Hindawi
BioMed Research International
Volume 2021, Article ID 6686283, 9 pages
https://doi.org/10.1155/2021/6686283

Research Article

Phenetic and Molecular Diversity of Nitrogen Fixating Plant Growth Promoting *Azotobacter* Isolated from Semiarid Regions of India

Devendra Jain,[1] **Jyoti Sharma**,[1] **Gunnjeet Kaur**,[1,2] **Ali Asger Bhojiya**,[1,3] **Surya Chauhan**,[1] **Vimal Sharma**,[1] **Archna Suman**,[4] **Santosh Ranjan Mohanty**,[5] **and Elina Maharjan**[6]

[1]*Department of Molecular Biology and Biotechnology, Rajasthan College of Agriculture, Maharana Pratap University of Agriculture and Technology, Udaipur, 313001 Rajasthan, India*
[2]*School of Agricultural Sciences, Dr. K. N. Modi University, Newai, 304021, Tonk, India*
[3]*Department of Agriculture and Veterinary Sciences, Mewar University, Gangrar, 312901, Chittorgarh, India*
[4]*Division of Microbiology, ICAR-Indian Agricultural Research Institute, 11001, New Delhi, India*
[5]*All India Network Project on Soil Biodiversity and Biofertilizers, Indian Institute of Soil Science, Indian Council of Agricultural Research, 462038, Bhopal, M.P, India*
[6]*Central Department of Microbiology, Tribhuvan University, Kirtipur, Kathmandu, Nepal*

Correspondence should be addressed to Devendra Jain; devroshan@gmail.com and Elina Maharjan; mrjn.elina@gmail.com

Received 5 October 2020; Revised 20 December 2020; Accepted 29 December 2020; Published 11 January 2021

Academic Editor: Marta Laranjo

In the present study, 24 *Azotobacter* strains were isolated from soils of different areas of southern Rajasthan and characterized at biochemical, functional, and molecular levels. The isolated *Azotobacter* strains were gram negative and cyst forming when viewed under the microscope. These strains were also screened for their plant growth promoting activities and the ability of these isolates to survive under abiotic stress conditions *viz.* salt, pH, temperature, and drought stress. All the isolates showed IAA, siderophore, HCN, and ammonia production, whereas seven *Azotobacter* strains showed phosphate solubilization. Amplified Ribosomal DNA Restriction Analysis (ARDRA) revealed significant diversity among *Azotobacter* strains and the dendrogram obtained differentiated twenty-four of the strains into two major clusters at a similarity coefficient of 0.64. Qualitative and quantitative N_2 fixation abilities of these strains were also detrained, and the amounts of acetylene reduced by *Azotobacter* strains were in the range of 1.31 to 846.56 nmol C_2H_4 mg protein^{-1} h^{-1}. The strains showing high nitrogen fixation ability with multiple PGP activities were selected for further pot studies, and these *Azotobacter* strains significantly increased the various plant growth parameters of maize plantlets. Furthermore, the best *Azotobacter* isolates were subjected to 16S rRNA sequencing and confirmed their identities as *Azotobacter* sp. The indigenous *Azotobacter* strains with multiple PGP activities could be further used for commercial production.

1. Introduction

In agriculture, nitrogen deficiency directly influences the yield and profitability of crop plants worldwide and can be overcome by the application of inorganic chemical fertilizers [1]. The increased use of nitrogen based inorganic chemical fertilizers causes serious adverse effects on the physicochemical properties of soil, i.e., degradation of soil organic carbon (SOC) and soil acidification [2]. Plant growth promoting rhizobacteria (PGPR) provides a promising sustainable solution for increasing agricultural productivity by encouraging plant growth and using plenty of growth-promoting pathways,

beneficial plant microbe interactions, etc. and thereby decreases the use of these inorganic chemical based fertilizers [3]. In agriculture, many plant growth promoting microorganisms such as *Azotobacter*, *Rhizobium*, and *Pseudomonas* are used as biofertilizers, which not only provide plant nutrition but also maintain the soil health.

Nitrogen fixing bacteria, especially from the genus *Azotobacter*, holds an important role in soil fertility [4], since it can provide fixed N_2 to plants and promote plant growth by providing other necessary nutrients to plants. *Azotobacter* is gram-negative, nonsymbiotic diazotrophic bacteria that belongs to the *Azotobacteriaceae* family that can fix an average of 20 kg N/ha/per year which can be further increased through inoculation of potent strains [5].

The ecological distribution of *Azotobacter* spp. is a complicated subject that depends on soil characteristics and climate conditions [6]. There are around six species in the genus *Azotobacter* and among them *Azotobacter chroococcum* dominant in Indian soils [7]. Several researchers around the world have successfully used *Azotobacter* as PGPB in biological N_2 fixation, increased nutrient availability in the rhizosphere, and induced root surface not only yielding attributes but also reduced production costs [8, 9].

Biotic and abiotic stress significantly affects crop production and can be overcome by the application of plant growth promoting rhizobacteria, which stimulates plant growth by employing a plethora of growth-promoting mechanisms [10]. *Azotobacter* not only has the capacity to fix atmospheric nitrogen but also has various PGP activities, which makes *Azotobacter* the most effective and widespread among the PGPR community [11].

Despite the availability of *Azotobacter* strains, there is still a need to isolate and characterize native potent indigenous strains adapted to the local environment, which can not only contribute to the formulation of effective bioinoculants but also have a more competitive ability to survive in field conditions [12]. Hence, the aim of this study was to isolate, screen, and characterize the local *Azotobacter* present in the rhizospheric soil of different districts of Rajasthan for various plant growth promoting attributes and to evaluate their nitrogen-fixing ability by the Acetylene Reduction Assay (ARA).

2. Materials and Methods

2.1. Isolation of Azotobacter. Isolation of *Azotobacter* sp. from rhizospheric soils was done by the procedure described by Upadhyay et al. [13]. The rhizospheric soil samples collected from the districts of Rajasthan viz. Udaipur, Banswara, Dungarpur, Rajsamand, and Chittorgarh and were stored at 4°C until processed (Supplementary data sheet Table: S1). Rhizosphere soil was diluted up to 10^{-7} dilutions under aseptic conditions, and *Azotobacter* strains were isolated on Nitrogen free Jensen's medium [14].

