different salt concentrations was tested by streaking each isolate on Jensen's Medium (Agar) containing 1–5% (w/v) NaCl. The tolerance of the isolates to acid or basic pH was examined in a liquid tryptone yeast extract (TY) medium with pH adjusted between 4 (1 N HCl) and 12 (I N Na_2CO_3), at an increment of 2 pH units. Temperature tolerance of all strains was tested on solid *Azotobacter* Agar plates by incubating at 20, 30, 40, and 50 ^{0}C.

2.3. Morphology and Biochemical Characterization

The *Azotobacter* isolates were further grown on nutrient agar for morphological characterization (color, shape motility of the bacteria, and size). Gram stain of the isolates was done following standard microbial methods (Collee and Miles, 1989). The biochemical tests, such as oxidase, catalase, urease, indole production, methyl red, Voges-Proskauer (acetoin production), nitrate reduction, citrate utilization, hydrogen sulfide production, carbohydrate metabolism, carbohydrate fermentation, arginine dihydrolase, extracellular enzyme activity, i.e., hydrolysis of starch, lipid, tween 80, cholesterol, protein (gelatine and casein), pectin and chitin and lecithin, were studied following standard methods described by Patel *et al.* (2013).

2.4. Molecular identification

The *Azotobacter spp.* was cultured in a 15-mL LB broth at 200 rpm at 30 ^{0}C. The genomic DNA of the isolate was extracted using the CangWei century bacteria Gen DNA kit. The 16S rRNA gene of the isolate was amplified using primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACC TTGTTACGACTT-3′) (Weisburg *et al.,* 1991). PCR conditions were as follows: initial denaturation for 2 min at 94 C, 30 cycles of 30 s at 94 ^{0}C, 30 s at 55 ^{0}C, and 30 s at 72 ^{0}C. The amplified fragments were recovered from agarose gel using the Universal DNA Purification kit (Tiangen, China) and sequenced by the International Institute of Tropical Agriculture, Ibadan. The 16S rRNA gene sequences were matched with those from the NCBI BLAST search (https://www.ncbi.nlm.nih.gov/). The sequences of bacteria with high similarity to the isolate were used for phylogenetic tree analysis by using the ClustalX and MEGA 5.0 software to identify bacterial attribution. The sequences obtained were aligned with the relevant *Azotobacter* sequences retrieved from the Gene bank by using the CLUSTAL W program in the MEGA 5.2 software (Tamura *et al*., 2007). Aligned sequences were analyzed using the same software to construct phylogenetic trees by using the neighbor-joining method, with bootstrap values based on 1000 replications.

2.5. Plant Growth Promoting Ability

The *Azotobacter* strains were evaluated for their ability to enhance plant growth by solubilizing inorganic phosphate and production of indole acetic acid (IAA). The quantitative phosphorus solubilization capacity of *Azotobacter* isolates was determined in Nautiyal broth containing 0.5% tri-calcium phosphate (pH 7) on a rotary shaker incubate at 28 ◦C for 8 days (Nautiyal, 1999). The drop in pH of the medium was recorded and the available phosphorous was analyzed using the protocol of Watanabe and Olsen (1965) and solubilization efficiency was calculated. Isolated *Azotobacter* strains were tested for the production of IAA following the procedure as adopted by Hayat *et al*., (2013).

3. Results

3.1. Isolation and morpho-biochemical characterization of isolates of *Azotobacter*

A total of four (4) *Azotobacter* strains were isolated from nodules of Oryza sativa L. harvest from different field locations and designated as AR-1, AR-2, AR-3, and AR-4. All isolates formed raised and off-white colonies on the Jensen medium.

