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ORIGINAL ARTICLE

Phenotypic and molecular characterization of native *Azospirillum* strains from rice fields to improve crop productivity

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Abstract Beneficial microorganisms have been considered as an important tool for crop improvement. Native isolates of *Azospirillum* spp. were obtained from the rhizospheres of different rice fields. Phenotypic, biochemical and molecular characterizations of these isolates led to the identification of six efficient strain of *Azospirillum*. PCR amplification of the *nif* genes (*nif*H, *nif*D and *nif*K) and protein profile of *Azospirillum* strains revealed inter-generic and inter-specific diversity among the strains. In vitro nitrogen fixation performance and the plant growth promotion activities, viz. siderophore, HCN, salicylic acid, IAA, GA, zeatin, ABA, NH_3, phosphorus metabolism, ACC deaminase and iron tolerance were found to vary among the *Azospirillum* strains. The effect of *Azospirillum* formulations on growth of rice var. *Khandagiri* under field condition was evaluated, which revealed that the native formulation of *Azospirillum* of CRRI field (As6) was most effective to elevate endogenous nutrient content, and improved growth and better yield are the result. The 16S rRNA sequence revealed novelty of native *Azospirillum lipoferum* (As6) (JQ796078) in the NCBI database.

Keywords *Azospirillum* · Biofertilizer · Crop improvement · Plant growth · Rice rhizosphere · Plant yield

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Introduction

Microorganisms were used as an important tool in sustainable agriculture since the last two decades in various parts of the world. The use of plant growth-promoting rhizobacteria (PGPR) in agriculture is an important practice in order to increase the fertility of soil. The use of PGPR is steadily increased in agriculture and offers an attractive way to replace chemical fertilizers, pesticides and supplements (Freitas et al. 2007). Rice (*Oryza sativa* L., Family Poaceae) is the earliest crop gathered, consumed and cultivated worldwide, i.e. in >122 countries (excluding Antarctica) for >10,000 years (yr) (Kenmore 2003; Zeigler and Adams 2008). Rice production typically depends on nitrogen (N) applications because the rhizospheric soil of rice-growing area is deficient in N, globally (Choudhury and Kennedy 2004; Bashan and de-Bashan 2010). Chemical fertilizers degrade soil and environment, reduce fertilizer use efficiency of the crops and inherently pollute the terrestrial ecologies. Nitrogen fertilizer application in anaerobic (flooded) rice would lead to emission of nitrogen oxides (especially N_2O) ranging from 0.02–0.04 % from the soil atmosphere (Briones et al. 2002; Shrestha and Maskey 2005; Bhattacharjee et al. 2008), whereas microbes keep the biogeochemical processes like C, N, S, P, K, Fe, Zn and Mn cycles operational in the soil and metabolize the xenobiotic compounds, plant growth factors, toxins, inhibitors, etc. to sustain plant growth and development (Sahrawat 2000; Choudhury and Kennedy 2004; Subba Rao 2007; Bashan and de-Bashan 2010).

Current environmental protection relies on ecologically clean technique of crop production that maximizes use of natural sources of bound N. In addition, scarcity and the increasing cost of non-renewable chemical fertilizers necessitated the greater use of renewable indigenous biological N_2-fixation system as source of N. Thus, biological fixation of atmospheric N, especially non-symbiotic N_2-fixation in the soil, has been the subject of continuing interest in the recent decades especially for low-input agriculture. Associative (free-living) nitrogen-fixing bacteria are present around roots or inside plant tissues, fix nitrogen and contribute to plant nitrogen nutrition significantly in tropical grasses (C_4 Gramineae) and wetland rice. The *Azospirillum* spp. are widespread and effective nitrogen-fixing bacteria in the rhizosphere of many graminaceous species, and *Azospirillum lipoferum*, *Azospirillum brasilense* and *Azospirillum amazonense* are highly potent nitrogen-fixing organism in the rice rhizosphere (Postgate 1982; Alexander 1991; Watanabe 2010; Subba Rao 2007). Biological (mainly associative) nitrogen fixation (BNF) amounting 19–47 % of the total N 1.8 t/ha (i.e. up to 22 %) by *Azospirillum* spp. in rice ecologies would sustain production, sparing the environment (Choudhury and Kennedy 2004; Shrestha and Maskey 2005; Saikia and Jain 2007; Zaki et al. 2009; Bashan and de-Bashan 2010; Kannan and Ponmurugan 2010).

As *Azospirillum* is microaerophilic, it can function efficiently in flooded rice fields where N application is a difficult proposition. However, the functionality of BNF is highly location-specific, and therefore, resident strains would be better suited (Choudhury and Kennedy 2004; Zaki et al. 2009; Bashan and de-Bashan 2010; Kannan and Ponmurugan 2010). Unfortunately, probably no efficient and dependable BNF formulation for rice for laterite soils and eastern India has been developed to date. Therefore, native *Azospirillum* isolates were obtained from the rhizosphere of popular high-yielding rice (*O. sativa* var. *Khandagiri* (drought tolerant) and *Pooja*) of different localities. The phenotype and genotype of *Azospirillum* isolates were characterized, and their PGP functions along with in vitro nitrogen fixation performance were studied. *Azospirillum* formulation was compared with available commercial biofertilizers of *Azospirillum* spp. (commercial name not mentioned) under field condition for improved growth and better yield of rice (*O. sativa*) var. *Khandagiri* in the Central Farm, OUAT, Odisha.

Materials and methods

Isolation of *Azospirillum* from different soil samples

The tentative *Azospirillum* bacteria were isolated from different rice rhizospheres of different locations in Odisha, India, viz. System Rice Intensification (SRI) field (OUAT), Long-Term Fertilizer Experiment (LTFE) field (OUAT), Central Rice Research Institute (CRRI) field 1 and field 2 (Cuttack), Majhisahi (OUAT experimental field, Dhenkanal) and Badamba (OUAT experimental field, Cuttack) where rice is cultivated for more than 20 yr, and the fields are maintained regularly to a basic fertility level by applying N/P/K (60:30:30 kg/ha). The soil was scrapped off from the rice plant roots and blotted to optimum on a filter paper, and 1 g of soil was diluted with 9 mL of sterile distilled water (autoclaved at 120 °C, 1.1-kPa pressure, 15 min) and serially diluted up to 10^{-5} level. From 10^{-4} dilution, 100-μl extract was taken for plating in specific enrichment media, i.e. Okon's media modified by Lakshmi-Kumari et al. (1980) for tentative *Azospirillum* spp.

Phenotypical and biochemical characterization of tentative isolates of *Azospirillum* spp.

Bacterial cultures diluted in water were smeared on slides (Sahoo et al. 2013), and Gram's stain and spore stain (malachite green) of the isolates were done following standard microbial methods (Collee and Miles 1989). Morphological characteristics, viz. shape, size, motility and Gram's stain of the isolated bacteria were examined under a phase contrast light microscope (×100 objective). Various physiological and biochemical tests such as oxidase, catalase, urease, indole production; methyl red; Voges-Proskauer (acetoin production); nitrate reduction; citrate utilization; hydrogen sulphide production; carbohydrate metabolism; carbohydrate fermentation; dihydrolase and extracellular enzyme activity, i.e. hydrolysis of polysaccharides; lipid (tributyrin and vegetable oil); Tween 80; cholesterol; and protein (gelatine and casein) were studied following standard methods (Bergey's manual) (Patel et al. 2013).