2.2. Morphological Characterization of Azotobacter Isolates. Morphological characterization based on colony characteristics *viz.* shape, size, appearance, color, gram staining, pigment production [15], and cyst formation [16] among isolated *Azotobacter* strains was observed.

2.3. Screening of Plant Growth Promoting (PGP) Attributes and Abiotic Stress Tolerance of Azotobacter. All *Azotobacter* strains were further screened for multiple plant growth promoting activities *viz.* IAA production, siderophore production, ammonia production, HCN production, phosphate solubilization, and different abiotic stress tolerance *viz.* pH tolerance, temperature tolerance, salinity tolerance, and drought tolerance according to the methodologies published in our previous research [17, 18].

2.4. Molecular Characterization Using ARDRA of the 16S rDNA Region. The total genomic DNA of *Azotobacter* strains was carried out following the method outlined by Jain et al. [19]. The DNA patterns of the restriction digested 16S rDNA region were amplified and analyzed on agarose gel for all *Azotobacter* isolates as outlined by Jain et al. [19] and Kour et al. [17]. The 16S rDNA amplicons were digested with *Hinf*I, *Alu*I, and *Taq*I restriction endonucleases [20]. Data analysis was performed using the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System) software and SIMUQUAL Jaccards similarity coefficient [21].

2.5. Qualitative and Quantitative Estimation of Nitrogen Fixation. The qualitative nitrogen fixing ability of *Azotobacter* was detected in petri plates containing nitrogen-free malate media (NFM) with bromothymol blue (BTB) dye, and the positive strains produced a blue color zone around the inoculated strains. The amount of total nitrogen produced by *Azotobacter* in Jensen's broth was evaluated by micro-Kjeldahl method as described by Jackson [22]. Furthermore, *Azotobacter* isolates were screened for acetylene reduction ability (ARA) according to the methodologies of Marag and Suman [23], and the ARA results were expressed in nanomoles of ethylene produced per mg protein per hour.

2.6. In Vitro Studies on the Effect of Azotobacter on the Growth and Yield of Maize Seedlings. To study the effect of *Azotobacter* on maize (variety FEM-2), the pot experiments with selected *Azotobacter* strains with multiple PGP and high ARA activities were conducted in triplicate in a complete randomized design (CRD) as per our previous research [17]. The seeds treated with *Azotobacter* inoculant were sown whereas uninoculated control was also maintained. Different plant growth parameters, i.e., shoot length, root length, root number, leaf number, and chlorophyll content, were recorded after 30 days of germination.

2.7. Sequencing and Phylogenetic Analysis of 16S rDNA of Potent Azotobacter Strains. The PCR amplified 16S rDNA regions of efficient *Azotobacter* were sequenced using an automated DNA Sequencer (ABI model 377, Applied Biosystems) as per the method described by Jain et al. [18]. The 16S rDNA sequences were edited before the BLAST using the BioEdit software package. The sequences obtained in the study were compared with previously submitted sequences of the nucleotide database GenBank at National Centre for Biotechnology (NCBI) using the nucleotide BLAST (blastn)

Table 1: PGPR activities of *Azotobacter* isolates.

Isolates	IAA (μg/ml)	PSI (cm)	SPI (cm)	HCN production	NH_3 production
Azo1	26.5 ± 1.31	1.14 ± 0.06	1.5 ± 0.08	++	++
Azo2	25 ± 1.21	NS	1.5 ± 0.09	++	++
Azo3	24.5 ± 1.22	NS	1.45 ± 0.07	+++	+++
Azo4	34 ± 1.8	1.6 ± 0.09	1.2 ± 0.06	+++	+++
Azo5	25 ± 1.25	NS	1.4 ± 0.07	+	++
Azo6	26 ± 1.34	NS	1.08 ± 0.05	+++	++
Azo7	33.5 ± 1.68	1.43 ± 0.07	1.17 ± 0.06	+++	+++
Azo8	25 ± 1.26	NS	1.25 ± 0.07	+++	+++
Azo9	17 ± 0.91	NS	1.42 ± 0.09	+++	++
Azo10	31 ± 1.56	NS	1.46 ± 0.09	+++	+++
Azo11	24.5 ± 1.26	NS	1.46 ± 0.05	+++	+++
Azo12	20 ± 1.11	NS	1.58 ± 0.08	++	++
Azo13	20.5 ± 1.04	NS	1.5 ± 0.08	+++	++
Azo14	26 ± 1.33	1.78 ± 0.10	1.67 ± 0.08	+	++
Azo15	28.5 ± 1.42	3.61 ± 0.18	1.29 ± 0.06	+++	+++
Azo16	16.5 ± 0.87	NS	1.66 ± 0.09	++	++
Azo17	31.5 ± 1.59	NS	1.45 ± 0.06	+++	+++
Azo18	27 ± 1.37	NS	1.29 ± 0.06	+++	++
Azo19	26.5 ± 1.33	NS	1.35 ± 0.07	+++	++
Azo20	22.5 ± 1.13	1.67 ± 0.08	1.64 ± 0.08	++	+
Azo21	20 ± 1.12	NS	1.81 ± 0.09	+++	+++
Azo22	22 ± 1.11	NS	1.29 ± 0.06	+++	++
Azo23	24 ± 1.24	NS	1.53 ± 0.07	+	+++
Azo24	21 ± 1.05	1.26 ± 0.07	1.44 ± 0.08	+++	+

Value ± SD. NS: no solubilization; PSI: phosphorus solubilizing index; SPI: siderophore production index.

[24]. The 16S rDNA consensus sequences were aligned using an online tool CLUSTAL-W [25]. This alignment was further used for phylogenetic tree construction through the MEGA 6.06 software using the Maximum Likelihood method [26].

3. Results and Discussion

3.1. Isolation and Characterization of the Azotobacter Strains. In the present study, 24 *Azotobacter* strains were isolated from different rhizospheric soil samples of Southern Rajasthan on Jensen's medium. The morphological characteristics of *Azotobacter* strains are summarized in Table S2 (Supplementary data sheet). Based on cell morphology and pigment production, these isolates were preliminary characterized as *Azotobacter*. All these isolates were able to grow in nitrogen-free medium without producing water-soluble pigments. Microscopic studies confirmed that all these strains are cyst forming gram-negative rods when examined under the microscope. The present findings were corroborated with those of Khosravi and Dolatabad [27] who isolated *Azotobacter* from rhizospheric soils from arid and semiarid regions of Iran.

