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Application of rice (*Oryza sativa* L.) root endophytic diazotrophic *Azotobacter* sp. strain Avi2 (MCC 3432) can increase rice yield under green house and field condition

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ABSTRACT

Use of plant-associated beneficial microbes, especially endophytes are getting popular day by day as they occupy a relatively privileged niche inside different plant tissues with lesser competition for food and shelter than rhizosphere. The effects of different physical factors like temperature, rainfall, and seasonal variation and UV radiation on plant growth promoting endophytic communities are less pronounced than those on the rhizospheric and phylloplane microbes. This present work has been compromised with further utilization of an indigenous rice (*Oryza sativa* L.) root endophytic *Azotobacter* sp. strain Avi2 (MCC 3432) (*AzA*) as a bio-formulation for sustainable rice production based on several physiological parameters (plant height, root length/weight, leaf area, yield, chlorophyll contain), *in-vitro* comparative plant growth promoting assays, greenhouse and field experiments (dry and wet season). Treatments with *AzA* exhibited higher yield as well as maximal chlorophyll fluorescence (Fm) of flag leaves in flowering and grain filling stages indicating higher photosynthetic rates. Scanning electron microscopic image of rice roots demonstrated accumulation of bacterial biofilm at the junction of primary and lateral root confirming the root-colonizing ability of the bacterium. The results of the study were quite encouraging as *AzA* exhibited better vegetative and reproductive growth of rice in pot and field experiment compared to formulated rhizospheric *Azotobacter* sp. (commercial product). Apart from that plants treated with *AzA* (supplemented 50% nitrogenous fertilizer of recommended dose) exhibited similar yield parameters when it was compared with the recommended dose of fertilizer (RDF; 120:60:60 mg N:P:K kg^{-1} soil/without any bacterial). Therefore, it can be concluded that application of this plant growth promoting endophyte can reduce a substantial amount of N-fertilizer for field application.

1. Introduction

In an agricultural prioritize country like India, food security to feed around 1.3 billion population along with declining inclination of cultivated land would be a great threat, as the horizontal expansion of crop production is very limited. Therefore, vertical growth by enhancement of productivity and protection of crops, especially the cereal like rice which feeds 85% Indian population is most desired (Banik et al., 2016a). To feed ever increasing world's population around 8.1 billion, 800 million tonnes (mt) rice production would be necessary to meet the requirement by 2025. Rice, *Oryza sativa* L, one of the members of *Poaceae* family is an economically important crop and cultivated in more than hundred countries along the globe. The major contributors are China (209503037 tonnes), India (158,756,871 tonnes), Indonesia (77,297,509 tonnes), Bangladesh (52,590,000 tonnes) and many tropical and subtropical climatic countries (Vietnam, Thailand, Myanmar, Philippines, Japan etc.) cultivate it (FAOSTAT, 2013). Rice consumed as a staple food for a diverse part of the world's human population and paddy as fodder. In India, rice area is ~44.6 million hectares distributed as 52.6% irrigated, 32.4% rain fed, 12% upland and 3% submerged regions yielding ~80 mt (paddy) and average productivity is ~1.9 tonnes $hectare^{-1}$ (Muthayya et al., 2014). Nitrogen, one of the

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indispensable limiting macro element for plant growth and development, as it is a major component of chlorophyll, amino acid, ATP, nitrogenous heterocyclic bases (purines and pyrimidines) etc. Nonetheless, it is the most abundant element (present in the form of N_2 gas) in earth's atmosphere but plants can utilize its reduced form only. Rice production is highly dependent on nitrogen fertilizers and requires about 10 mt nitrogen per annum as the most soil of the world is nitrogen deficient which would restrict rice production, especially for the nitrogen-responsive high yielding varieties (Abrol et al., 1999). Besides having detrimental effects on the environment, half of the nitrogen fertilizer is lost through denitrification, ammonia volatilization, leaching, and rain-washes (Zhao et al., 2016). Therefore, biological nitrogen fixation (BNF) by microorganisms that accounts for 0.4–80 kg nitrogen hectare^{-1} (19–47% of required nitrogen) is the best proposition to replenish the nitrogen deficiency. Apart from this, the biologically fixed nitrogen is not readily liable for loss through natural processes as it is not accumulated at the production site and readily assimilated by the plants or by the bacteria. So, the indigenous rice associated microbiota, especially the endophytic, epiphytic and rhizoplanic diazotrophs should be explored to obtain efficient nitrogen-fixing microbes which can be used either directly or genetically improved to enhance and sustain production under the nitrogen-deficient condition and reduce chemical nitrogenous fertilizer application (Banik et al., 2016a). Predominant among the members of *Proteobacteria* in diverse rice ecologies plays a very crucial role in the uplifting of plants health and production. Among all, different members of *Proteobacteria.* like *Azotobacter, Pseudomonas, Acinetobacter, Gluconacetobacter, Aeromonas, Enterobacter, Azoarcus, Klebsiella,* Burkholderia, *Pantoea, Stenotrophomonas* spp. etc were the predominant. During our previous investigation, 35 multiple plant growth promoting (PGP) endophytic and epiphytic diazotrophs from different parts (leaf, stem and root) of three cultivated (*Oryza sativa* L. var. *Swarna /Swarna-Sub1/Sabita*) and one wild rice (*Oryza eichingeri*) genotypes were isolated and characterized through partial polyphasic taxonomy and PGP traits. The summarized results indicated that among all the isolates particularly *Azotobacter* sp. strain Avi2 (NCBI gene bank acc. no- KP099933), a root endophyte of cv. Swarna, was selected as the most potent, based on different preliminary *in-vitro* PGP traits. The strain forms an oval shaped cyst, brown pigment and exopolysaccharide on Ashby's mannitol agar medium after 48 h of incubation (Banik et al., 2016a, b; Ghosh et al., 2016a, b; Naskar et al., 2016).

The present work demonstrates further development and utilization of *Azotobacter* sp. strain Avi2 (*AzA*) strain as a formulation for sustainable rice production based on several biochemical- physiological tests, *in-vitro* comparative plant growth promoting assays, pot in green house and field experiments. The study was undertaken to know whether the native bacterial strain *AzA* can sustain rice production under half dose of nitrogenous fertilizer which is agronomically practiced.

2. Materials and methods

2.1. Location of experimental site

Field and green house experiments were carried out at ICAR-National Rice Research Institute, Cuttack, Odisha, India (GPS geoplaner Location- 45 Q 388682 2262018, 20.45340 °N 85.93279 °E Elevation = 24.4 m), situated at the apex of Mahanadi River delta, with rice genotype Swarna (Supplementary Fig. S1). The city is situated to a close proximity of Bay of Bengal (around 85 km) which influences its tropical wet and dry climate with three varied seasons: summer (March to June), rainy (July to October) and winter (November to February) with an average annual rainfall around 140 cm. The texture of soil at the experimental site categorised under Aeric Endoaquept soil type having 0.02% N, 11 mg kg^{-1} P, 99 kg ha^{-1} K, 1.02% organic carbon, 481 ug g^{-1} microbial biomass carbon (MBC), 38 ug g^{-1} fluorescein diacetate hydrolysis (FDA), 38.6 ug g^{-1}dehydrogenase activity (DHA) and pH around 5.16 (Kumar et al., 2017).

2.2. Comparative 16S rDNA based phylogenetic analysis

Phylogenetic tree based on 16S rDNA sequences (NCBI acc. no-KP099933) was constructed comparing with the 16S rRNA gene sequences from NCBI GenBank with nearest (BLAST score ≥99%) type strains of *Azotobacter* sp. and an outgroup (*Bacillus megaterium*^{T} ATCC 14581) by Neighbor-joining (Kimura 2-parameter model) method with 1000 replicates of bootstrap, using MEGA 7 software. The sequences were aligned with Clustal W 1.6 and the tree was drawn to scale, through branch lengths with the similar units as those of the evolutionary distances used to deduce the phylogenetic relationship. All the gaps and missing data were eliminated (Banik et al., 2016a).