The *Azotobacter* isolates were subjected to morphological characterization. On Jensen's medium, the *Azotobacter* isolates produced circular (0.60– 1.00 mm diameter), brown, off-white colonies (Table 1). Morphological characteristics, viz shape, size, motility, and Gram stain of the bacteria, were checked under a phase contrast microscope (100X objective), and the characteristics of the 4 colonies were observed distinctly (Table 2). The biochemical assay for oxidase, phosphatase, nitrate reduction, catalase, carbohydrate utilization, carbohydrate fermentation, nitrate reduction, and citrate utilization was evaluated for all tested isolates. Isolates obtained from different rice fields showed diverse biochemical profiles (Table 2). Among the *Azotobacter spp*., three species were *A. vinelandii*, namely, *A. vinelandii* (AR-1), *A. vinelandii* (AR-2), *A. vinelandii* (AR-3) and *A. chroococum* (AR-4).

3.2. Abiotic Stress Tolerance

The stress tolerance of the isolated *Azotobacter* strains is presented in Table 1. The table showed that all strains are able to grow at temperatures between 30 and 40 ◦ C in the pH range of 6–10, but cannot grow at 4, 12. All strains can tolerate NaCl concentrations of 1 – 3% but cannot tolerate concentrations of 4% and above.

Table 1 Differential stress tolerance features of isolated *Azotobacter* strains

Stress conditions	**Growth in/at**		**Isolates**		
		AR-1	AR-2	AR-3	AR-4
	$20^{O}C$	-	-	-	-
TEMP. TOLERANCE	$30^{O}C$	+	+	+	+
	$40^{O}C$	+	+	+	+
	$50^{O}C$	-	-	-	-
	NACL (1%)	+	+	+	+
SALT TOLERANCE	NACL (2%)	+	+	+	+
	NACL (3%)	+	+	+	+
	NACL (4%)	-	-	-	-
	NACL (5%)	-	-	-	-
	4	-	-	-	-
	6	+	+	+	+
PH	8	+	+	+	+
	10	+	+	+	+
	12	-	-	-	-

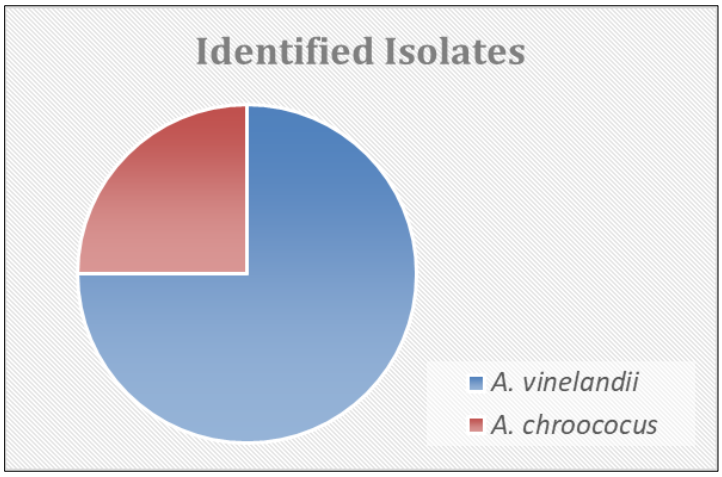

Figure 1 Different *Azotobacter* isolates from nodules of rice were identified as *Azotobacter vinelandii* (3) and *Azotobacter chroococum* (1)

Table 2 Morpho-Biochemical Test Characteristics of the bacteria isolated from Jenson's medium to identify the tentative *Azotobacter* isolates