3.2. Screening of Plant Growth Promoting (PGP) Attributes and Abiotic Stress Tolerance of Azotobacter Strains. All 24 *Azotobacter* strains were subjected to various plant growth promoting activities, and the results are summarized in Table 1. All 24 *Azotobacter* strains were able to produce IAA ranging from 16.5 to 34 $\mu g\,ml^{-1}$ in media supplemented with tryptophan. The maximum IAA production was observed by Azo4 (34 $\mu g\,ml^{-1}$) followed by Azo7 (33.5 $\mu g\,ml^{-1}$) whereas the least by Azo16 (16.5 $\mu g\,ml^{-1}$). In the present study, IAA production by *Azotobacter* strains were slightly higher as compared with an earlier report from Pandey et al. [11] where 23 μg/ml of IAA production was recorded in *Azotobacter* strain AU1.

Out of the 24 strains, only seven were phosphate solubilizers, and the phosphate solubilization index (PSI) ranged between 1.14 and 3.61, and the strain Azo15 showed the highest solubilization index, i.e., 3.61. Nosrati et al. [28] studied the phosphate solubilization potentials of twenty *Azotobacter* isolates and reported the PSI for these strains between 3.5 ± 0.1 and 1.4 ± 0.1 which was also observed in the present investigation.

In the present study, all 24 strains were found to be positive for siderophore production, which was determined by their ability to produce the distinct orange halo on the

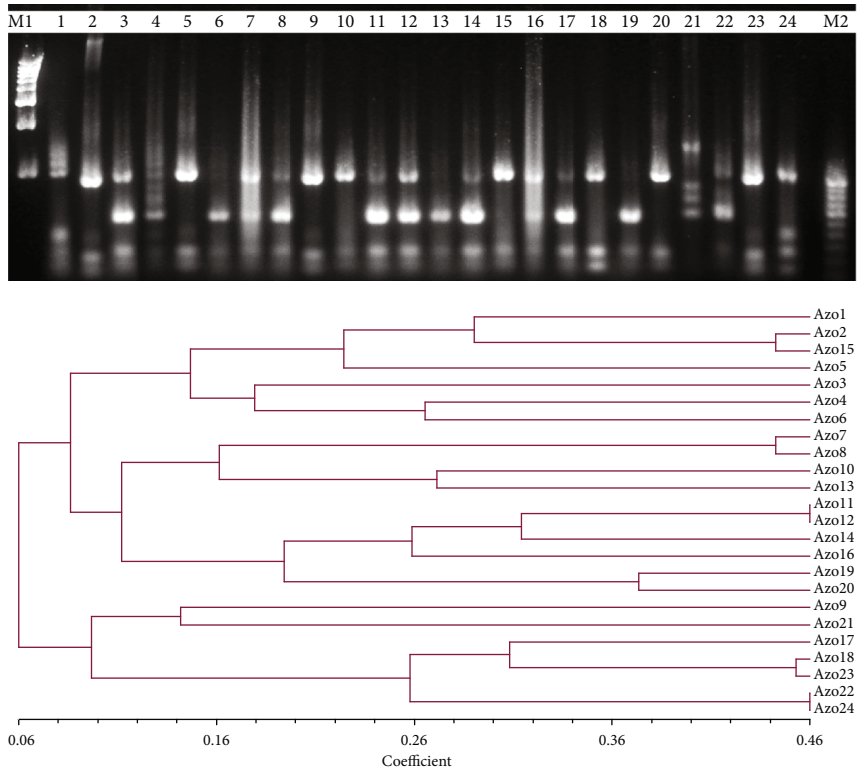

Figure 1: (a) Gel showing ARDRA pattern of 24 *Azotobacter* strains by *Hinf*I. (b) Dendrogram generated using UPGMA cluster analysis.

chrome azurol S (CAS) agar media. The highest siderophore production index was shown by Azo21 (1.81) whereas the least by Azo6 (1.08). Ferreira et al. [29] reported that *A. vinelandii* produced the highest levels of siderophore (80–140 μmol l^{-1}) after 72 h incubation.

All *Azotobacter* strains were able to produce HCN, and out of 24 isolates, 16 isolates exhibited strong (+++) production, 5 isolates as moderate (++), and 3 isolates as weak (+). Whereas all *Azotobacter* strains were ammonia producers and 10 were recorded as strong (+++) ammonia producers, 12 as moderate (++) ammonia producers, and 2 showed poor (+) ammonia production. Chennappa et al. [30] studied the PGP activities of ten *Azotobacter* strains and reported that all strains were able to produce HCN and ammonia, which also supports finding of the present investigation.

The ability of *Azotobacter* strains to tolerate abiotic stress was also evaluated, and the results are summarized in supplementary data sheet (Table S3-S6). Among the 24 strains of *Azotobacter*, 6 strains exhibited growth at pH 4.0; all the strains were able to grow at pH 6.0, whereas 4 strains were able to grow at pH 8.0 and one strain at pH 10.0. Similar results were reported by Jimenez et al. [31] where pH 6.0-7.0 were determined as optimum for *Azotobacter* strains isolated from different crop plant soil samples.

Azotobacter growth was also determined for salt tolerance using varying concentration of NaCl in Jensen broth. At 1-4% NaCl concentration, all 24 strains showed profuse growth, and at 5% NaCl concentration, 20 strains showed profuse growth, and 4 strains showed moderate growth, whereas at 6% NaCl concentration, 7 strains showed profuse growth, and the remaining 17 strains showed moderate growth. At 7% NaCl concentration, 3 strains showed moderate, and 13 strains showed week growth, and at 8% NaCl concentration, 3 strains showed moderate, and 1 strain showed weak growth. At 9%NaCl concentration, 2 strains showed moderate, and 2 showed weak growth, whereas at 10% salt concentration, only two strains showed weak growth. The above result agreed with the findings of Chennappa et al. [30] who studied four *Azotobacter* species for salt tolerance and reported that *A. chroococcum*, *A. vinelandii*, and *A. salinestris* can tolerate up to 8% NaCl concentration; however, the cell counts were reduced with high concentration of NaCl.