2.3. In-vitro comparative assessment of plant growth promotion by bacteria

2.3.1. Preparation of seedlings for bacterial inoculation

Oryza sativa L. var *Swarna* seeds grains were dehusked and surface sterilized with 70% ethanol followed by 1% Chloramine-T for 5 min each. Afterward, the seeds were aseptically transferred in a Petri dish containing Murashige and Skoog (MS) agar (1.5%) medium supplemented with 2% sucrose (Murashige and Skoog, 1962; Banik et al., 2016b) and incubated in darkness for 3 d at 30 ± 2 °C for germination. After 3 d, sterile seedlings at 2 leaves stage were chosen to inoculate with the experimental bacterial *AzA* (Banik et al., 2016b).

2.3.2. Preparation of cells for bacterization of seedlings

Cells from the exponentially growing culture in Ashby's nitrogen-free (ANF) medium (Castillo et al., 2011) was grown at 30 ± 2 °C and centrifuged at 3000*g* for 5 min., pellets were washed twice in HEPES buffer (0.1 M) and the turbidity of each bacterial suspension was adjusted to 1 at 600 nm (haemocytometer count 1.2 × 10^8 cells mL^{-1}) (Banik et al., 2014). Bacterial suspensions were soaked separately in the Petri dishes containing six rice seedlings (cv. Swarna) per set and incubated for 6 h at 30 ± 2 °C in an incubator. Seedlings treated with only HEPES buffer (0.1 M) were maintained as a control. After 6 h of incubation, the bacteria treated seedlings were aseptically transferred in tubes (30 × 3 cm dia.) containing 40 mL sterile 1.5% nitrogen free agar. The tubes were incubated in a plant growth chamber for 12 d at 30 ± 2 °C on 14 h light and 10 h dark cycle. The plants were harvested after 12 d and the growth parameters i.e. primary root length, shoot length, fresh weight and dry weight were recorded (Banik et al., 2016b).

2.3.3. Effect of different doses Azotobacter sp. strain Avi2 on the growth of rice seedlings

To study the effect of the *AzA* concentration, seedlings were treated with two different concentrations, after 2 and 5 d of germination and the data were recorded. Surface sterilized seeds were soaked individually in two different concentration of 24 h old culture (grown at 30 ± 2 °C, counted by haemocytometer) i.e. 1.2 × 10^8 cells mL^{-1} (T1) (Banik et al., 2014) and 1.2 × 10^5 cells mL^{-1} (T2) for 6 h at 30 ± 1 °C. *AzA* treated seedlings were transferred in culture-tubes (20 height, 5 cm dia.) containing 35 mL sterile 1% agar planted 1 seedling culture^{-1} tube for each treatment and incubated for 10 d at 30 ± 2 °C. Seedlings soaked in sterile ANF medium were kept as control i.e. treatment '0′ (T0). After 5 d some of the control seedlings were treated separately with 24 h old culture (for 6 h) containing 1.2 × 10^8 cells mL^{-1} *AzA* (T3) and 1.2 × 10^5 cells mL^{-1} (T4) and incubated along with T0, T1, and T2 on 14 h light 10 h^{-1} dark cycle. After 10 d, different plant growth parameters like the length of shoot and root, number of secondary roots, fresh and dry weight were recorded. The experiments were conducted with three independent replications.

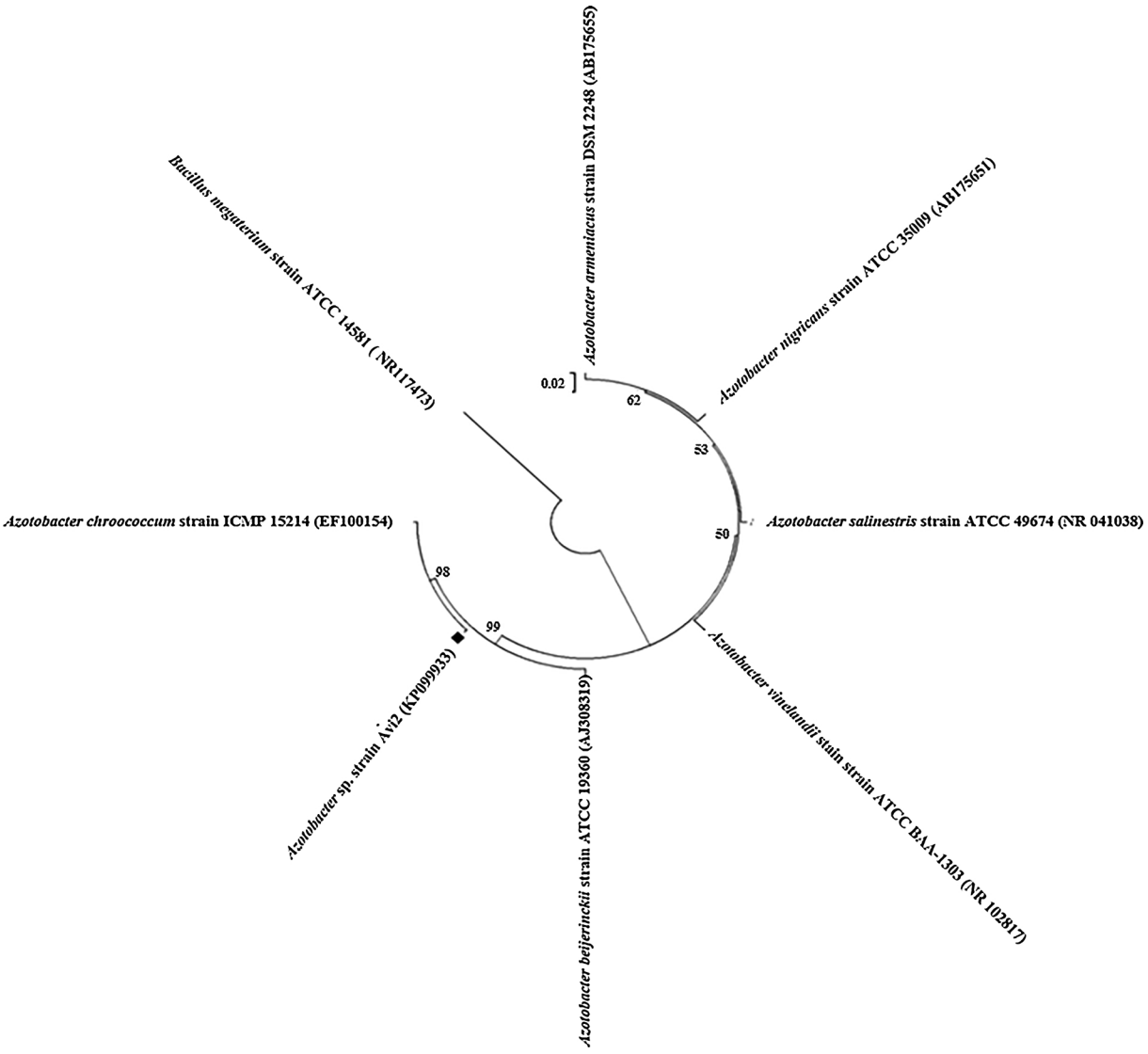

Fig. 1. Phylogenetic tree based on 16S rDNA sequences showing the relative position of *Azotobacter* sp. Avi2 with the closest type strains. The tree was constructed using MEGA 7 software using neighbour-joining method (Kimura 2-parameter model). Bootstrap values shown at nodes are expressed as percentage of 500 replications (if greater than 50%). Bar indicates 0.02 substitutions per site. The optimal tree with the sum of branch length = 0.31659629 is shown.