In vitro nitrogen fixation performance and by plant growth promotion functions of identified *Azospirillum* species from rice fields

Nitrogen fixation efficiency in culture by *Azospirillum* isolates were assessed by acetylene reduction assay (ARA) in the laboratory (Hardy et al. 1968) cultivated on semisolid N-free malate medium (g/l water: DL-malic acid 5.0, K_2HPO_4 0.5, KOH 4.0, $MgSO_4.7H_2O$ 0.1, NaCl 0.2, $CaCl_2$ 0.01, $FeSO_4.7H_2O$ 0.05, Na_2MoO_4 0.002, $MnSO_4.4H_2O$ 0.01, bromothymol blue (0.5 % ethanolic) 2.0 mL, pH 7.0, agar 1.8 (Okon et al. 1977) slants, respectively. ARA was performed as described by Sahoo et al. (2013). Acetylene was generated immediately before use from calcium carbide and contaminated ethylene (if any) was detected immediately through the GC (Hardy et al. 1968). Ethylene production was estimated as nmole C_2H_4/mg bacteria/h. The *Azospirillum* spp. of the locally available most efficient formulations were

isolated and assessed with the experimental isolates for comparison. The siderophore, hydrogen cyanide (HCN) and salicylic acid (SA) were estimated by adopting the method of Reddy et al. (2008). The IAA production from different isolates were estimated by method described by Ahmad et al. (2005). Estimation of GA3 and ABA from bacterial isolates was done following the procedure of Tien et al. 1979. The cytokinin like substances was identified by method of Strzelczyk et al. (1994).

Isolation and analysis of genomic DNA, plasmid DNA and cellular protein from *Azospirillum* isolates

Genomic DNA was isolated from overnight-grown bacterial cultures in nutrient broth on a shaker at 150 rpm and 30± 0.1 °C as per the method described by Jimenez et al. (2011). Plasmids of the bacteria were isolated from these broth cultures by following the method of Jensen et al. (1994). The profile was visualized as fluorescent bands through a UV transilluminator (312 nm) and photographed through a gel photo documentation system. The cellular bacterial proteins were extracted by the method described by Bhaduri and Demchick (1983).

PCR amplification of *nifH*, *nifK* and *nifD* genes of *Azospirillum* isolates

The genomic DNA was amplified using full-length *nif*H, *nif*K and *nif* D primers designed by using Primer3 software. The forward and the reverse primers were F5′-ATGGCTATGCGT CAATGCGC-3′ and R5′-TCAGACTTCTTCGGCGGTTT-3′, F5′-ATGAGCCAGCAAGTCGATAA3′ and R5′-TGGT GCTGGACCATGCGATT-3′, and F5′-ATGACCGGTATG TCGCGCCA-3′ and R5′-CGGCGGTCGCGGACT-3′, respectively. The primers were designed from the sequences of the *nif* gene cluster (GenBank accession numbers GenBank: M20568.1). The PCR reaction mixture contained template DNA (100 ng), *Taq* polymerase (3 U/μl), 10X *Taq* polymerase buffer (100 mM Tris (pH 9), 500 mM KCl, 15 mM $MgCl_2$, 0.1 % gelatin), dNTP mix (10 mM) and 1 μm (100 ng) of each primer. The amplification conditions were 94 °C for 4 min, 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1.30 s for 30 cycles and 72 °C for 8 min.

16S rRNA gene sequencing and analysis of phylogeny

The 16S rRNA gene of the *Azospirillum* spp. was amplified from genomic DNA through PCR using the forward primer (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer (5′-TTACCTTGTTACGACTTAAGTCGTAACAAGGTAA CC-3′). The PCR reaction mixture contained template DNA (150 ng), *Taq* polymerase (1.5 U/μl), 10X *Taq* polymerase buffer (100 mM Tris (pH 9), 500 mM KCl, 15 mM $MgCl_2$, 0.1 % gelatin), dNTP mix (10 mM) and 10 μm of each primer. The PCR reaction conditions were 94 °C for 4 min, 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1.30 s for 30 cycles and then 72 °C for 8 min. The amplified PCR products were sequenced and analyzed using BLAST of ncbi.nlm.nih.gov database, and the phylogenetic tree was constructed after multiple sequence alignment using cluster algorithm of CLUSTALW software (Yushmanov and Chumakov 1988) followed by tree construction using neighbour joining method in TREECON software based on the matrix of pair distances between the sequences. In the bootstrap, a multiple alignment was resembled 100 times.

Field experiments

Biofertilizers were formulated aseptically under a laminar air flow using native efficient rhizospheric *Azospirillum* spp. (selected from ARA and PGPR assay) comprising (grams per kilogram) sterile (autoclaved) charcoal powder 700, $CaCO_3$ 100, gum acacia 20 and liquid culture 180 (180 mL containing 10^9 cfu/mL), i.e. a final population of 2×10^8-cfu/g formulation (according to the Bureau of Indian Standards (BIS) guidelines). Commercial biofertilizer formulations of *Azospirillum vinelandii* were procured from the local market, and vermicompost was collected from the College of Agriculture, OUAT, Odisha, India and tested along with the experimental formulations.

The field experiments were carried out with rice variety *Khandagiri* in the central farm of Orissa University of Agriculture and Technology to unveil the native efficient *Azospirillum* strains for formulation and production of potent indigenous biofertilizers for commercial exploitation in lateritic soil. The experimental fields are lowland, and rice is grown in *Kharif* season of each year. The crop was harvested after 90 days, and the pre-post harvest observations like plant height (centimetres), tiller/hill (number), effective tiller/hill (number), panicle length (centimetres), leaf area (square centimetres), panicle length (centimetres), root length (centimetres), root dry weight (grams), root volume (millilitre), panicle weight (grams), grain yield/plant (grams), filled grain/panicle (number) and 1,000 grain weight (grams) were recorded. The endogenous nutrient ions like nitrogen (N), phosphorus (P), potassium (K), sulphur (S), calcium (Ca) and magnesium (Mg) were estimated from each plant tissue following the methods described by Sahoo et al. (2013).

Statistical analysis

All statistical analyses were performed using the graph and prism software. The experimental data were mean values from three independent series, each with three replicates, and the results were presented as means±standard error (SE) based on

three replications. The statistical significance at $P<0.05$ has been calculated.

Results

Isolation and characterization of *Azospirillum* isolates

The isolated bacteria displayed a specific colony on Okon's medium which produces circular, wrinkled, dark pink, white, fluorescent, flat, plicate, low convex character, gummy, slimy, non-slimy colony, with size ranging from 0.60–1.00 mm (supplementary Table 1). Morphological characteristics, viz. shape, size, length and breadth were studied microscopically, and the characteristics of all the 35 colonies were different (supplementary Table 2). The isolates obtained from the different locations were responding differentially with respect to a particular biochemical assay (Supplementary Fig. 1A–F).