All 24 strains were subjected to temperature stress, and at 20°C, poor growth was noted in 8 *Azotobacter* strains whereas moderate growth was observed in 16 isolates. At 30°C, all 24 *Azotobacter* strains showed strong growth whereas at 35°C and 40°C only 2 strains and 1 strain, respectively, showed strong growth. At 45°C, 20 *Azotobacter* strains showed poor growth, and 4 isolates failed to grow at this temperature. Similar results are corroborated by Chennapa et al. [30] where they studied temperature tolerance in *Azotobacter* and reported that the strains of *A. chroococcum* were found to be tolerant to the temperature range of 35-45°C.

Azotobacter growth was determined for drought stress using varying concentration of PEG in Jensen broth. At 10%, 20%, 30%, and 40% PEG concentration, Azo18, Azo11, Azo10, and Azo7 strains showed moderate to high growth, respectively. Reports are available on PGP bacteria tolerance to drought stress (20-40%) induced by PEG by Marulanda et al. [32]. Ali et al. [33] reported drought tolerance among thirty-two selected PGP bacterial isolates by PEG (6000) and hence could withstand under water stress conditions.

Table 2: Qualitative and quantitative estimation of nitrogen fixation by *Azotobacter* strains.

Isolates	N_2 fixation using NFMM with BTB*	N_2 fixed** (mg g^{-1} sucrose)	ARA*** (nmol C_2H_4 mg $protein^{-1}$ hr^{-1})
Azo1	Positive	24.197 ± 0.97	846.561 ± 40.02
Azo2	Positive	6.672 ± 0.3	6.138 ± 0.29
Azo3	Positive	4.411 ± 0.22	46.804 ± 2.39
Azo4	Positive	7.519 ± 0.36	70.582 ± 3.8
Azo5	Positive	20.239 ± 0.95	47.968 ± 2.06
Azo6	Negative	6.672 ± 0.3	2.106 ± 0.25
Azo7	Positive	15.435 ± 0.71	343.947 ± 18.01
Azo8	Positive	6.672 ± 0.2	85.926 ± 4.01
Azo9	Positive	8.085 ± 0.39	55.344 ± 2.46
Azo10	Positive	4.411 ± 0.2	45.397 ± 2.20
Azo11	Positive	9.216 ± 0.41	139.788 ± 7
Azo12	Negative	8.368 ± 0.44	10.529 ± 0.64
Azo13	Positive	12.890 ± 0.67	261.143 ± 11.08
Azo14	Positive	17.413 ± 0.69	66.455 ± 3.01
Azo15	Positive	14.021 ± 0.7	283.122 ± 14
Azo16	Negative	6.954 ± 0.29	1.3122 ± 0.06
Azo17	Positive	6.389 ± 0.3	80.614 ± 4.1
Azo18	Positive	10.063 ± 0.5	159.048 ± 6
Azo19	Positive	6.106 ± 0.32	4.317 ± 0.3
Azo20	Positive	17.130 ± 0.85	668.519 ± 33.01
Azo21	Positive	5.824 ± 0.3	4.359 ± 0.2
Azo22	Negative	3.173 ± 0.13	8.265 ± 0.5
Azo23	Positive	5.824 ± 0.3	46.402 ± 2.5
Azo24	Positive	4.976 ± 0.2	45.513 ± 1.9
CD at 5%	—	0.748152188	17.03123459
CV%	—	4.695825426	7.468182458

Value ± standard deviation. *Positive *Azotobacter* strains produced a blue color zone on the medium indicating the nitrogen fixation; **using micro-Kjeldhal method; ***acetylene reduction assay.

3.3. Molecular Characterization Using ARDRA. The genetic diversity among the twenty-four *Azotobacter* isolates was assessed by the PCR-RFLP of 16S rDNA. Three restriction endonucleases *viz. Hinf*I, *Alu*I, and *Taq*I produced 57 banding patterns of varying sizes in 24 *Azotobacter* strains upon digestion of the 16S rDNA amplicon (Figure 1(a)). On the basis of restriction digestion by *Hinf*I, 21 different patterns were obtained, whereas digestion by *Taq*I and *Alu*I produced 16 and 20 different patterns, respectively. Based on UPGMA clustering analysis, the *Azotobacter* strains showed significant molecular diversity, and the dendrogram obtained differentiated 24 strains into 2 major clusters A and B comprising 17 and 7 strains, respectively, at the similarly coefficient of 0.10 (Figure 1(b)). Similarity indices established on the basis 57 bands of the three restriction enzymes ranged from 0.04 to 0.45. Genetic relationship among 24 *Azotobacter* strains was also visualized by performing PCA, and the *Azotobacter* strains occupied the same position in two-dimensional and three-dimensional scaling as observed in the dendrogram. Therefore, different fingerprinting profiles were obtained which showed significant genetic diversity of *Azotobacter* strains selected from different districts of southern Rajasthan.

Rubio et al. [34] identified genetic diversity among *A. chroococcum*, *A. salinestris*, and *A. Armeniacus* by PCR-RFLP of 16S rDNA sequences using the enzymes *Rsa*I and *Hha*I. Mazinani and Asgharzadeh [35] also reported the molecular identification of three *Azotobacter* sp., i.e., *A. chroococcum*, *A. vinelandii*, and *A. beijernckii* using *Rsa*I, *Hpa*II, and *Hha*I. Khosravi and Dolatabad [27] studied the molecular differentiation and diversity analysis of *Azotobacter* species and reported that the ARDRA technique with *Hpa*II, BOX, and REP PCR based markers was able to differentiate between *A. chroococcum* and *A. salinestris*.

3.4. Detection of Nitrogen Fixing Activity. To identify the free nitrogen fixers among these isolates, they were preliminary screened on nitrogen-free malate agar medium (NFMM) containing bromothymol blue (BTB) as an indicator. Out of 24 isolates, twenty *Azotobacter* strains produced a blue color zone on the medium indicating the fixation of nitrogen by them. The studies by Suleiman et al. [36] and Gothwal et al. [37] also reported a similar result. Furthermore, the amounts of total N_2 fixed by *Azotobacter* strains ranged between 3.17 and 24.19 mg g^{-1} sucrose using micro-Kjeldhal method (Table 2). The highest amount of N_2 was fixed by Azo1 while the lowest by Azo22. Upadhyay et al. [13] reported the amounts of N_2 fixed by different *Azotobacter* strains ranged from 18.88 to 6.04 mg g^{-1}sucrose and is in close agreement with the results of N_2 fixation in the present study.