2.3.4. Comparative plant growth assessment of with native of Azotobacter sp. versus commercial Azotobacter sp

The plant growth promoting activity of the *Azotobacter* sp. strain Avi2 was also compared with *Azotobacter* sp. (*AzC*) isolated from commercial bio-fertilizer formulation (Premium AZOTO Plus, Hindustan Insecticide Ltd., India). Surface sterilized rice seeds cv. Swarna were aseptically transferred into MS medium supplemented with 2% sucrose and incubated in darkness for 3 d at 30 ± 2 °C for germination. After 3 d, seedlings were checked for contamination and only the sterile seedlings were aseptically removed from the Petri dish and separately treated 6 h with 1.2×10^8 cells mL^{-1} *AzA* and commercial *AzC*. The following day, the seedlings treated with *AzA* (T1) and *AzC* (T2) were aseptically transferred in a culture-tube (20 cm in height, 5 cm in diameter) containing 35 mL of sterile MS medium in laminar air flow. Seedlings soaked in sterile Ashby's N-free medium were kept as control (T0). All treatments were further incubated in a plant growth chamber for 15 d at 30 ± 2 °C on 14 h light 10 h^{-1} dark cycles. After 15 d of incubation, roots were surface sterilized by 1% chloramine T for 5 min and 100 mg roots were crushed in sterile HEPES buffer (0.1 M) for serial dilution and spreader on Ashby's N-free medium to study the bacterial population dynamics. The identity of re-isolated bacteria were confirmed by 16S rRNA gene amplification to support Koch's postulates. All the experiment was conducted with three replications (Bashan et al., 2014; Banik et al., 2016a).

2.4. Scanning electron microscopic (SEM) observation of root colonized Azotobacter sp. strain Avi2

The roots of 21 d old bacterial inoculated rice seedlings were rinsed five times with sterile HEPES buffer (0.1 M) and subsequently cut into 3 mm segments. The root fragments were successively dehydrated at 4 °C by transferring selected section from lower to higher concentration (30, 40, 50, 60 and 70%) of ethanol and then fixed using 4% glutaraldehyde in 70% ethanol for 3 h at 4 °C. Roots were rinsed with 70% ethanol for five times to remove the surface bounded glutaraldehyde and further dehydrated up to 100% keeping the sections in different concentration (80, 90 and 100%) of alcohol for 15 min. each. The samples were mounted on metal stubs, coated with gold-palladium and photographed using a scanning electron microscope (Hitachi, model S530) (Banik et al., 2014).

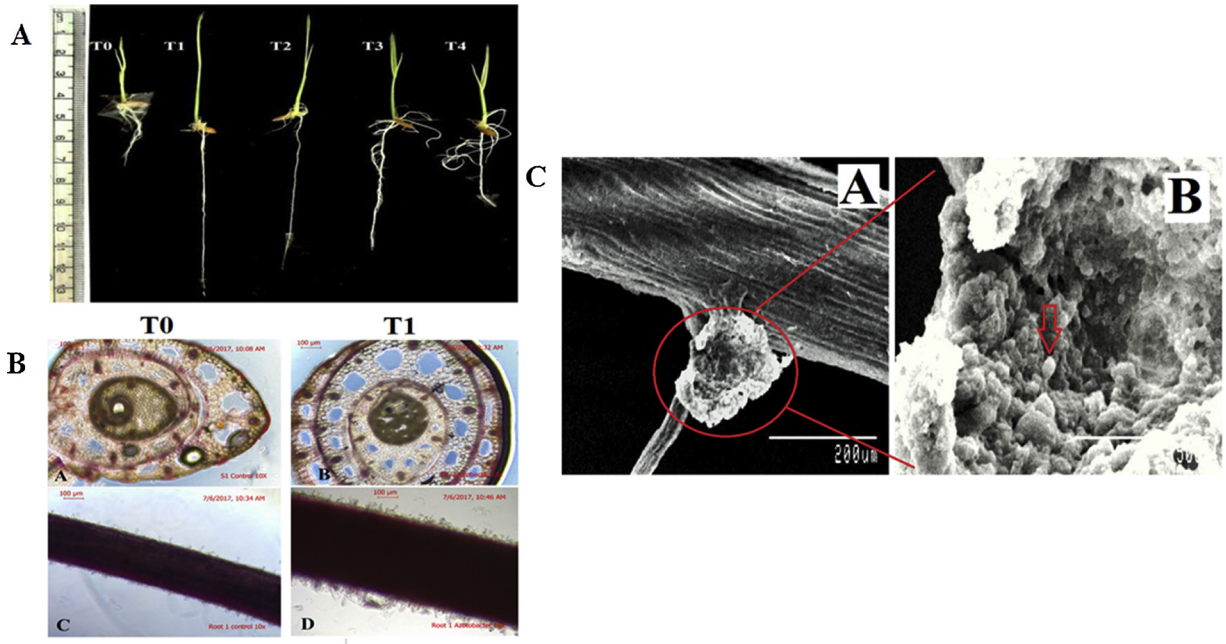

Fig. 2. A. Effect of different doses *Azotobacter* sp. Avi2 on rice seedlings. T0- Seedlings treated with sterile Ashby's nitrogen-free medium (control), T1- Seedlings treated with 1.2×10^8 cells mL^{-1} *Azotobacter* sp. Avi2 strain, T2- Seedlings treated with 1.2×10^5 cells mL^{-1} *Azotobacter* sp. Avi2 strain, T3- Seedlings treated with 1.2×10^8 cells mL^{-1} *Azotobacter* sp. Avi2 strain after 5 d of the previous treatment, T4- Seedlings treated with 1.2×10^5 cells mL^{-1} *Azotobacter* sp. Avi2 strain after 5 d of previous treatment. B. Transverse section of rice root. C. Scanning electron microscopic image of root (cv. Swarna) colonized *Azotobacter* sp. Avi2. C-A. Formation of biofilm after 21 days of inoculation (O.D.-1, 1.2×10^8 cells mL^{-1}) by *Azotobacter* sp. Avi2 at the junction of primary and lateral root at 200x magnification. C-B. enhanced bacterial colonization in biofilm at 10000x magnification showing bacterial cells (red arrow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

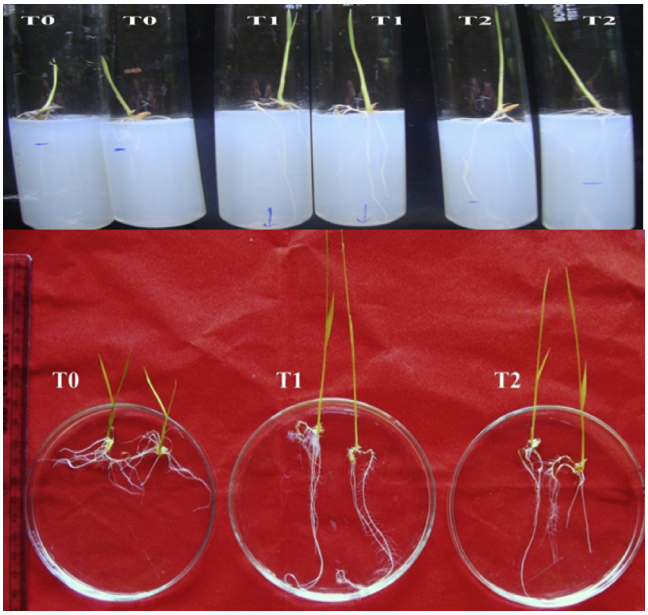

Fig. 3. Comparative growth promotion effects of *Azotobacter* sp. Avi2 and commercial *Azotobacter* sp. on rice seedlings grow on 1.5% nitrogen free agar medium. T0- Seedlings treated with sterile Ashby's nitrogen-free medium were kept as control, T1- Seedlings treated with 1.2×10^8 cells mL^{-1} *Azotobacter* sp. Avi2 strain for 15 d, T2- Seedlings treated with 1.2×10^8 cells mL^{-1} commercial *Azotobacter* sp. formulation for 15 d.