		Isolates		
Tests	AR-1	AR-2	AR-3	AR-4
Gram reaction	-	-	-	-
Motility	+	+	+	+
Color	Brown	Brown	Brown	Off white
Shape	Circular	Circular	Circular	Circular
Size	0.65 – 0.70mm	0.60 – 0.70mm	0.65 – 0.70mm	0.90 – 1.0mm
Oxidase	+	+	+	+
Phosphatase	+	+	+	+
Utilization of Carbon Source	+	+	+	-
Rhamnose	+	+	+	-
Caprylate	+	+	+	-
Meso-inositol	+	+	+	+
Mannitol	+	+	+	-
NO_3 Reaction Test	+	+	+	-
H_2S Production	+	+	+	+
Utilization as Sole Carbon Source	+	+	+	+
Fructose	+	+	+	+
Sucrose	+	+	+	+
Glucose	+	+	+	+
Glutamate	+	+	+	+
Oxaloacetate	+	+	+	+
D-galactose	+	+	+	+
Glycerol	+	+	+	+
Presumed bacteria	*Azotobacter vinelandii*	*Azotobacter vinelandii*	*Azotobacter vinelandii*	*Azotobacter chroococcum*

Table 3 Composition of cellular protein of *Azotobacter* isolates

Isolates	Molecular weight (kDa)
AR-1	107.260, 97.516, 83.247, 74.547, 67.365, 65.000, 56.520, 51.613, 50.513, 49.137, 43.725, 41.804, 42.469, 40.670, 39.340, 31.348, 32.459, 278308
AR-2	107.130, 98.567, 82.247, 75.547, 67.760, 63.786, 65.134, 57.626, 47.891, 46.893, 45.500, 43.198, 41.283, 39.153, 37.181, 32.485, 30.459, 27.462
AR-3	90.369, 65.432, 54.381, 49.231, 46.415, 41.843, 39.153, 37.500, 35.043, 32.623, 27.923, 26.538, 23.769
AR-4	83.822, 74.858, 67.760, 55.134, 51.949, 49.231, 46.415, 44.631, 41.469, 39.324, 37.500, 35.190, 32.900, 31.802, 30.459, 28.077, 26.846