Identification, in vitro nitrogen fixation and PGP functions of isolates

The bacterial isolates were obtained in axenic culture from different rice fields (Table 1). The bacterial isolates from the different rice fields were identified as *Azospirillum* spp. (19), *Pseudomonas* sp. (8), *Beijerinckia* sp. (6), *Derxia* sp. (1) and *Aquaspirillum* sp. (1) (Fig. 1a). The nitrogen fixation efficiency (ARA) of the *Azospirillum* spp. varied between 58.88 and 161.22 nmole C_2H_4/mg bacteria/h, and the *A. lipoferum* CRRI1As6 of the CRRI1 field fixed the highest nitrogen at 161.22 nmole C_2H_4/mg bacteria/h and was more effective than the respective commercial formulations (Com As) (133.51 nmole C_2H_4/mg bacteria/h). Furthermore, the *Azospirillum* spp. of the CRRI fields was more efficient in fixing nitrogen than the other bacteria (Fig. 1b). PGP functions such as production of siderophore, HCN, salicylic acid, IAA, GA_3, zeatin, ABA, and NH_3; phosphorus release; and ACC deaminase and iron tolerance of the *Azospirillum* isolates were evaluated. The PGP activity of the tested bacterial isolates was observed to be greatly variable. The *A. lipoferum* (As6) illustrated paramount all plant growth promotion functions such as siderophore, HCN, salicylic acid, IAA, GA3, zeatin, NH3, phosphorus solubilisation, ACC deaminase and iron tolerance with respect to others (Fig. 1c).

Molecular characterization of efficient *A. lipoferum* isolates

The genomic DNA profile of the tested isolates of *Azospirillum* showed that As6 (isolated from the CRRI1 field) exhibited the heaviest band of molecular weight size of 33.55-kb gDNA as compared to the other isolates. The size of the gDNA of As11 (isolated from Badamba field) was 29.34 kb which was the least among the tested isolates and was more or less similar to isolate As10 (isolated from the Majhisahi field) (Fig. 2a, Table 2). The plasmid DNA profile of *Azospirillum* isolates showed that the highest number of plasmid DNA of As6 (isolated from the CRRI1 field) and As3 (isolated from the LTFE field) was four among the tested isolates (Fig. 2b, Table 2). The protein profiles of the different *Azospirillum* isolates showed that they were broadly comparable with some minor variations. The organisms produce 5–38 bands, with size ranging from a minimum of 14.85 to a maximum 205 kDa (Fig. 2c, Table 3). The *nif* gene of the *A. lipoferum* species obtained from the rice fields was evaluated to assess *nif* diversity. The PCR-amplified genes *nif*H, *nif*D and *nif*K produced the expected size of 0.87, 1.4 and 1.5 kb, respectively (Fig. 2 d–f).

16S rRNA sequencing of *A. lipoferum* isolates

The amplified fragment of the 16S rRNA of the *A. lipoferum* isolates was sequenced and aligned with other partial 16S rRNA sequences required for multiple alignments from the gene bank. All the sequences were subjected to multiple sequence alignment using cluster algorithm. The 16S rRNA sequence and bootstrap analysis revealed that the sequences were matched 100 % with the 16S rRNA sequence of *A. lipoferum.* The 16S rRNA sequence of *A. lipoferum* (CRRI As6) was submitted to the NCBI gene bank with the accession number catalogued as JQ796078 (Fig. 3).

Effect of *Azospirillum* formulations on growth and yield parameters and endogenous nutrient content of rice plants under field conditions

The isolates of *A. lipoferum* including commercial *A. lipoferum* isolate were selected on the basis of their nitrogen-fixing performance in ARA and growth promotion activities for the preparation of biofertilizers. The biofertilizers were formulated by using efficient *A. lipoferum*, viz. As1, As3, As6, As7, As10 and As12 to evaluate the growth and yield parameters of rice (*O. sativa* L. var. *Khandagiri*) in the lateritic rice fields in OUAT, Bhubaneswar. The population dynamics in all the fields were found to be varied, and the treatment with As6 showed superior population, i.e. 5.88×10^5 cfu g^{-1} during the time of harvest (Table 4). The effects of the *Azospirillum* formulations in the field experiments were conducted in rice the variety *Khandagiri* at OUAT fields, and the growth parameters like plant height, number of tiller/hill, effective tiller/hill, panicle length, root dry weight, root volume, leaf area, panicle length, panicle weight, grain yield, filled grain/panicle and 1,000 grain weight were recorded for each treatment. The treatment T6 (As6) showed the highest response in all the tested growth parameters as compared to the rest of the treatments (Fig. 4a). From the field experiments, the rice plants

Table 1 Identification of *Azospirillum* spp. from different isolates

Location	Isolate no.	Name of bacteria
SRI field (Central Farm, OUAT)	As1	*Azospirillum lipoferum* (As1)
	As1a	*Beijerinckia* spp.
	As2	*Azospirillum* spp. (As2)
	As2a	*Azospirillum lipoferum* (As2a)
	As2b	*Pseudomonas* spp.
	As2c	*Beijerinckia* spp.
LTFE field (Central Farm, OUAT)	As3	*Azospirillum lipoferum* (As3)
	As3a	*Aquaspirillum* spp.
	As3b	*Pseudomonas* spp.
	As3c	*Beijerinckia* spp.
	As3d	*Derxia* spp.
CRRI field 1	As4	*Azospirillum brasilense* (As4)
	As4a	*Pseudomonas* spp.
	As5	*Azospirillum lipoferum* (As5)
	As5a	*Pseudomonas* spp.
	As6	*Azospirillum lipoferum* (As6)
	As6a	*Azospirillum lipoferum* (As6a)
	As6b	*Pseudomonas* spp.
CRRI field 2	As7	*Azospirillum lipoferum* (As7)
	As7a	*Pseudomonas* spp.
	As8	*Azospirillum lipoferum* (As8)
	As8a	*Azospirillum* spp. (As8a)
	As8b	*Azospirillum* spp. (As8b)
	As9	*Azospirillum* spp. (As9)
Majhisahi, Dhenkanal (OUAT experimental field)	As9a	*Pseudomonas* spp.
	As9b	*Beijerinckia* spp.
	As10	*Azospirillum brasilense* (As10)
	As10a	*Azospirillum brasilense* (As10a)
	As10b	*Azospirillum brasilense* (As10b)
Badamba, Cuttack (OUAT experimental field)	As11	*Azospirillum lipoferum* (As11)
	As11a	*Beijerinckia* spp.
	As12	*Azospirillum lipoferum* (As12)
	As12a	*Azospirillum* spp. (As12a)
	As13	*Pseudomonas* spp.
	As14	*Beijerinckia* spp.

treated with As6 formulations demonstrated higher content of nutrients and crude protein with respect to the others treatments (Fig. 4b).

Discussion

All together 35 bacteria were isolated from Okon's medium, and they were phenotyped by morphophysiological and biochemical characters (Smibert and Krieg 1995; Baldani et al. 2005; Kennedy 2005a; Kennedy 2005b; Kennedy et al. 2005; Palleroni 2005; Pot and Gills 2005). Among them, 19 isolates formed non-slimy, wrinkled colonies of different shape, size, margin and elevation and were motile, aerobic, Gram negative, rods of 2.1–3.8 × 1.0–1.2 μm size (supplementary Tables 1 and 2). The biochemical characters of these isolates showed that out of 11 discernible tests, oxidase, phosphatase, nitrate reduction, utilization of glucose, α-ketoglutarate, mannitol, fructose, galactose and arabinose were positive, whereas rhamnose and sucrose utilization was negative (Supplementary Fig. 1a–f). However, few isolates showed negative for glucose and α-ketoglutarate along with rhamnose and sucrose utilization (Supplementary Fig. 1a–f). Nevertheless, the phenotypic characters confirmed the identity of the organisms as *Azospirillum* spp. (Smibert and Krieg 1995; Baldani et al.