2.5. In vitro comparative PGP activity assay on rice seedlings in laboratory and green house

2.5.1. Preparation of bacterial inoculum

The *AzA* strain was grown in ANF medium and 1.2×10^8 cells mL^{-1} (O.D. was used 1) for inoculation into the rice seedlings. *AzC* was grown like the experimental strain (*AzA*) and adjusted to 1.2×10^8 cells mL^{-1} (Couillerot et al., 2013).

2.5.2. Preparation of pots for green house experiment

For pot experiments, the plastic pots (20 cm top dia. × 10 cm h) were washed with 1% teepol followed by sterile water to remove traces of detergent, finally with 50% (w/v) bleaching powder and dried under sun. After drying the inner wall of pots were washed with 70% formaldehyde and filled up to 5 cm below the rim) with about 3 kg sterile sand and soil mixture (1:1 by weight autoclaved at 121 °C, 1 h, 3 d consecutively) (Yadav et al., 2014)

2.5.3. Evaluation of Azotobacter sp. Strain Avi2 in pots in green house

Healthy, 21 d old rice (cv. Swarna) seedlings were washed thoroughly in tap water followed by sterile tap water and dipped separately in bacterial suspensions (1.2×10^8 cells mL^{-1}) for 6 h and transplanted in different pots with three replications (degree of freedom = 12, one seedling $hill^{-1}$, three plants pot^{-1}, there replication $treatment^{-1}$) each viz. control

(T0): without fertilizer and seedlings soaked in sterile ANF media before transplanting,

(T1): seedlings treated with 1.2×10^8 cells mL^{-1} of *AzA* cells,

(T2): seedlings treated with 1.2×10^5 cells mL^{-1} of *AzA*, and

(T3): seedlings were initially treated with sterile tap water followed by growing in

recommended dose of fertilizers (RDF; 120:60:60 mg N:P:K kg^{-1} soil),

(T4): seedlings were treated with 1.2×10^8 cells mL^{-1} of *AzA* which was further

supplemented with half "N" of recommended doses of fertilizers (60:60:60 mg

N:P:K kg^{-1} soil) were applied.

(T5): seedlings were treated with 1.2×10^8 cells mL^{-1} of *AzC* and

Table 1
Effect of *Azotobacter* sp. Avi2 (*AzA*), commercial *Azotobacter* sp. (*AzC*) and recommended dose of fertilizers (RDF) on growth of rice genotype Swarna on pots in green house.

Growth parameters	T 0	T1	T2	T3	T4	T5	T6	CV(%)	Tukey HSD at 5%
Plant height (cm)	$43.58^{C} \pm 0.84$	$55.78^{AB} \pm 1.26$	$54.59^{AB} \pm 1.02$	$57.64^{A} \pm 1.21$	$58.30^{A} \pm 1.10$	$52.93^{B} \pm 1.07$	$56.02^{AB} \pm 0.8$	6.82	4.5854
Tiller hill^{-1} (no.)	$2.33^{C} \pm 0.23$	$3.58^{AB} \pm 0.23$	$2.67^{BC} \pm 0.22$	$4.25^{A} \pm 0.21$	$4.50^{A} \pm 0.28$	$4.25^{A} \pm 0.13$	$4.33^{A} \pm 0.14$	20.72	0.9526
Effective tiller hill^{-1} (no.)	$1.33^{D} \pm 0.14$	$3.17^{B} \pm 0.24$	$2.33^{C} \pm 0.19$	$3.75^{AB} \pm 0.18$	$4.17^{A} \pm 0.27$	$3.08^{BC} \pm 0.19$	$3.83^{AB} \pm 0.11$	21.67	0.8327
Root length (cm)	$18.13^{C} \pm 0.17$	$30.23^{A} \pm 0.52$	$30.27^{A} \pm 0.32$	$24.80^{B} \pm 0.55$	$28.60^{A} \pm 0.56$	$25.70^{B} \pm 0.15$	$26.30^{B} \pm 0.32$	2.53	1.8979
Root wet wt. (g)	$4.76^{F} \pm 0.04$	$11.58^{CD} \pm 0.21$	$10.53^{DE} \pm 0.16$	$9.48^{E} \pm 0.18$	$20.82^{A} \pm 0.38$	$11.76^{C} \pm 0.07$	$15.54^{B} \pm 0.09$	3.08	1.0607
Root dr. wt. (g)	$1.05^{E} \pm 0.02$	$2.18^{CD} \pm 0.01$	$1.95^{D} \pm 0.02$	$2.71^{B} \pm 0.11$	$3.68^{A} \pm 0.04$	$2.12^{CD} \pm 0.05$	$2.33^{C} \pm 0.04$	4.09	0.2674
Root volume (cm^3)	$9.20^{F} \pm 0.05$	$16.37^{C} \pm 0.18$	$15.53^{D} \pm 0.12$	$18.93^{B} \pm 0.20$	$28.37^{A} \pm 0.14$	$14.53^{E} \pm 0.12$	$19.43^{B} \pm 0.09$	1.24	0.6215
Flag leaf area (cm^2)	$62.51^{G} \pm 0.48$	$83.63^{E} \pm 0.26$	$80.67^{F} \pm 0.32$	$114.79^{C} \pm 0.71$	$147.67^{A} \pm 0.4$	$117.34^{B} \pm 0.44$	$96.94^{D} \pm 0.24$	0.71	2.051
Second leaf area (cm^2)	$71.64^{G} \pm 0.79$	$101.74^{D} \pm 1.08$	$95.30^{E} \pm 0.63$	$118.87^{A} \pm 0.95$	$114.70^{B} \pm 0.42$	$79.72^{F} \pm 0.88$	$108.79^{C} \pm 0.6$	1.38	3.897
Third leaf area (cm^2)	$68.26^{E} \pm 0.97$	$88.67^{D} \pm 0.63$	$88.31^{D} \pm 0.82$	$111.90^{A} \pm 0.27$	$111.33^{A} \pm 1.17$	$96.57^{C} \pm 0.53$	$101.91^{B} \pm 0.87$	1.53	4.1528
Panicle length (cm)	14.72 ± 1.06	17.9 ± 0.3	18.13 ± 0.2	18.09 ± 0.6	17.1 ± 1.86	17.42 ± 0.26	17.83 ± 0.38	15.49	NS
Panicle weight (g)	$1.10^{E} \pm 0.01$	$1.78^{D} \pm 0.02$	$1.76^{D} \pm 0.01$	$2.15^{B} \pm 0.008$	$2.53^{A} \pm 0.02$	$1.88^{C} \pm 0.02$	$1.87^{C} \pm 0.01$	1.71	0.091
Grain weight plant^{-1} (g)	$3.40^{F} \pm 0.13$	$6.12^{E} \pm 0.10$	$6.03^{E} \pm 0.05$	$11.20^{B} \pm 0.15$	$12.94^{A} \pm 0.19$	$9.09^{D} \pm 0.13$	$10.42^{C} \pm 0.13$	2.93	0.7074
Grain yield plant^{-1}(no)	$192.00^{E} \pm 6.92$	$308.00^{D} \pm 4.61$	$305.33^{D} \pm 3.52$	$503.33^{B} \pm 7.26$	$565.00^{A} \pm 7.63$	$436.67^{C} \pm 6.00$	$476.67^{B} \pm 6.66$	2.87	32.66
Filled grain panicle^{-1}(no)	$64.00^{E} \pm 2.30$	$77.00^{D} \pm 1.15$	$76.33^{D} \pm 0.88$	$100.67^{B} \pm 1.45$	$113.00^{A} \pm 1.52$	$87.33^{C} \pm 1.20$	$95.33^{B} \pm 1.33$	3.03	7.5989
Chaps panicle^{-1}(no)	$8.00^{BC} \pm 0.57$	$2.00^{D} \pm 0.57$	$2.00^{D} \pm 0.57$	$9.00^{B} \pm 0.57$	$5.67^{C} \pm 0.88$	$15.00^{A} \pm 0.57$	$7.67^{BC} \pm 0.88$	15.99	3.2201
1000 grain wt. (g)	$17.69^{F} \pm 0.08$	$19.85^{E} \pm 0.05$	$19.76^{E} \pm 0.07$	$22.26^{B} \pm 0.05$	$22.90^{A} \pm 0.04$	$20.81^{D} \pm 0.03$	$21.85^{C} \pm 0.03$	0.49	0.2882
Chlorophyll a (mg g^{-1} wt. leaf tissue)	$0.85^{F} \pm 0.007$	$1.79^{D} \pm 0.03$	$1.83^{D} \pm 0.01$	$1.60^{E} \pm 0.01$	$2.71^{B} \pm 0.03$	$2.11^{C} \pm 0.01$	$2.96^{A} \pm 0.01$	1.98	0.1121
Chlorophyll b (mg g^{-1} wt. leaf tissue)	$0.25^{E} \pm 0.001$	$0.61^{D} \pm 0.03$	$0.61^{D} \pm 0.001$	$0.58^{D} \pm 0.001$	$1.00^{B} \pm 0.001$	$0.75^{C} \pm 0.008$	$1.08^{A} \pm 0.01$	2.25	0.0449
Chlorophyll a + b (mg g^{-1} wt. leaf tissue)	$1.09^{F} \pm 0.007$	$2.40^{D} \pm 0.05$	$2.44^{D} \pm 0.01$	$2.18^{E} \pm 0.02$	$3.71^{B} \pm 0.03$	$2.86^{C} \pm 0.02$	$4.04^{A} \pm 0.02$	1.88	0.1434