Acetylene reduction assay (nitrogenase activity) was used as an estimation of the rate of nitrogen fixation by *Azotobacter* [38]. The *Azotobacter* strains showed the ARA activity in the range from 1.312 ± 0.06 to 846.561 ± 40.02 nmol C_2H_4 produced mg $protein^{-1}$ h^{-1}. Maximum nitrogenase of 846.561 ± 40.02 nmol C_2H_4 produced mg $protein^{-1}$ h^{-1} was observed for isolate Azo1, and the least ARA activity of 1.312 ± 0.06 nmol C_2H_4 mg $protein^{-1}$ h^{-1} was observed for Azo16. Nosrati et al. [28] recorded amounts of acetylene reduced by *A. vinelandii* isolates in the range of 12.1 to 326.4 nmol C_2H_4 h^{-1} $vial^{-1}$.

3.5. In Vitro Studies on the Effect of Azotobacter on the Growth and Yield of Maize Seedlings. The pot culture experiment was conducted in plastic pots filled with sterile planting mixture. To study the plant growth promoting activity of *Azotobacter* isolates, maize cultivable variety (FEM-2) seeds were treated with *Azotobacter* inoculant (seed bacterization method) and sown. Fourteen *Azotobacter* strains were selected for pot studies based on their higher ARA values. Pot experiment data recorded after 30 days of germination are summarized in Table 3.

Higher plant growth was observed in maize seedlings raised from bacterized seeds with selected *Azotobacter* isolates compared with absolute control. This indicates the

Table 3: *In vitro* studies on the effect of *Azotobacter* on growth and yield of maize seedling.

S. no.	Treatment	Average shoot length (cm)	Average root length (cm)	Average root number	Average leaf number	Total chlorophyll (μg/ml)
1	Control	12.5 ± 0.12^{cd}	31.5 ± 0.66^{ef}	9.66 ± 0.58^{f}	5 ± 1.0^{a}	30.14 ± 0.95^{i}
2	Azo1	18.9 ± 0.20^{a}	38.5 ± 0.95^{c}	18.33 ± 1.15^{a}	6.3 ± 0.58^{a}	52.83 ± 1.90^{ab}
3	Azo3	12 ± 0.50^{d}	31.65 ± 0.96^{ef}	10.67 ± 0.58^{ef}	5.3 ± 1.53^{a}	31.56 ± 1.49^{hi}
4	Azo5	12 ± 0.38^{d}	32.45 ± 1.05^{ef}	11.67 ± 0.58^{def}	5 ± 0.0^{a}	34.71 ± 1.20^{fghi}
5	Azo7	17.95 ± 0.51^{a}	38.69 ± 1.03^{c}	17.67 ± 0.58^{ab}	5.67 ± 0.58^{a}	48.50 ± 1.15^{bc}
6	Azo9	13.56 ± 0.52^{bcd}	31.12 ± 0.98^{f}	10.33 ± 1.53^{ef}	6.3 ± 0.58^{a}	35.72 ± 1.27^{fgh}
7	Azo11	14.23 ± 1.16^{bc}	32.78 ± 1.07^{ef}	10.67 ± 1.15^{ef}	5.3 ± 0.58^{a}	32.49 ± 1.98^{ghi}
8	Azo13	18.2 ± 0.62^{a}	47.23 ± 0.94^{ab}	18.33 ± 1.53^{a}	6.3 ± 0.58^{a}	47.1 ± 1.86^{cd}
9	Azo14	11.88 ± 0.58^{d}	32.3 ± 1.12^{ef}	10.67 ± 1.53^{ef}	5 ± 0.0^{a}	39.2 ± 2.05^{ef}
10	Azo15	17.68 ± 0.51^{a}	45.95 ± 1.90^{b}	16.33 ± 1.15^{abc}	5.3 ± 0.58^{a}	42.85 ± 2.26^{de}
11	Azo17	14.59 ± 1.02^{b}	32.96 ± 1.10^{ef}	13.67 ± 0.58^{cde}	5.67 ± 0.58^{a}	37.87 ± 2.15^{efg}
12	Azo18	13.26 ± 0.54^{bcd}	38.16 ± 0.93^{c}	13.33 ± 0.58^{cde}	6.3 ± 0.58^{a}	37.13 ± 2.05^{fg}
13	Azo20	18.49 ± 0.62^{a}	49.58 ± 1.15^{a}	16.67 ± 2.08^{abc}	6.67 ± 0.58^{a}	54.39 ± 2.90^{a}
14	Azo23	13.85 ± 1.05^{bcd}	37.23 ± 1.2^{cd}	14.33 ± 0.58^{bcd}	5.3 ± 0.58^{a}	38.67 ± 1.08^{ef}
15	Azo24	14.34 ± 1.04^{bc}	34.54 ± 1.24^{de}	11.66 ± 1.53^{def}	5 ± 0.0^{a}	34.76 ± 2.05^{fghi}
	CD at 5%	1.110036913	1.91982297	1.731219988	1.128863211	3.009485161
	CV%	4.454842219	3.103901796	7.611017199	11.95775004	4.513783099

Each value is a mean of 3 replicates from 2 experiments. Mean ± SD followed by the same letter in a column of each treatment is not a significant difference at $p = 0.05$ by the Tukey–Kramer HSD test. Data are recorded after 30 days of germination.