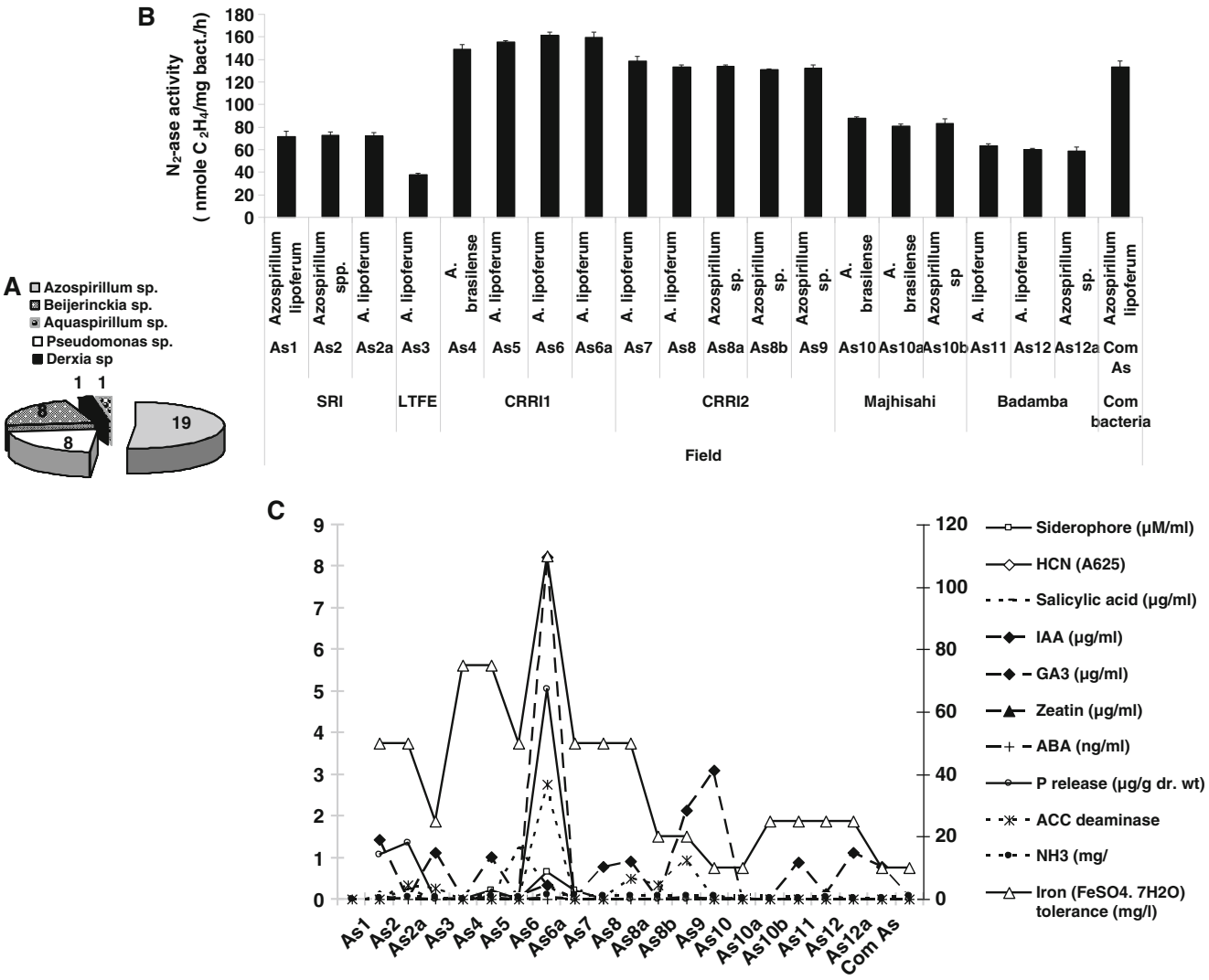

Fig. 1 Identification of nitrogen-fixing performance (ARA) and plant growth promotion functions of bacterial species including *Azospirillum* spp. from the different rice fields. Different bacterial isolates obtained from the different rice rhizospheres were identified as *Azospirillum* spp. (19), *Pseudomonas* sp. (8), *Beijerinckia* sp. (6), *Derxia* sp. (1), *Aquaspirillum* sp. (1) (**a**). Nitrogen-fixing performance of bacterial species indicating higher amount of nitrogen (161.22 nmole C_2H_4/mg bacteria/h) in pure culture was fixed by *A. lipoferum* CRRI1As6 of CRRI1 field fixed as compared to the other isolates (**b**). The plant growth-promoting (PGP) activity of the isolated bacterial species showed that siderophore, hydrogen cyanide and salicylic acid, indole-3-acetic acid, gibberellic acid, cytokinin and abscisic acid were higher in *A. lipoferum* (As6) (**c**)

2005; Kennedy 2005a; Kennedy 2005b; Kennedy et al. 2005; Palleroni 2005; Pot and Gills 2005). Furthermore, the morphological, physiological and biochemical characters identified the species belonging to *Azospirillum* with isolate numbers As1, As2a, As3, As5, As6, As6a, As7, As8, As11 and As12 as *A. lipoferum* and As4, As10, As10a and As10b as *A. brasilense* (Smibert and Krieg 1995; Kennedy et al. 2005; Hill and Sawers 2009), and the species of the other isolates remained unknown which need further analysis.

The PGP activities of the identified isolates of bacteria were observed to be highly variable among the tested isolates of bacteria, and not all the PGP functions were expressed by all the isolates studied (Fig. 1a–b), suggesting that all PGPR may not be equally effective, i.e. may not possess polyvalent functions to support plant growth (Bashan and de-Bashan 2010; Hayat et al. 2010; Saharan and Nehra 2011). Besides, almost all functions were at higher or next-higher levels of the PGP functions of the *Azospirillum* As6, viz. siderophore (0.65 µM/mL), HCN (0.02 A625), salicylic acid (0.31 µg/mL), IAA (8.23 µg/mL), GA3 (0.34 µg/mL), zeatin (0.06 µg/mL), NH_3 (1.49 mg/10 mL), phosphorus release (67.12 µg/mL), ACC deaminase (36.36 nM α-KBT/mg/h) and iron tolerance (110 mg/L) and nominal ABA (0.05 ng/mL) (Fig. 1c). The results proved that the organism would be a superior PGPR with polyvalent functions. It is well