Treatments.
T0: Seedlings soaked in sterile ANF media before transplanting.
T1: Seedlings treated with 1.2 × 108 cells mL^{-1} of AzA cells.
T2: Seedlings treated with 1.2 × 105 cells mL^{-1} of AzA.
T3: Seedlings were initially treated with sterile tap water followed by growing in recommended dose of fertilizers (RDF; 120:60:60 mg N:P:K kg^{-1} soil).
T4: seedlings were treated with 1.2 × 108 cells mL^{-1} of AzA which was further supplemented with half "N" of recommended doses of fertilizers (60:60:60 mg N:P:K kg^{-1} soil).
T5: Seedlings were treated with 1.2 × 108 cells mL^{-1} of AzC and half "N" of recommended dose of fertilizers (60:60:60 mg N:P:K kg^{-1} soil).
T6: seedlings were treated with 1.2 × 10^8 cells mL^{-1} of *AzA* and full dose P-K fertilizers (60:60 mg P: K kg^{-1} soil).

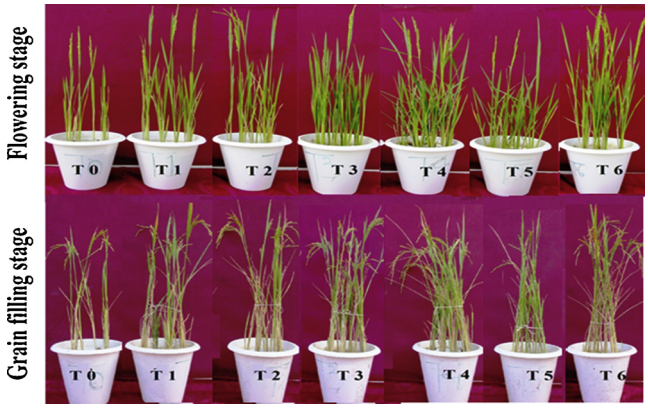

Fig. 4. Comparative plant growth (cv. Swarna) treated with different concentrations of *Azotobacter* sp. Avi2, commercial *Azotobacter* sp. and recommended doses of fertilizer. **T0**- seedlings treated with sterile Ashby's nitrogen-free medium, **T1**- seedlings treated with 1.2 × 10^8 cells mL^{-1} *Azotobacter* sp. Avi2 cells, **T2**- seedlings treated with 1.2 × 10^5 cells mL^{-1} *Azotobacter* sp. Avi2 cells, **T3**- seedlings treated with sterile tap water grown with recommended doses of fertilizers (RDF; 120:60:60 mg N:P:K kg^{-1} soil), **T4**- seedlings treated with 1.2 × 10^8 cells mL^{-1} *Azotobacter* sp. Avi2, and grown with half recommended N dose (60:60:60 mg N:P:K kg^{-1} soil), **T5**- seedlings treated with 1.2 × 10^8 cells mL^{-1} commercial *Azotobacter* sp. and grown with half N of recommended dose (60:60:60 mg N:P:K kg^{-1} soil), **T6**- seedlings treated with 1.2 × 10^8 cells mL^{-1} *Azotobacter* sp. Avi2 and grown with full dose P-K fertilizers, without N (60:60 mg P:K kg^{-1} soil).

half "N" of

recommended dose of fertilizers (60:60:60 mg N:P:K kg^{-1} soil) and for

(T6): seedlings were treated with 1.2 × 10^8 cells mL^{-1} of *AzA* and full dose P-K

fertilizers (60:60 mg P: K kg^{-1} soil) (Yadav et al., 2014).

2.5.4. Assessment of growth parameters

The mature crop was harvested after 145 d of the transplantation and different growth parameters like plant height (cm), panicle length (cm), tiller hill^{-1} (no), effective tiller hill^{-1} (no), leaf area (square cm) and panicle length (cm) were measured prior to harvest. The root length (cm), root volume (mL), root dry weight (g), panicle weight (g), grain yield plant^{-1} (g), filled grain panicle^{-1} (no.) and 1000 grain weight (g) were recorded after harvest.

2.5.5. Measurement of chlorophyll fluorescence and chlorophyll content

Chlorophyll fluorescence of fully expanded flag leaves was measured at pre-dawn of the day from three different plants of same treatment using a Plant Efficiency analyser, Handy PEA (Hansatech Instruments Ltd., Norfolk, UK). Leaves were maintained in darkness for 30 min before recording chlorophyll fluorescence. The irradiance saturating pulse was set at 1500 μM/ (m^2s^{-1}) to capture the maximal intensity of source the light. Various chlorophyll fluorescence parameters like maximal fluorescence (F_m), minimal fluorescence (F_o), variable fluorescence ($Fv = F_m - F_o$), the maximum photochemical efficiency of PSII (F_v/F_m) and performance index (PI) were calculated using the software supplied by the manufacturer. After measuring the photosynthetic rate and chlorophyll fluorescence characteristics from the flag leaf, the same leaves were used for the determination of chlorophyll content, which encompassed both chlorophyll *a* and chlorophyll b. 100 mg of finely chopped fresh leaves were placed in a flacon tube containing 20 mL 80% acetone and kept in a refrigerator at 4 °C for 24 h (Panda et al., 2008). The chlorophyll content was

Table 2
Different parameters of chlorophyll fluorescence at flowering (F) and grain filling (GF) NS- non-significant.