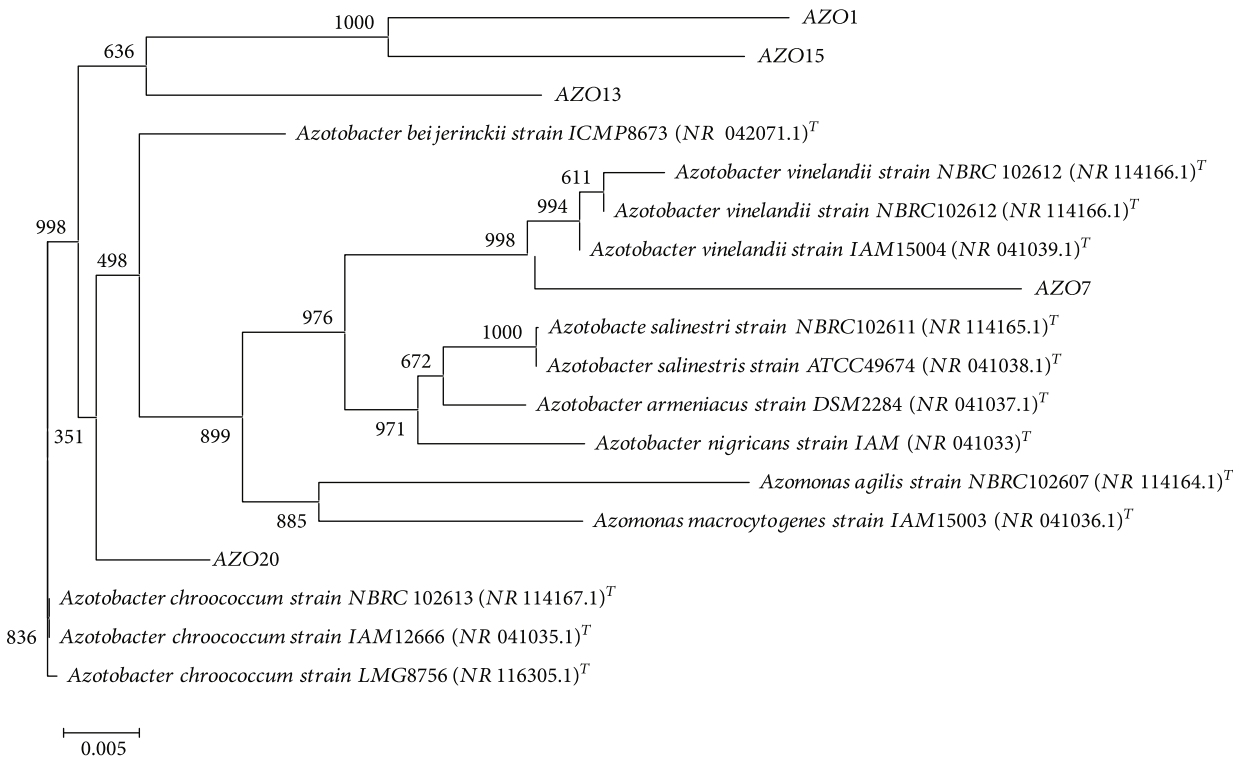

Figure 2: Phylogenetic tree of native *Azotobacter* strains based on the 16S rDNA with closely related type strains using the MEGA 6.06 software. GenBank accession numbers (in parenthesis) are listed after the species names. Bootstrap values are indicated at branching points. The scale bar indicates five base substitutions for 100 nucleotide positions.

Table 4: Identification of native *Azotobacter* strains by 16S rDNA sequencing.

Strain	Molecular identity	GenBank accession no.	Closest type strain Species (strain)	Accession no.
Azo1	*Azotobacter chroococcum*	MT312860	*Azotobacterchroococcum* strain LMG 8756	NR_116305
Azo7	*Azotobacter vinelandii*	MT312861	*Azotobactervinelandii* strain NBRC 102612	NR_114166
Azo13	*Azotobacter chroococcum*	MT312862	*Azotobacterchroococcum* strain NBRC 102613	NR_114167
Azo15	*Azotobacter chroococcum*	MT312863	*Azotobacterchroococcum* strain IAM 12666	NR_041035
Azo20	*Azotobacter chroococcum*	MT312864	*Azotobacterchroococcum* strain NBRC 102613	NR_114167

positive effect of *Azotobacter* strains on maize plantlets. Shoot length, root length, root number, and total chlorophyll content have been significantly increased in the maize plantlets inoculated with the selected *Azotobacter* strains as compared to uninoculated control.

Shoot length increases by 51.20% in pots inoculated with Azo1 followed by Azo20 (47.92%). Root length increases by 57.39% in pots inoculated with Azo20 followed by Azo13 (49.94%). Root number increases by 89.75% in pots inoculated with Azo1 and Azo13. Leaf number increases by 33.4% in pot inoculated with Azo20 followed by Azo1 and Azo13 (26.0%). Chlorophyll content increased by 80.46% in pots inoculated with Azo20 followed by Azo1 (75.28%). All *Azotobacter* strains significantly influenced the observed parameters as compared to uninoculated control and contributed to plant growth. Similar finding was observed by Mahato and Neupane [39] who reported *Azotobacter* seed bacterization in maize stimulated the growth of treated plants as characterized by the increase of root and shoot length. Romero-Perdomo et al. [40] reported *Azotobacter* bacterial inoculation in cotton positively influenced plant growth parameters reducing 50% nitrogen fertilization dose. These findings advocated the application of *Azotobacter* for the improvement of plant growth due to their intrinsic ability of fixing atmospheric nitrogen and expressing plant growth-promoting substances.

3.6. Molecular Identification of the Isolates. The full length sequence of 16S rDNA gene of the most promising *Azotobacter* strains based on the multiple PGP activities, nitrogen fixation ability, and *in vitro* performance *viz.* Azo1, Azo7, Azo13, Azo15, and Azo20 were sequenced and analyzed using the nucleotide BLAST tool. Based on the BLAST results, these strains showed the greatest sequence identity with the previously reported type strains of *Azotobacter chroococcum* and *Azotobacter vinelandii* (Figure 2). Indian soils are previously reported to have both *chroococcum* and *vinelandii* species of *Azotobacter* [11, 41] also supporting the present study. The molecular identities and NCBI GenBank accession number assigned to these strains are presented in Table 4.