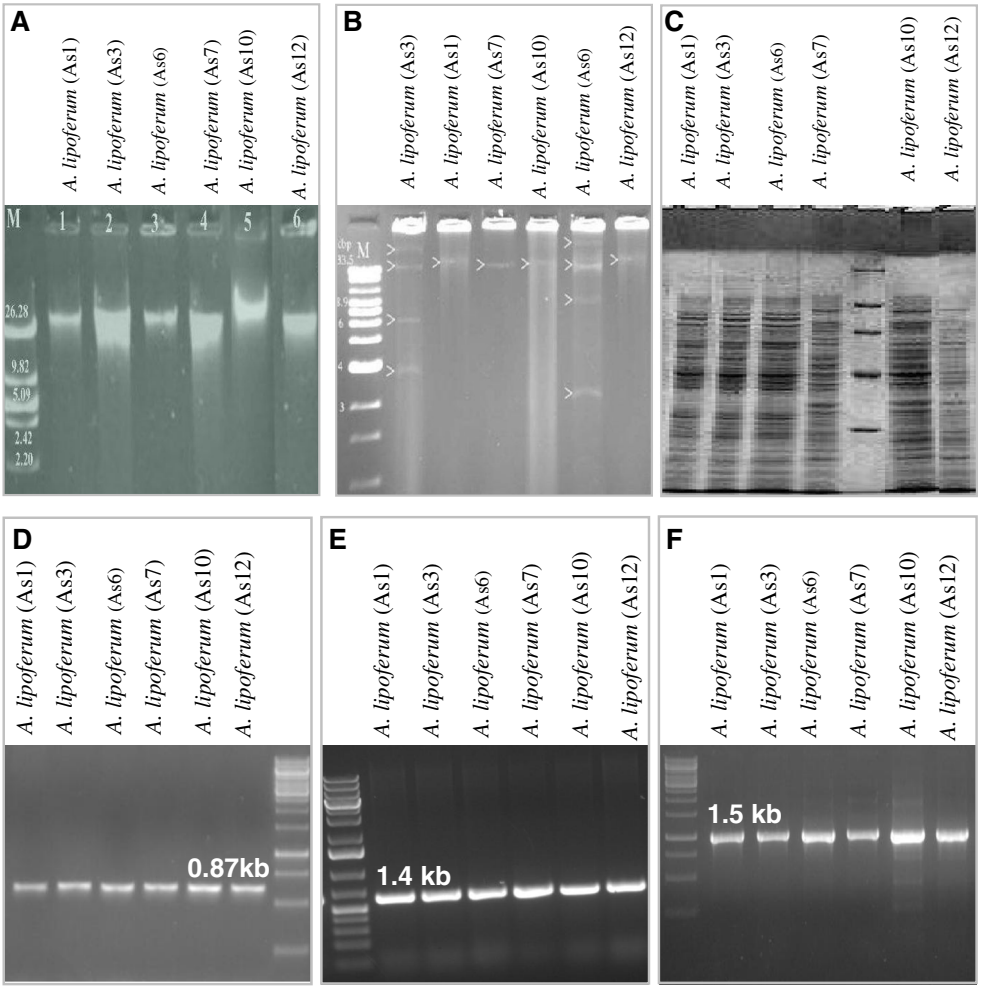

Fig. 2 Molecular characterization of *A. lipoferum* spp. isolated from different rice fields. Genomic DNA, plasmid DNA and cellular proteins profile of *A. lipoferum* isolated from different rice fields show that As6 (isolated from CRRI1 field) exhibited the heaviest band of molecular weight size of 33.553 kb gDNA as compared to the other isolates, and the least were As10 and As11 with 29.342 kb (**a**). The highest number of plasmid DNA, i.e. four was observed in As6 (isolated from the CRRI1 field) and As3 (isolated from the LTFE field) isolates (**b**). The organisms produce 5–38 bands with size range from 14.848 to 205 kDa (**c**). The PCR-amplified genes *nif*H, *nif*D and *nif*K produced the expected size of 0.87, 1.4 and 1.5 kb, respectively (**d**–**f**)

established that the PGP functions of PGPR *Azospirillum* spp. are not universal, rather highly strain-specific and vary both qualitatively and quantitatively (Choudhury and Kennedy 2004; Karadeniz et al. 2006; Perrig 2007; Bashan and de-Bashan 2010; Hayat et al. 2010; Saharan and Nehra 2011; Samuel and Muthukkaruppan 2011). However, the quantitative PGP factors of the *Azospirillum* spp. in the present study (Fig. 1c) were evident by the production of IAA (16.5–38 μg/mg protein, etc.), GAa/GA3 (1.64–4.17 μg/25 mL, 48.8–150.4 μg/mL, etc.), cytokinin metabolism (0.75–2.37 μg/mL), P solubilization (74.23 μg/g) and NH_3 production (1.65 mg/10 mL) by *Azospirillum* spp. (Dangar and Basu 1991; Choudhury and Kennedy 2004; Karadeniz et al. 2006; Perrig 2007; Bashan and de-Bashan 2010; Hayat et al. 2010; Saharan and Nehra 2011). The results of the other PGP factors, viz. HCN, complied with that of A625 (0.02–0.04) but were lower than the siderophore (0.66–9.45 μM/mL) and salicylic acid (0.15–8.12 mg/mL) produced by PGPR *Pseudomonas* spp. of rice (Reddy et al. 2008). The siderophore production by the *Azospirillum* spp. would help acquire Fe which is required for nitrogenase reductase (Fe protein) (Hartmann 1988; Rubio et al. 2005). The Fe tolerance limit of the *Azospirillum* spp. was variable and relatively lower than those of several other PGPR, but the tolerance limit of the BNFs of the present study (range over all 10.1–180.2, Az3 180.2 and As6 110.4 mg/l) corroborated to that of *Azospirillum* spp. (90 μg/mL) of other rice soil (Samuel and Muthukkaruppan 2011; Saharan and Nehra 2011). Higher Fe

Table 2 Genomic DNA and plasmid DNA composition of selected *Azospirillum* isolates

Isolate no.	Genomic DNA (kbp)	Plasmid	
		Number	Molecular weight (kbp)
As1	30.108	1	50.458
As3	30.108	4	67.417, 43.778, 6.290, 3.990
As6	33.553	4	73.583, 44.292, 9.064, 3.434
As7	29.725	1	45.319
As10	29.342	1	49.431
As12	29.342	1	53.542

Table 3 Composition of cellular protein of *Azospirillum* isolates

Isolates	Molecular weight (kDa)
As1	180.847, 122.348, 107.529, 91.121, 77.599, 72.127, 67.743, 60.350, 56.907, 53.615, 50.025, 46.724, 45.520, 43.344, 41.982, 40.495, 39.051, 37.489, 34.923, 33.307, 30.569, 28.087, 26.413, 23.826, 21.543, 19.870, 17.587, 15.000
As1	119.212, 90.342, 77.599, 71.476, 66.000, 60.818, 56.907, 53.283, 49.248, 46.311, 43.344, 41.815, 40.986, 39.528, 37.798, 35.668, 33.599, 32.726, 31.429, 30.712, 28.087, 26.413, 23.978, 22.000, 20.630, 18.196, 16.065
As6	172.931, 140.917, 116.158, 104.842, 95.039, 88.018, 79. 755, 71.476, 66.571, 62.275, 57.720, 54.650, 51.422, 48.753, 47.367, 45.328, 43.172, 41.648, 39.688, 38.109, 37.029, 36.118, 34.775, 33.161, 31.860, 30.712, 29.570, 28.239, 27.326, 25.652, 23.522, 22.152, 21.087, 19.717, 18.043, 15.913, 14.848
As7	174.900, 116.158, 103.536, 95.826, 87.249, 80.485, 72.787, 67.743, 57.720, 53.953, 51.422, 48.998, 47.149, 45.328, 43.000, 40.986, 39.368, 38.109, 35.818, 34.627, 32.581, 31.860, 30.569, 29.285, 27.935, 26.870, 25.348, 23.370, 22.000, 20.935, 18.043, 16.217, 14.848
As10	205.000, 97.400, 66.000, 43.000, 29.000
As12	93.468, 78.312, 68.343, 61.294, 57.310, 53.615, 49.503, 47.367, 45.910, 44.045, 42.658, 41.151, 39.368, 38.265, 35.668, 33.453, 32.726, 30.712, 28.543, 26.870, 25.652, 24.283, 21.848, 20.326, 17.739, 15.913