Parameter	Growth Stage	T 0	T1	T2	T3	T4	T5	T6	CV (%)	Tukey HSD at 5%
F_0	F	247 ± 8.71	240 ± 1.73	244 ± 1.52	241 ± 3.21	237.67 ± 5.17	238.33 ± 2.66	238.33 ± 2.72	3.02	NS
	GF	271 ± 9.81	278.33 ± 12.45	254 ± 6.50	253 ± 6.65	267.33 ± 8.96	270.67 ± 8.21	265.67 ± 11.25	6.19	NS
F_m	F	$1194.33^{B} \pm 4.91$	$1355.33^{A} \pm 7.35$	$1351.67^{A} \pm 1.76$	$1338^{A} \pm 5.03$	$1281.33^{AB} \pm 5.62$	$1261.33^{AB} \pm 16.17$	$1310.67^{AB} \pm 19.54$	3.66	136
	GF	$1274.00^{AB} \pm 41.30$	$1295.33^{A} \pm 43.17$	$1212.33^{AB} \pm 21.83$	$1094.67^{B} \pm 19.91$	$1293^{A} \pm 36.05$	$1282.67^{AB} \pm 14.33$	$1295.67^{A} \pm 64.39$	5.41	193.3
F_v	F	$944^{B} \pm 7.23$	$1123.33^{A} \pm 6.88$	$1107.67^{A} \pm 1.45$	$1097^{A} \pm 2.08$	$1043.67^{AB} \pm 5.14$	$1023^{AB} \pm 13.61$	$1072.33^{A} \pm 19.17$	4.09	123.7
	GF	$1003^{A} \pm 31.50$	$1017^{A} \pm 32$	$958.33^{AB} \pm 16.91$	$841.67^{B} \pm 16.67$	$1025.67^{A} \pm 28.52$	$1012^{A} \pm 13.31$	$1030^{A} \pm 55.96$	5.66	159.2
F_v/F_m	F	$0.77^{B} \pm 0.005$	$0.82^{A} \pm 0.0008$	$0.82^{A} \pm 0.0008$	$0.82^{A} \pm 0.001$	$0.81^{A} \pm 0.004$	$0.81^{A} \pm 0.0005$	$0.82^{A} \pm 0.005$	0.76	0.018
	GF	$0.79^{AB} \pm 0.0008$	$0.79^{AB} \pm 0.004$	$0.79^{AB} \pm 0.002$	$0.77^{B} \pm 0.004$	$0.79^{A} \pm 0.003$	$0.79^{AB} \pm 0.005$	$0.79^{A} \pm 0.006$	1.04	0.023
PI	F	$2.79^{B} \pm 0.009$	$5.56^{AB} \pm 0.250$	$6.12^{A} \pm 0.559$	$5.69^{A} \pm 0.677$	$6.59^{A} \pm 0.833$	$6.98^{A} \pm 0.201$	$5.85^{A} \pm 0.710$	17.69	2.858
	GF	2.62 ± 0.31	2.74 ± 0.2	2.92 ± 0.17	2.89 ± 0.18	3.55 ± 0.44	3.24 ± 0.55	3.18 ± 0.51	22.82	NS

(Treatments are same as Table 1).

determined spectrophotometrically following Porra (2005).

2.6. Field evaluation of Azotobacter sp. strain Avi2

Field experiment (each field size- 29 m × 2.2 m, distance between plant to plant and line to line 15 × 15 cm, transplanted 2 seedlings $hill^{-1}$, total row × column- 9 × 49) was conducted in *Rabi* (dry) and *Kharif* (wet) seasons, with four replications for each treatment (degree of freedom = 15) using *AzA* strain. The layout (Randomized complete block design) of the fields was generated using the online server of ICAR- Indian Agricultural Statistical Research Institute (Design Resources Server- http://iasri.res.in/design/), New Delhi. Rice (*Oryza sativa* L. var. *Swarna*) seedlings were grown 21 d in seedbed and seedling were dipped separately in bacterial suspensions (1.2×10^8 cells mL^{-1}) for 6 h and transplanted in different pots with four replications each viz.

Treatment 1 (T0) without any fertilizer where seedlings were soaked in sterile

Ashby's nitrogen-free medium before transplanting,

Treatment 2 (T1) containing seedlings treated with 1.2×10^8 cells mL^{-1} of *AzA* cells,

Treatment 3 (T2) seedlings were initially treated with sterile tap water which was

further given recommended dose of fertilizers (RDF; 120:60:60 mg N:P:K kg^{-1} soil),

Treatment 4 (T3) seedlings were treated with 1.2×10^8 cells mL^{-1} of *AzA* which was

further given half "N" of recommended dose of fertilizers (60:60:60 mg N:P:K kg^{-1}

soil),

Treatment 5 (T4) seedlings were treated with 1.2×10^8 cells mL^{-1} of commercial

bio-fertilizer formulations of *AzC*,

Treatment 6 (T5) seedlings were treated with 1.2×10^8 cells ml^{-1} of *AzA* and was

further given full dose P-K fertilizers (60:60 mg P:K kg^{-1} soil) but without N

fertilizer (Das et al., 2003).

Plant height, tiller number and chlorophyll contain were measure at flowering stage (at 70 days, as it is metabolically most active stage) and rest of the yield parameters like panicle length, panicle weight, grain weight etc. were calculated at harvest time (at 145 days).

2.7. Statistical analysis

Plant samples were analysed by selecting plants from one squire meter of each experimental plots (central area of each plot to reduce the boudary effect) and normalized using z-transformation. All the experimental results were scrutinized using online server of Statistical Computing for NARS, Indian Agricultural Statistics Research Institute (IASR), New Delhi, India. The mean difference comparison between the treatments was analyzed by analysis of variance (ANOVA) and subsequently by Tukey's HSD at 5% level (Banik et al., 2017).

3. Results

3.1. Phylogenetic analysis

From 16S rDNA sequence analysis through Ribosomal Data project (RDP) database and NCBI, the strain was classified as the genus *Azotobacter* (Fig. 1). The strain Avi2 showed the closest sequence similarity with *Azotobacter chroococcum*^{T} ATCC9043 (99%), followed by *Azotobacter beijerinckii*^{T} ATCC 19360 (99%). The evolutionary history was determined using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.31659629 is shown. There were a total of 1338 positions in the final dataset (Fig. 1). The strain was