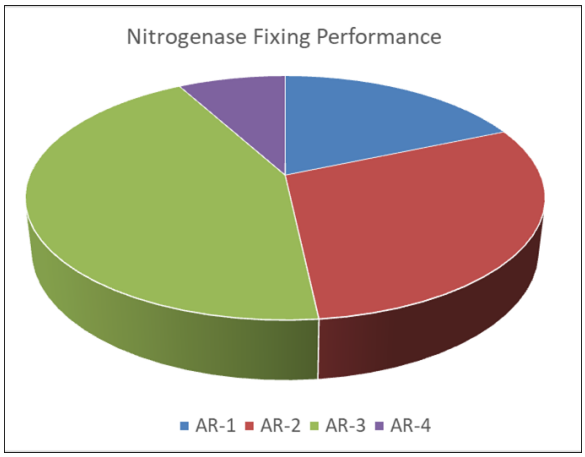

Figure 2 Nitrogen Fixing Performance of *Azotobacter spp.*

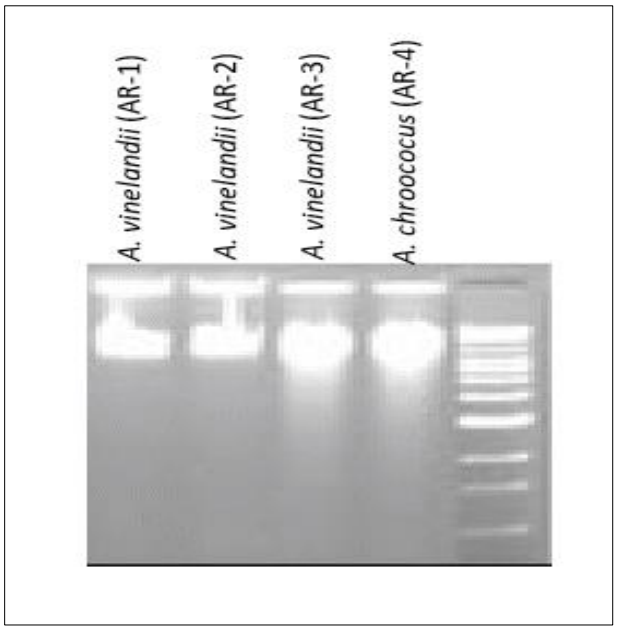

Figure 3 Genomic DNA of A. vinelandii isolated from different rice fields

Table 4 Genomic DNA and plasmid composition of selected *Azotobacter* isolates

Isolates	Genomic DNA (mDa)	Plasmid no.	Molecular weight (mDa)
AR-1	18.553	8	87.458, 45.833, 5.105, 3.685, 3.102, 2.554, 1.546, 1.107
AR-2	20.208	4	126.000, 33.500, 8.153, 3.702
AR-3	24.423	8	64.333, 45.833, 5.035, 3.073, 2.630, 2.218, 1.421, 0.961
AR-4	22.342	2	90.542, 12.861, 5.285

Table 5 Plant growth promoting characteristics of *Azotobacter* isolates

Isolates	P- Solubilization	Ph	IAA with Tryptophan (µg mL^{-1})	IAA without Tryptophan (µg mL^{-1})
AR-1	142±40	4.50	53.6±1.8	9.0±1.0
AR-2	156±20	5.34	63.5±2.1	12.6±1.5
AR-3	78.8±17	4.89	70.0±3.9	25.3±0.7
AR-4	288±34	6.32	46.1±2.4	11.7±1.4

3.3. Plant Growth Promoting Ability of Isolated Strains

All four *Azotobacter* isolates were positive for phosphorus solubilization (Table 5), and solubilized inorganic phosphate (tricalcium phosphate) in the range of 56–290 µg mL−1, decreasing the pH of the broth medium from 7.0 to 4.5 after 8 days of incubation, indicating the ability of the isolates to synthesize organic acids responsible for creating acidic conditions in the media. The ability of the *Azotobacter* isolates for the synthesis of Indole-3-Acetic Acid (IAA) was determined by incubating isolates in pure culture (LB broth) in the presence (500 µg mL^{-1}) and absence of L-tryptophan (precursor of IAA production). Without tryptophan, IAA was synthesized in the range of 9–25 µg mL^{-1}, whereas significant differences in IAA production were recorded with the addition of tryptophan (46–70 µg mL^{-1}), indicating the ability of tryptophan to act as a precursor in IAA production.

3.4. 16S rRNA gene sequencing and analysis of phylogeny for A. vinelandii (AR-4) strain

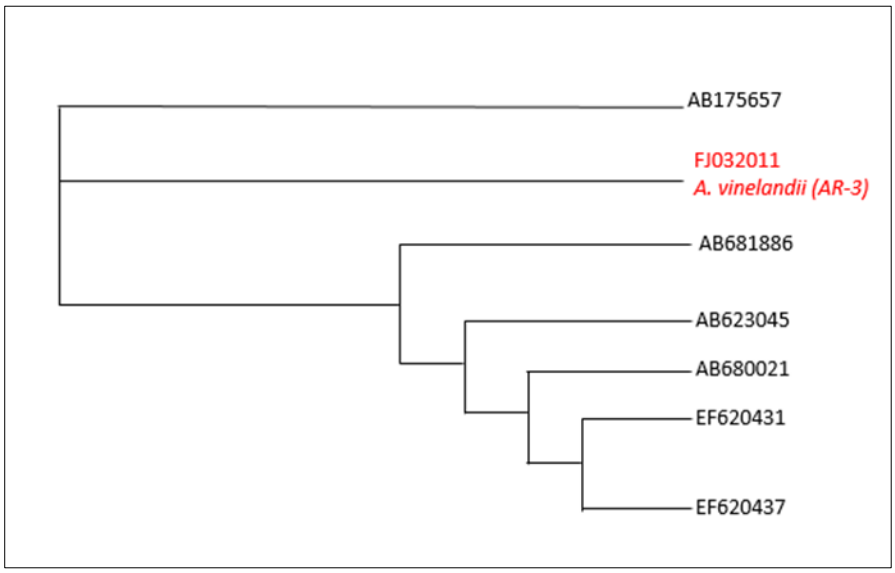

Figure 4 Phylogram based on 16S rRNA sequence of different species of *Azotobacter*

16S rRNA sequencing of *A. vinelandii* (AR-4) isolated from rice fields was performed. The amplified fragment of 16S rRNA of *A. vinelandii* (AR-4) was sequenced, and bootstrap analysis revealed that the sequences matched 100 % with the 16S rRNA sequence of *A. vinelandii* with the accession number JQ796077.