tolerance of the As6 isolate would allow them to establish in the laterite soil, and the polyvalent functions would be the reason of better performance of the isolates in the lateritic fields of OUAT (Fig. 1c). Nevertheless, ABA metabolism by As6 (0.05 ng/mL) was much lower than those of the other BNFs (0.07–7.70 ng/mL) (Perrig et al. 2007). The metabolism of plant hormones, viz. indoles (IAA), enhance rice root growth, elongation, surface area, dry matter and development of lateral roots, root hairs, etc.; GAs promote cell division, elongation, etc.; cytokinins enhance cell division, shoot and root morphogenesis, chloroplast maturation, cell enlargement, auxiliary bud release, etc.; and ABA helps the rice plants tolerate drought (Bashan and de-Bashan 2010; Hayat et al. 2010; Saharan and Nehra 2011) which would be the beneficial factors and would be the make As6 the best suited PGPR for rice improvement. Similarly, the production of siderophores (metal (Fe) chelator) and toxins (HCN) would protect the plants by controlling the various microbes, pests and pathogens, whereas the salicylic acid would help the plants endure drought (Bashan and de-Bashan 2010; Hayat et al. 2010; Saharan and Nehra 2011). Thus, several PGP characters along with nitrogen fixation and P solubilization of the *Azospirillum* As6 would render the most effective BNF (PGPR) among all the isolates of the rice rhizosphere of the present study.

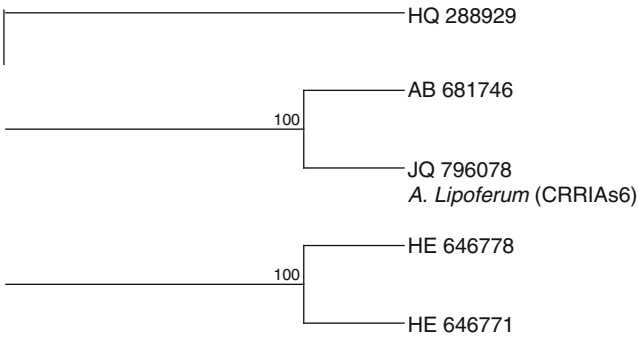

Fig. 3 Phylogram based on 16S rRNA sequence of different species of *Azospirillum*. The NCBI phylogram showed the identity (100 %) of the 16S rRNA gene of our native *A. lipoferum* (JQ796078: *Azospirillum lipoferum*, CRRI As6) with the different strains of *A. lipoferum* (AB681746: *Azospirillum lipoferum*). Similar trends were also reflected by comparing native *A. vinelandii* with other strains of *A. lipoferum*, viz., HQ 288929, HE 646778 and HE 646771 in the phylogram

Azospirillum spp. which were used for field trials were As1, As3, As6, As7, As10 and As12 (Table 1) which possessed genomes of 29.342–33.553 kbp size (Fig. 2a, Table 4), and their plasmid composition was found to vary among the isolates, viz. As1, As3, As6, As7, As10 and As12 which possessed one (50.458 kbp), four (67. 417, 43.778, 6.290 and 3.990 kbp), four (73.583, 44.292, 9.064 and 3.434 kbp), one (45.319 kbp), one (49.431 kbp) and one (53.542 kbp) plasmids, respectively (Fig. 2b, Table 4). However, multiple chromosomes of 4.8×10^3–9.7×10^3 kbp and several megaplasmids and smaller plasmids, some of which linear in the *Azospirillum* spp. through 16S rDNA analysis, were recorded (Caballero-Mellado 1999; Martin-Didonet 2000; SantAnna 2011); however, Kaneko et al. (2010) reported a single chromosome (3,311.395 kbp) and six plasmids of 261.596–1,455.109-kbp sizes. The reports indicated that the genomic configuration of *Azospirillum* is complex despite the results corroborating the genome and plasmid compositions of *Azospirillum* spp. of the present study. The cellular protein profiles of the selected BNFs were not identical in the *Azospirillum* spp., and they produced imprint of proteins of 14.848–205.000 MDa (Fig. 2c, Table 3). The results proved physiological and (or) metabolic distance among the members of that genus. However, proteomic information is an important molecular tool for differentiation of microbes which have been extensively used for strain differentiation of the *Azospirillum* spp. (Mot and Vanderhyden 1989; Kluepfel 1993; Khan et al. 2003).

The performance of the *Azospirillum* in the lateritic field soil of OUAT was better (although mostly insignificant) and broadly corroborated the effects of the field experiments (Table 4). The PGPR functions, including nitrogen fixation, of the native microbial guilds supported and improved plant growth over the field treatments. However, the nominal effects of the controls, viz. NPK (103–127 % but panicle length 95 %), vermicompost (102–121 % but root length 94 %) and commercial formulations of *Azospirillum* (Com As)-treated plots were grossly comparable (Table 4). Furthermore, effects of *Azospirillum* CRRI1As6 on most of the characters did not

Table 4 Population dynamics of *Azospirillum* spp. in the treated fields

Treatment	*Azospirillum* population in pots (×105 cfu/g soil) in various days							CD (*P*=0.05)
	0	15	30	45	60	75	90	
Control	0	0	0	0	0	0	0	0
NPK	0	0	0	0	0	0	0	0
Vermicompost	0	1.91	2.13	2.21	2.45	2.47	2.45	0.76
SRIAs1	0	3.11	3.81	3.24	3.76	3.93	3.53	0.79
LTFE As3	0	2.93	3.45	3.98	3.35	3.11	3.86	0.79
CRRI1As6	0	5.55	5.87	5.09	5.17	5.89	5.88	0.97
CRRI2 As7	0	3.71	3.71	4.23	3.78	3.08	3.43	0.92
MajhiAs10	0	4.21	4.56	4.27	4.23	4.12	4.09	0.88
BadamA12	0	3.22	4.54	4.71	3.32	3.56	3.10	0.99
ComAz0	0	2.50	4.11	3.76	4.23	4.44	4.90	0.78
CD, *P*=0.05	0	0.49	0.65	0.77	0.51	0.62	0.41	0