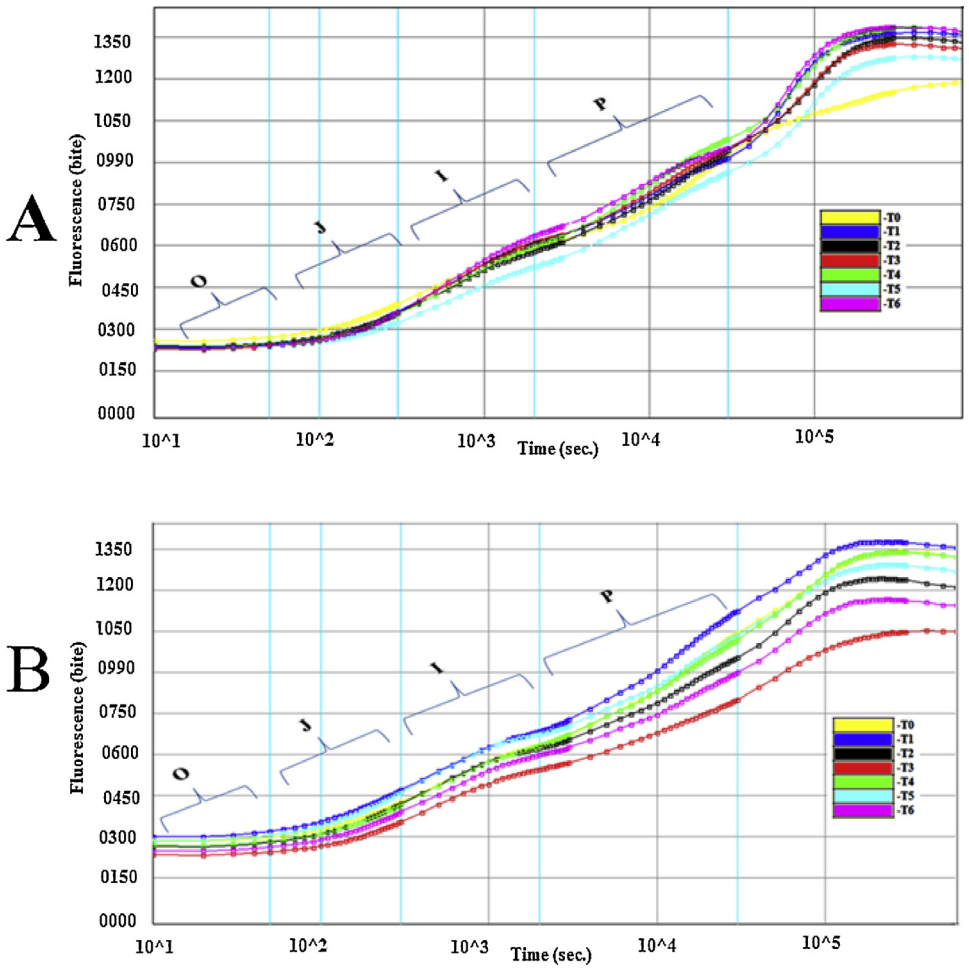

Fig. 5. Distribution of steady state (Fs) chlorophyll fluorescence emission at flowering (A) and grain filling (B) stages of flag leaf (treatments ref. as Fig. 5). Open symbols [▫] show distribution of fluorescence recorded through FluorCam image, while closed symbols represent the same values corrected for the leaf inclination [▫]. The 3D corrected data were calculated by renormalizing the fluorescence signal by cosine of the angle between the normal to the leaf surface and the optical axes of the camera.

submitted to Microbial Culture Collection, National Center for Cell Science, India and assigned the accession number MCC 3432.

3.2. Effect of different doses Azotobacter sp. strain Avi2 on rice seedling growth

Effectiveness of the *AzA*, on 2 d and 5 d old rice seedlings (cv. Swarna) revealed that higher dose of inoculum (1.2×10^8 cells mL^{-1}) produced higher root (6.3 ± 0.06 cm), shoot (5.33 ± 0.09 cm) length and biomass (fresh weight of 6 seedlings 0.85 g and dry weight of 6 seedlings 0.09 g) compared to uninoculated control (seedlings are treated with sterile Ashby's broth media) (fresh weight of 6 seedlings 0.48 g and dry weight of 6 seedlings 0.04) seedlings. Whereas lower dose of inoculums (1.2×10^5 cells mL^{-1}) (fresh weight of 6 seedlings 0.77 g and dry weight of 6 seedlings 0.08 g) produced less biomass than the higher inoculum. Whereas, 5 d old sterile seedlings gained more root and shoot length, and biomass when treated with two different concentration of inocula after for 10 d growth (Fig. 2A and Table S1). The transverse section of root appeared much more developed under the microscope than the control with a depressed number of root hairs (Fig. 2B).

3.2.1. Scanning electron microscopy of root colonized by Azotobacter sp. strain Avi2

Scanning electron microscopic image of 21 d old root of rice seedlings, colonized *Azotobacter* sp. strain Avi2 showed accumulation of bacterial biofilm at the junction of primary root and lateral root, especially at the zone of elongation confirming the root-colonizing ability of the bacterium (Fig. 2C)

3.3. Comparative of PGP activity of Azotobacter sp. strain Avi2 and a commercial Azotobacter sp. in laboratory, pot and field tests

3.3.1. Laboratory culture

Plant growth promoting attributes of *AzA* strain was compared with the commercial bio-fertilizer of *AzC*. After 15 d incubations at 30 ± 2 °C on 14 h light and 10 h dark cycle, it was observed that *AzA* increased root length by 12%, shoot length by 17%, fresh weight by 13% and dry weight by 33% over the commercial strain (Fig. 3 and Supplementary Table S2).

3.3.2. Plant growth attributes in pot culture

Plant height increased progressively with time and reached the maximum at flowering stage. The rate of increase in height of the plant was maximum between 45 and 90 d and tabulated in Table 1 for the greenhouse experiment. Plant height, Tiller number, Root wet/dry weight, leaf area, panicle weight, number of filled grains area were maximum in T4. Numbers of chaps were very low in the only bacteria treated plants only (T1and T2), while it was maximum in T5 (Table 1, Fig. 4 and Supplementary Fig. S2).

Table 3
Effect of *Azotobacter* sp. Avi2, commercial *Azotobacter* sp. and recommended dose of fertilizer on rice plant in different physiological combinations grown on *Rabi* season (dry season) and *Kharif* (wet season) in fields.