4. Discussion

In this study, we have characterized 4 bacteria isolates from nodules of rice. The characterization covered morphological, biochemical, and molecular which indicated that the isolates belonged to the genus *Azotobacter*. The diversity within the *Azotobacter* population made them capable to adapt to stressful environments for survival. The nodule-*Azotobacter* symbiosis is affected by factors such as changes in temperatures, pH, and soil salinity, which restrict symbiotic nitrogen fixation, and the strains capable of tolerating the extreme conditions would survive efficiently. All the isolates can tolerate NaCl up to 3.0% and be able to grow between 30 and 40 ◦C with a pH between 4 and 10 indicating that the isolates were alkali and salt tolerant, and exhibiting survival adaptations against the stressful abiotic conditions.

The morphological and biochemical characters identified the isolates, viz. AR-1, AR-2, and AR-3 as *A. vinelandii* and AR-4 as *A. chroococcum*. The selected isolates of *Azotobacter spp* (Fig. 1) were evaluated by their potential of NFE (ARA) was highly variable ranged from 21.56 to 118.05 nmol $C_2H_4mg^{-1}$ bacteria h^{-1} (Fig. 2). The *A. vinelandii* AR-3 isolate was key performer compared to other indigenous isolates. The NFE of the *A. vinelandii* (AR-3) was superior to the endophytic *Azotobacter spp*. reported by Dragana *et* al., (2015) which fixed 79.6– 91.50 nmol $C_2H_4h^{-1}$ $culture^{-1}$ or 5.03–15.1 nmol C_2H_4 h^{-1} $culture^{-1}$ reported by Barua *et al*., (2012). The finding of the present study confirmed the augmented nitrogen-fixing ability of native *A. vinelandii* isolates and the potential of the *Azotobacter* isolates to be used for plant growth promotion (Sabutal *et al*., 2009). The four isolates of *Azotobacter spp.* viz. AR-1, AR-2, AR-3, and AR-4 possessed one genome each of 18.553–24.423 kbp sizes and their plasmid composition was variable viz. 2–8 plasmids of variable sizes (Table 4). However Setubal *et al*. (2009) recorded a single circular genome sequence of *A. vinelandii* 5365318 bp, and Hill and Sawers (2000) reported the genome size of *A. vinelandii* was 4.5–4.7 mbp of 80–100 copies. The 2–8 plasmid in the organisms corroborated the reports of 2–6 plasmids in different other *Azotobacter spp*. (Kennedy *et al*. 2005; Setubal *et al*. 2009). The 16S rRNA of the most potent *A. vinelandii* (AR-3) has a genomic DNA of 24.423 mDa which was compared with *Azotobacter spp*. reported earlier (Kennedy *et al*. 2005; Setubal *et al*. 2009), and the phylogram provided molecular identity among the closely related species of *Azotobacter* genus (Fig. 3).

All isolates have shown plant growth-promoting characteristics through the solubilization of inorganic phosphate and synthesizing IAA.

5. Conclusion

The finding thus concludes that the native Azotobacter spp. of the rice rhizosphere is highly diverse and enhances the growth and production of rice (Oryza sativa) under adverse conditions. Hence, they have the ability to be used as biofertilizers to enhance yield and N_2-fixation, thus rice farmers can explore the use of it as a very high potential input.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

All the Authors declare that no competing interest exists and we all agree to publish the manuscript.

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