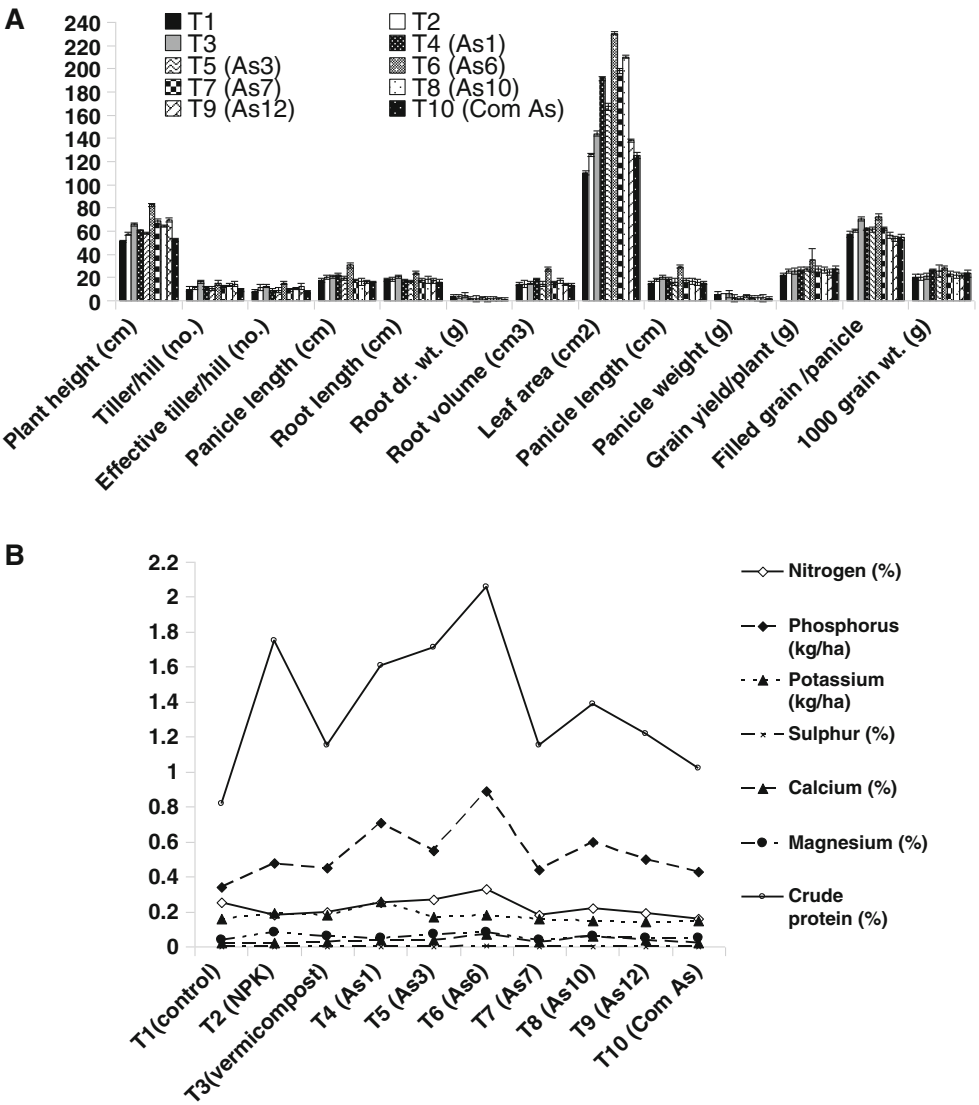

Fig. 4 Evaluation of *Azospirillum* formulations on rice variety *Khandagiri* at Central Farm of OUAT, Bhubaneswar and the determination of nutrient profile of the crop. The growth parameters such as plant height, number of tiller/hill, effective tiller/hill, panicle length, root dry weight, root volume, leaf area, panicle length, panicle weight, grain yield, filled grain/panicle and 1,000 grain weight (**a**) and nutrient content, viz., N, P, K, S, Ca and Mg with crude proteins in rice crop were higher in the treatment with As6 (T_6 As6) (**b**)

differ significantly, despite that the tiller number (168 %), effective tiller (184 %), panicle length (152 %), root dry weight (146 %), leaf area (216 %) and panicle weight (157 %) were more increased by the former, but plant height (166 %), root length (130 %), root volume (151 %) and grain weight (133 %) were more increased by the later (Fig. 4a). The results indicated that BNFs enhanced growth and yield of the rice var. *Khandagiri*, but more nitrogen-fixing organisms customarily might not be superior in the field, i.e. interaction of the resident microbes would modify functionality of the inoculants (Choudhury and Kennedy 2004; Perrig 2007; Bashan and de-Bashan 2010; Saharan and Nehra 2011). Absence of interference would exert more precise effects than the field treatments (Table 4). Treatments of the formulations among the fields had differential effects on the nutrient contents, viz. N, P, K, S, Ca, Mg and protein of the plants; the *Azospirillum* CRRI1 As6 was more effective. Nevertheless, unlike growth components, *Azospirillum* CRRI1 As6 supported more nutrient acquisition in the plants, and the commercial formulations were inferior to the native isolates (Fig. 4b). The results supported the proposition of indifference or differential positive impact of diverse BNF strains on productivity, and the effect depends on the metabolic difference of the plants (Choudhury and Kennedy 2004; Singh 2006; Zaki et al. 2009; Kannan and Ponmurugan 2010).

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Conflict of interest The authors declare that they have no conflict of interest.

References

Ahmad F, Ahmad I, Khan MS (2005) Indole acetic acid production by the indigenous isolates of Azotobacter and fluorescent Pseudomonas in the presence and absence of tryptophan. Turkish J Biol 29:29–34

Alexander M (1991) Introduction to soil microbiology. Krieger, NY, USA, 467p

Baldani JI, Krieg NR, Baldani VLD, Hartmannand A, Dobereiner J (2005) Genus II. *Azospirillum*. In: Brenner DJ, Krieg NR, Staley JT (eds) Bergey's manual of systematic bacteriology, vol 2C. Springer, New York, USA, pp 7–26

Bashan Y, de-Bashan LE (2010) How the plant growth-promoting bacterium *Azospirillum* promotes plant growth—a critical assessment. Adv Agron 108:77–136

Bhaduri S, Demchick PH (1983) Simple and rapid method for disruption of bacteria for protein studies. Applied Environmental Microbiology 46:941–943

Bhattacharjee RB, Singh A, Mukhopadhyay SN (2008) Use of nitrogen-fixing bacteria as biofertilizer for non-legumes: prospects and challenges. Appl Microbiol Biotechnol 80:199–209

Briones AM, Okabe S, Umemiya Y, Ramsingh NB, Reichardt W, Okuyama H (2002) Effect of different cultivars on population of ammonia-oxidizing bacteria in root environment of rice. Appl Environ Microbial 68:3067–3075

Caballero-Mellado J, Lopez-Reyes L, Bustillos-Cristales R (1999) Presence of 16S rRNA genes in multiple replicons in *Azospirillum brasilense*. FEMS Microbiol Lett 178:283–288

Choudhury ATMA, Kennedy IR (2004) Prospects and potentials for systems of biological nitrogen fixation in sustainable rice production. Biol Fertil Soils 39:219–227

Collee JG, Miles PS (1989) Tests for identification of bacteria. In: Collee JG, Duguid JP, Fraser AG, Marmion BP (eds) Practical medical microbiology. Churchil Livingstone, NY, USA, pp 141–160

Dangar TK, Basu PS (1991) Abscisic acid production in culture by some Rhizobium spp. of leguminous trees and pulses. Folia Microbiol 36: 527–532

Freitas ADS, Vieira CL, Santos CERS, Stamford NP, Lyra MCCP (2007) Caracterizac¸a˜o de rizo´bios isolados de Jacatupe´ cultivado em solo salino no Estado de Pernanbuco, Brasil. Bragantia 66:497–504

Hardy RWF, Holsten RD, Jackson EK, Burns RC (1968) The acetylene-ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiol 43:1185–1207

Hartmann A (1988) Ecophysiological aspects of growth and nitrogen fixation in Azospirillum spp. Plant and Soil l l0:225–238

Hayat R, Ali S, Amara U, Khalid R, Ahmed I (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol 60:579–598

Hill S, Sawers G (2000) *Azotobacter*. In: Lederberg J (ed) Encyclopedia of microbiology, vol. 1A-Cth edn. Academic, NY, USA, pp 359–371

Jensen RB, Dam M, Gerdes K (1994) Partitioning of plasmid R1. The parA operon is autoreguated by ParR and its transcription is highly stimulated by a downstream activating element. J Mol Biol 236: 1299–1309

Jimenez DJ, Montana JS, Martinez MM (2011) Characterization of free nitrogen fixing bacteria of the genus Azotobacter in organic vegetable-grown colombian soils. Brazilian J Microbiol 42:846–858.