In the present study, biochemical and molecular characterization of *Azotobacter* strains was reported from the soils of Southern Rajasthan, and based on 16S rDNA sequencing, the potent strain was characterized as *Azotobacter chroococcum* and *Azotobacter vinelandii*. In this research, additional molecular techniques including ARDRA revealed significant genetic diversity among *Azotobacter* strains studied in this study. The plant growth promoting traits and nitrogen fixation by local *Azotobacter* strains is very critical for the selection of such strains for biofertilizer formulations in order to replace the ineffective strains. The results indicated that inoculation with multi-PGP *Azotobacter* strains significantly improved the plant growth under *in vitro* conditions and may be used for commercial production. Hence, dedicated field studies are required to confirm the efficacy of these *Azotobacter* strains.

Data Availability

The data used to support the findings of this study are included within the supplementary information file(s).

Conflicts of Interest

No potential conflict of interest was reported by the authors.

Authors' Contributions

DJ, SRM, and EM conceived and designed the experiments. JS, GK, AAB, and SC performed the laboratory experiments. AS performed the ARA. EM, DJ, and VS wrote the manuscript. All authors read and approved the final manuscript. DJ and JS contributed equally to the work.

Acknowledgments

The financial assistance from the All India Network Project on soil biodiversity and biofertilizers and the Rastriya Krishi VikasYojana (RKVY) research project is highly acknowledged.

Supplementary Materials

Table S1: location of different isolated *Azotobacter* strains from Rajasthan. Table S2: morphological characterization of *Azotobacter* isolates. Table S3: pH tolerance in *Azotobacter* isolates. Table S4: temperature stress tolerance in *Azotobacter* isolates. Table S5: salinity tolerance in *Azotobacter* isolates. Table S6: drought tolerance in *Azotobacter* isolates *(Supplementary Materials)*

References

[1] X. E. Pantazi, D. Moshou, and D. Bochtis, *Intelligent Data Mining and Fusion Systems in Agriculture. Utilization of multisensors and data fusion in precision agriculture*, Academic Press, San Diego (CA), 2020.

[2] L. Liu, C. Zhu, S. Xu, Y. Li, H. X. Zheng, and R. Shi, "Combined application of organic and inorganic nitrogen fertilizers affects soil prokaryotic communities compositions," *Agronomy*, vol. 10, no. 1, p. 132, 2020.

[3] S. Timmusk, L. Behers, J. Muthoni, A. Muraya, and A. C. Aronsson, "Perspectives and challenges of microbial application for crop improvement," *Frontiers in Plant Science*, vol. 8, p. 49, 2017.

[4] S. E. Fischer, S. I. Fischer, S. Magris, and G. B. Mori, "Isolation and characterization of bacteria from the rhizosphere of wheat," *World Journal of Microbiology and Biotechnology*, vol. 23, no. 7, pp. 895–903, 2007.

[5] E. R. Eid, M. N. Awad, and H. A. Hamouda, "Evaluate effectiveness of bio and mineral fertilization on the growth parameters and marketable cut flowers of *Matthio laincana* L.," *American-Eurasian Journal of Agricultural and Environmental Science*, vol. 5, pp. 509–518, 2009.

[6] J. González-López, "Microorganismos diazotrofos asociados a raíces de plantas no leguminosas," in *Interacción Planta-Microorganismo*, J. González-López and C. Lluch, Eds., Biología del Nitrógeno, Rueda, Madrid, Spain, 1992.

[7] S. Martyniuk and M. Martyniuk, "Occurrence of *Azotobacter spp.* in some polish soils," *Polish Journal of Environmental Studies*, vol. 12, pp. 371–374, 2003.

[8] A. Namvar and T. Khandan, "Response of wheat to mineral nitrogen fertilizer and biofertilizer (*Azotobacter* sp. and *Azospirillum* sp.) inoculation under different levels of weed interference," *Ekologija*, vol. 59, pp. 85–94, 2013.

[9] A. Amiri and M. Rafiee, "Effect of soil inoculation with *Azospirillum* and *Azotobacter* bacteria on nitrogen use efficiency and agronomic characteristics of corn," *Annals of Biological Research*, vol. 4, pp. 77–79, 2013.

[10] J. Yang, J. W. Kloepper, and C. M. Ryu, "Rhizosphere bacteria help plants tolerate abiotic stress," *Trends in Plant Science*, vol. 14, no. 1, pp. 1–4, 2009.

[11] S. Pandey, S. Gupta, and N. Ramawat, "Unravelling the potential of microbes isolated from rhizospheric soil of chickpea (*Cicer arietinum*) as plant growth promoter," *3 Biotech*, vol. 9, no. 7, p. 277, 2019.

[12] A. Khalid, M. Arshad, and Z. A. Zahir, "Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat," *Journal of Applied Microbiology*, vol. 96, no. 3, pp. 473–480, 2004.

[13] S. Upadhyay, N. Kumar, V. K. Singh, and A. Singh, "Isolation, characterization and morphological study of *Azotobacter* isolates," *Journal of Applied and Natural Science*, vol. 7, no. 2, pp. 984–990, 2015.

[14] H. L. Jensen, "Non-symbiotic nitrogen fixation, in Soil Nitrogen Series of Agronomy," in *Mongr. No. 10*, W. V. Bartholomew and F. E. Clark, Eds., American Society of Agronomy Madison, Wisconsin, 1965.

[15] J. Bush and P. A. Wilson, "A Non-Gummy Chromogenic Strain of *Azotobacter vinelandii*," *Nature*, vol. 184, no. 4683, pp. 381-382, 1959.

[16] G. R. Vela and G. Cagle, "Formation of fragile cysts by a strain of *Azotobacter chroococcum*," *Journal of General Microbiology*, vol. 57, no. 3, pp. 365–368, 1969.

[17] R. Kour, D. Jain, A. A. Bhojiya et al., "Zinc biosorption, biochemical and molecular characterization of plant growth-promoting zinc-tolerant bacteria," *3 Biotech*, vol. 9, p. 421, 2019.

[18] D. Jain, S. Sanadhya, H. Saheewala et al., "Molecular diversity analysis of plant growth promoting *Rhizobium* isolated from groundnut and evaluation of their field efficacy," *Current Microbiology*, vol. 77, no. 8, pp. 1550–1557, 2020.

[19] D. Jain, S. D. Sunda, S. Sanadhya, D. J. Nath, and S. K. Khandelwal, "Molecular characterization and PCR-based screening of cry genes from *Bacillus thuringiensis* strains," *3 Biotech*, vol. 7, no. 1, 2017.

[20] C. A. Cihan, N. Tekin, B. Ozcan, and C. Cokmus, "The genetic diversity of genus *Bacillus* and the related genera revealed by 16s rRNA gene sequences and ARDRA analyses isolated from geothermal regions of Turkey," *Brazilian Journal of Microbiology*, vol. 43, no. 1, pp. 309–324, 2012.

[21] F. J. Rohlf, *NTSYSpc numerical taxonomy and multivariate analysis system version 2.0 user guide*, Applied Biostatistics Inc, Setauket, New York, 1998.

[22] M. L. Jackson, *Soil Chemical Analysis*, Prentice Hall of India Pvt. Ltd., New Delhi, 1967.

[23] P. S. Marag and A. Suman, "Growth stage and tissue specific colonization of endophytic bacteria having plant growth promoting traits in hybrid and composite maize (*Zea mays* L.)," *Microbiological Research*, vol. 214, pp. 101–113, 2018.

[24] S. F. Altschul, W. Gish, W. Miller, E. W. Myers, and D. J. Lipman, "Basic local alignment search tool," *Journal of Molecular Biology*, vol. 215, no. 3, pp. 403–410, 1990.

[25] J. D. Thompson, D. G. Higgins, and J. Gibson, "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice," *Nucleic Acids Research*, vol. 22, no. 22, pp. 4673–4680, 1994.

[26] K. Tamura, G. Stecher, D. Peterson, A. Filipski, and S. Kumar, "MEGA6: molecular evolutionary genetics analysis version 6.0.," *Molecular Biology and Evolution*, vol. 30, no. 12, pp. 2725–2729, 2013.

[27] H. Khosravi and H. K. Dolatabad, "Identification and molecular characterization of *Azotobacter chroococcum* and *Azotobacter salinestris* using ARDRA, REP, ERIC, and BOX," *Molecular Biology Reports*, vol. 47, no. 1, pp. 307–316, 2020.

[28] R. Nosrati, P. Owlia, H. Saderi, I. Rasooli, and M. A. Malboobi, "Phosphate solubilization characteristics of efficient nitrogen fixing soil *Azotobacter* strains," *Iranian journal of microbiology*, vol. 6, no. 4, pp. 285–295, 2014.

[29] C. M. H. Ferreira, Â. Vilas-Boas, C. A. Sousa, H. M. V. M. Soares, and E. V. Soares, "Comparison of five bacterial strains producing siderophores with ability to chelate iron under alkaline conditions," *AMB Express*, vol. 9, no. 1, p. 78, 2019.

[30] G. Chennappa, M. K. Naik, C. R. Adkar-Purushothama, Y. S. Amaresh, and M. Y. Sreenivasa, "PGP potential, abiotic stress tolerance and antifungal activity of *Azotobacter* strains isolated from paddy soils," *Indian Journal of Experimental Biology*, vol. 54, no. 5, pp. 322–331, 2016.

[31] D. J. Jiménez, J. S. Montaña, and M. M. Martínez, "Characterization of free nitrogen fixing bacteria of the genus *Azotobacter* in organic vegetable-grown Colombian soils," *Brazilian Journal of Microbiology*, vol. 42, no. 3, pp. 846–858, 2011.

[32] A. Marulanda, J. M. Barea, and R. Azcón, "Stimulation of plant growth and drought tolerance by native microorganisms (AM Fungi and bacteria) from dry environments: mechanisms related to bacterial effectiveness," *Journal of Plant Growth Regulation*, vol. 28, no. 2, pp. 115–124, 2009.

[33] S. Z. Ali, V. Sandhya, and L. V. Rao, "Isolation and characterization of drought-tolerant ACC deaminase and exopolysaccharide producing fluorescent *Pseudomonas* sp," *Annals of microbiology*, vol. 64, pp. 493–502, 2014.

[34] E. J. Rubio, M. S. Montecchia, M. Tosi, F. D. Cassan, A. Perticari, and O. S. Correa, "Genotypic characterization of azotobacteria isolated from Argentinean soils and plant-growth-promoting traits of selected strains with prospects for biofertilizer production," *The Scientific World Journal*, vol. 2013, Article ID 519603, 12 pages, 2013.

[35] Z. Mazinani and A. Asgharzadeh, "Genetic diversity of *Azotobacter* strains isolated from soils by amplified ribosomal DNA restriction analysis," *Cytology and Genetics*, vol. 48, no. 5, pp. 293–301, 2014.

[36] M. K. Suleiman, A. M. Quoreshi, N. R. Bhat, A. J. Manuvel, and M. T. Sivadasan, "Divulging diazotrophic bacterial community structure in Kuwait desert ecosystems and their N_2-fixation potential," *PLoS One*, vol. 14, no. 12, p. e0220679, 2019.

[37] R. K. Gothwal, V. K. Nigam, M. K. Mohan, D. Sasmal, and P. Ghosh, "Screening of nitrogen fixers from rhizospheric bacterial isolates associated with important desert plants," *Applied Ecology and Environmental Research*, vol. 6, no. 2, pp. 101–109, 2008.

[38] V. M. Hitchins and H. L. Sadoff, "Sequential metabolic events during encystment of *Azobacter vinelandii1*," *Journal of Bacteriology*, vol. 113, no. 3, pp. 1273–1279, 1973.

[39] S. Mahato and S. Neupane, "Comparative study of impact of *Azotobacter* and *Trichoderma* with other fertilizers on maize growth," *Journal of Maize Research and Development*, vol. 3, no. 1, pp. 1–16, 2017.

[40] F. Romero-Perdomo, J. Abril, M. Camelo et al., "*Azotobacter chroococcum* como biofertilizante bacteriano potencialmente util para el algodon (*Gossypium hirsutum* L.): efecto en la reducci on de la fertilizacion nitrogenada," *Revista Argentina de Microbiología*, vol. 49, no. 4, pp. 377–383, 2017.

[41] R. K. Sahoo, M. W. Ansari, M. Pradhan, T. K. Dangar, S. Mohanty, and N. Tuteja, "A novel *Azotobacter vinellandii* (SRIAz3) functions in salinity stress tolerance in rice," *Plant Signaling & Behavior*, vol. 9, no. 7, p. e29377, 2014.