Kaneko T, Minamisawa K, Isawa T, Nakatsukasa H, Mitsui H, Kawaharada Y, Nakamura Y, Watanabe A, Kawashima K, Ono A, Shimizu Y, Takahashi C, Minami C, Fujishiro T, Kohara M, Katoh M, Nakazaki N, Nakayama S, Yamada M, Tabata S, Sato S (2010) Complete genomic structure of the cultivated rice endophyte *Azospirillum* sp. B510. DNA Res 17:37–50

Kannan T, Ponmurugan P (2010) Response of paddy (*Oryza sativa* L.) varieties to *Azospirillum brasilense* inoculation. J Physiol 2:08–13

Kenmore P, (2003) Sustainable rice production, food security and enhanced livelihoods in "Rice Science: In: Mew TW, Brar DS, Peng S, Dawe D, Hardy B (eds.). Innovations and Impact for Livelihood. Beijing, China, p 27–34

Kennedy C (2005a) Genus I. *Beijernckia*. In: Staley JT, Boone DR, Brenner DJ, de Vos P, Garriety GM, Goodfellow M, Krieg NR, Rainey FA, Schlifer KH (eds) Bergey's manual of systematic bacteriology, vol 2C. Springer, New York, USA, pp 423–432

Kennedy C (2005b) Genus IV. *Derxia*. In: Staley JT, Boone DR, Brenner DJ, de Vos P, Garriety GM, Goodfellow M, Krieg NR, Rainey FA, Schlifer KH (eds) Bergey's manual of systematic bacteriology, vol 2C, eds. Springer, New York, USA, pp 671–674

Kennedy C, Rudnick P, MacDonald ML, Melton T (2005) Genus III. *Azotobacter*. In: Brenner DJ, Krieg NR, Staley JT (eds) Bergey's manual of systematic bacteriology, vol 2B. Springer, New York, USA, pp 384–402

Khan MR, Talukdar NC, Thakur D (2003) Detection of *Azospirillum* and PSB in rice rhizosphere soil by protein and antibiotic resistance profile and their effect on grain yield of rice. Indian J Biotechnol 2:246–250

Kluepfel DA (1993) The behaviour and tracking of bacteria in the rhizosphere. Annu Rev Phytopathol 31:441–472

Lakshmi-Kumari M, Lakshmi V, Nalini PA, Subba-Rao NS (1980) Reactions of Azospirillum to certain dyes and their usefulness in enumeration of the organisms. Curr Sci 49:438–439

Martin-Didonet CCG, Chubatsu LS, Souza EM, Kleina M, Rego FGM, Rigo LU, Yates MG, Pedrosa FO (2000) Genome structure of the genus *Azospirillum*. J Bact 182:4113–4116

Mot RD, Vanderhyden J (1989) Application of two-dimensional protein analysis for strain finger printing and mutant analysis of *Azospirillum* sp. Canadian J Microbiol 35:960–967

Okon Y, Albrecht SL, Burris RH (1977) Methods for growing Spirillum lipoferum and for counting it in pure culture and in association with plants. Appl Environ Microbiol 3:85–88

Palleroni NJ (2005) Genus I. Pseudomonas. In: Brenner DJ, Krieg NR, Staley JT, Garrity GM (eds) Bergey's manual of systematic bacteriology. Part B. The gammaproteobacteria. Springer, New York, p 323–379

Patel PH, Patel JP, Bhatt SA (2013) Characterization and phylogenetic relatedness of Azotobacter Salinestris. J Microbiol Biotechnol Res 3:65–70

Perrig D, Boiero ML, Masciarelli OA, Penna C, Ruiz OA, Cassan FD, Luna MV (2007) Plant-growth-promoting compounds produced by two agronomically important strains of *Azospirillum brasilense*, and implications for inoculants formulation. Appl Microbiol Biotechnol 75:1143–1150

Pot B, Gills M (2005) Genus III. *Aquaspirillum*. In: Staley JT, Boone DR, Brenner DJ, de Vos P, Garriet GM, Goodfellow M, Krieg NR, Rainey FA, Schlifer KH (eds) Bergey's manual of systematic bacteriology, 2cth edn. Springer, New York, USA, pp 801–823

Postgate JR (1982) The fundamentals of Nitrogen fixation. Cambridge University Press. p 60–102

Reddy BP, Reddy KRN, Rao MS, Rao KS (2008) Efficacy of antimicrobial metabolites of pseudomonas fluorescens against rice fungal pathogens. Curr Trends Biotechnol Pharm 2:178–182

Rubio MB, Hermosa MR, Keck E, Monte E (2005) Specific PCR assays for the detection and quantification of DNA from the biocontrol strain Trichoderma harzianum 2413 in soil. Microb Ecol 49:25–33

Saharan BS, Nehra V (2011) Plant growth promoting rhizobacteria: a critical review. Life Sci Medic Res 21:1–30

Sahoo RK, Ansari MW, Dangar TK, Mohanty S, Tuteja N (2013) Phenotypic and molecular characterization of efficient nitrogen fixing *Azotobacter* strains from rice fields for crop improvement. Protoplasma. doi:10.1007/s00709-013-0547-2

Sahrawat KL (2000) Macro and micronutrients removed by upland and lowland rice cultivars in West Africa. Commun Soil Sci Plant Anal 31:717–723

Saikia SP, Jain V (2007) Biological nitrogen fixation with non-legumes: an achievable target or a dogma? Curr Sci 92:317–322

Samuel S, Muthukkaruppan SM (2011) Characterization of plant growth promoting rhizobacteria and fungi associated with rice, mangrove and effluent contaminated soil. Curr Bot 2:22–25

SantAnna FH, Almeida LGP, Cecagno R, Reolon LA, Siqueira FM, Machado MRS, Vasconcelos ATR, Schrank IS (2011) Genomic insights into the versatility of the plant growth-promoting bacterium *Azospirillum amazonense*. BMC Genomics 12:409–423

Shrestha RK, Maskey SI (2005) Associative nitrogen fixation in lowland rice. Nepal Agric Res J 6:112–121

Singh MS (2006) Cereal crops response to *Azotobacter*—a review. Agric Rev 27:229–231

Smibert R, Krieg NR (1995) Phenotypic characterization. In: Gerhardt P, Murray RGE, Wood W, Krieg E (eds) Methods for general and molecular bacteriology. American Soc Microbiol, Washington DC, pp 607–654

Strzelczyk E, Kampert M, Li CY (1994) Cytokinin like substances and ethylene production by Azospirillum in media with different carbon sources. Microbiol Res 149:55–60

Subbarao NS (2007) Soil microorganisms and plant growth. Oxford IBH, New Delhi

Tien TM, Gaskins MH, Hubbell DH (1979) Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Appl Microbiol 37: 1016–1024

Watanabe A, Kawashima K, Ono A, Shimizu Y, Takahashi C, Minami C, Fujishiro T, Kohara M, Katoh M, Nakazaki N, Nakayama S, Yamada M, Tabata S, Sato S (2010) Complete genomic structure of the cultivated rice endophyte *Azospirillum* sp. B510. DNA Res 17:37–50

Yushmanov SV, Chumakov KM (1988) Algorithms of the maximum topological similarity phylogenetic trees construction. Mol Genet Microbiol Virol 3:9–15

Zaki N, Gomaa AM, Galal A, Farrag AA (2009) The associative impact of certain diazotrophs and farmyard manure on two rice varieties grown in a newly cultivated land. Res J Agric Biol Sci 5:185–190

Zeigler RS, Adams A (2008) The relevance of rice. Rice 1:3–10