Growth parameters	Growing Season	T0	T1	T2	T3	T4	T5	CV (%)	Tukey HSD at 5%
Plant height at 70d (cm)	*Rabi*	$37.91^{CD} \pm 1.09$	$42.71^{BC} \pm 1.27$	$45.68^{AB} \pm 1.39$	$44.62^{B} \pm 2.32$	$35.49^{D} \pm 1.96$	$44.12^{BC} \pm 1.18$	15.5	6.6
	Kharif	$35.82^{D} \pm 0.43$	$40.69^{C} \pm 0.23$	$46.10^{B} \pm 0.63$	$48.24^{B} \pm 0.68$	$37.07^{D} \pm 0.51$	$45.93^{B} \pm 0.64$	6	2.61
Tiller hill^{-1} at 70d (no.)	*Rabi*	$11.70^{C} \pm 0.39$	$13^{C} \pm 0.65$	$21.90^{A} \pm 1.11$	$21.60^{A} \pm 1.48$	$12.20^{C} \pm 0.70$	$18.05^{AB} \pm 1.22$	24.3	4
	Kharif	$11.45^{D} \pm 0.39$	$13.70^{C} \pm 0.44$	$23.60^{A} \pm 0.59$	$24.35^{A} \pm 0.5$	$13.05^{CD} \pm 0.4$	$19.45^{B} \pm 0.58$	11	1.942
Plant height at harvest (cm)	*Rabi*	$68.63^{E} \pm 0.26$	$71.83^{D} \pm 0.53$	$78.23^{B} \pm 1.22$	$75.83^{BC} \pm 0.29$	$71.53^{D} \pm 0.28$	$73.37^{CD} \pm 0.37$	1.18	2.6
	Kharif	$70.10^{C} \pm 0.46$	$73.50^{B} \pm 0.78$	$79.80^{A} \pm 1.51$	$79.90^{A} \pm 0.3$	$72.20^{BC} \pm 0.63$	$74.03^{B} \pm 0.54$	1.5	3.287
Root length (cm)	*Rabi*	$20.93^{D} \pm 0.43$	$31.17^{A} \pm 0.6$	$28.73^{ABC} \pm 0.86$	$29.27^{AB} \pm 0.53$	$27.77^{BC} \pm 0.63$	$26.20^{C} \pm 0.17$	3.7	2.9
	Kharif	$21.57^{E} \pm 0.2$	$32.63^{A} \pm 0.26$	$30.57^{B} \pm 0.2$	$33.60^{A} \pm 0.2$	$27.67^{CD} \pm 0.39$	$26.53^{D} \pm 0.2$	2.3	1.871
Root wet wt. (g)	*Rabi*	$32.17^{F} \pm 0.87$	$48.52^{CD} \pm 0.73$	$56.79^{B} \pm 0.84$	$66.73^{A} \pm 0.87$	$35.13^{EF} \pm 1.04$	$52.10^{C} \pm 1.03$	3.05	4.1
	Kharif	$32.56^{G} \pm 0.66$	$49.17^{D} \pm 0.47$	$58.11^{B} \pm 0.17$	$68.82^{A} \pm 0.26$	$35.51^{F} \pm 0.63$	$53.5^{C} \pm 0.24$	2	2.78
Root dr. wt. (g)	*Rabi*	$7.11^{E} \pm 0.05$	$9.28^{D} \pm 0.09$	$18.29^{B} \pm 0.12$	$23.73^{A} \pm 0.26$	$7.22^{E} \pm 0.02$	$12.89^{C} \pm 0.23$	4.34	1.6
	Kharif	$7.92^{F} \pm 0.02$	$9.94^{E} \pm 0.04$	$19.39^{B} \pm 0.29$	$24.43^{A} \pm 0.27$	$8^{F} \pm 0.03$	$14.22^{D} \pm 0.14$	3.1	1.239
Panicle length (cm)	*Rabi*	$20.20^{D} \pm 0.05$	$20.57^{D} \pm 0.18$	$22.40^{B} \pm 0.20$	$23.47^{A} \pm 0.14$	$20.33^{D} \pm 0.08$	$21.40^{C} \pm 0.20$	1.34	0.8
	Kharif	$20.90^{DE} \pm 0.11$	$21.47^{CDE} \pm 0.21$	$23.17^{AB} \pm 0.17$	$23.7^{A} \pm 0.2$	$20.57^{E} \pm 0.24$	$21.87^{CD} \pm 0.34$	1.7	1.091
Panicle weight (g)	*Rabi*	$2.48^{E} \pm 0.04$	$2.62^{D} \pm 0.02$	$3.78^{A} \pm 0.01$	$3.80^{A} \pm 0.01$	$2.16^{F} \pm 0.01$	$2.42^{E} \pm 0.02$	1.35	0.1
	Kharif	$2.55^{DE} \pm 0.01$	$2.66^{D} \pm 0.03$	$3.83^{A} \pm 0.01$	$3.85^{A} \pm 0.02$	$2.18^{F} \pm 0.01$	$2.47^{E} \pm 0.02$	1.3	0.11
Grain yield panicle^{-1}	*Rabi*	$149.33^{D} \pm 3.48$	$158.67^{D} \pm 2.96$	$230^{A} \pm 5.03$	$232.67^{A} \pm 3.52$	$145^{D} \pm 2.51$	$181.67^{C} \pm 3.17$	2.68	14
	Kharif	$155.67^{E} \pm 1.76$	$172.33^{D} \pm 1.7$	$235.67^{A} \pm 2.84$	$237.67^{A} \pm 0.88$	$161.33^{E} \pm 1.2$	$186.67^{C} \pm 1.2$	1.3	7.22
Chaps	*Rabi*	$22.33^{A} \pm 1.45$	$7.67^{B} \pm 1.20$	$19.33^{A} \pm 1.76$	$4.67^{B} \pm 0.88$	$8.67^{B} \pm 0.88$	$5.67^{B} \pm 1.45$	24.3	7.3
	Kharif	$16.67^{A} \pm 0.88$	$8.67^{CD} \pm 1.2$	$13.67^{ABC} \pm 0.88$	$7.33^{D} \pm 0.88$	$10^{BCD} \pm 1.52$	$7.33^{D} \pm 0.88$	16	5.395
1000 grain wt. (g)	*Rabi*	$16.82^{C} \pm 0.29$	$18.48^{B} \pm 0.14$	$16.60^{CD} \pm 0.11$	$20.83^{A} \pm 0.17$	$15.63^{E} \pm 0.16$	$18.63^{B} \pm 0.25$	1.52	0.8
	Kharif	$17.82^{D} \pm 0.05$	$18.93^{CD} \pm 0.01$	$20.27^{ABC} \pm 0.43$	$21.44^{A} \pm 0.27$	$18^{D} \pm 0.32$	$19.66^{BC} \pm 0.20$	2.8	1.614
Chlorophyll (mg g^{-1} wt. leaf tissue) 70d	*Rabi*	5.92 ± 0.40	5.75 ± 0.45	6.36 ± 0.14	6.25 ± 0.41	6.36 ± 0.18	5.66 ± 0.26	13.3	NS
	Kharif	$5.27^{D} \pm 0.08$	$5.38^{D} \pm 0.24$	$6.67^{ABC} \pm 0.06$	$6.94^{ABC} \pm 0.04$	$6.05^{CD} \pm 0.02$	$7.36^{A} \pm 0.34$	5.8	0.885

Treatments.
T0: Seedlings were soaked in sterile Ashby's nitrogen-free medium before transplanting.
T1: Seedlings treated with 1.2 × 108 cells mL^{-1} of AzA cells.
T2: Seedlings were initially treated with sterile tap water which was further given recommended dose of fertilizers (RDF; 120:60:60 mg N:P:K kg^{-1} soil).
T3: Seedlings were treated with 1.2 × 108 cells mL^{-1} of AzA which was further given half "N" of recommended dose of fertilizers (60:60:60 mg N:P:K kg^{-1} soil).
T4: Seedlings were treated with 1.2 × 108 cells mL^{-1} of commercial bio-fertilizer formulations of AzC.
T5: seedlings were treated with 1.2 × 108 cells ml^{-1} of AzA and was further given full dose P-K fertilizers (60:60 mg P:K kg^{-1} soil).

3.3.3. Chlorophyll content and chlorophyll fluorescence of flag leaf from different treatments

Flag leaf chlorophyll content of different treatments differed significantly (at $p < 0.005$). The T6 (4.04 mg g^{-1} leaf tissue) resulted in highest chlorophyll accumulation among the different treatments followed by T4 (3.71 mg g^{-1} leaf tissue), T5 (2.86 mg g^{-1} leaf tissue), T2 (2.44 mg g^{-1} leaf tissue), T1 (2.40 mg g^{-1} leaf tissue), T3 (2.18 mg g^{-1} leaf tissue) and T0 (1.09 mg g^{-1} leaf tissue). Leaf photosystem II (PSII) activity of rice flag leaves in pot culture revealed a difference in chlorophyll fluorescence of leaf adapted in darkness for 30 min measured through plant efficiency analyzer. Values (fluorescence units) of minimal fluorescence (F_0) at pre-flowering stage ranged between of 237.67 to 247 (T4 lowest and T0 highest) and at flowering, stage F_0 increased from 253 (T1 lowest) to 278.33 (T3 highest). Maximum fluorescence (F_m) at the pre-flowering stage was between of 1355.33 (T4 lowest) to 1194.33 (T0 highest) and at the flowering stage the range was of 1094.67 (T3 lowest) and to 1295.67 (T6 highest). Variable fluorescence (F_v) of different treatments at pre-flowering stage ranged between 944 (T0 lowest) to 1123.33 (T1 highest) and at the flowering stage, it lied between 1094.67 (T3 lowest) to 1295.67 (T6 highest). The maximum photochemical efficiency of PSII (F_v/F_m) at pre-flowering stage ranged between 0.77 (T0 lowest) to 0.82 (T1, T3, T2, T6 highest) and at the flowering stage, it ranged between 0.77 to 0.79 (T3 lowest). A proportion of activity of the water-splitting complex (F_v/F_o) on the donor side of the PSII was highest for T1 (4.65) and lowest for T0 (3.46) at pre-flowering stage (Table 2 and Fig. 5).

3.3.4. Field evaluation of different plant growth parameters by Azotobacter sp. strain Avi2

Different plant growth parameters were increased progressively and tabulated in Table 3. The rate of increase in height was maximum between 45 and 70 d and thereafter plant height increased slowly with time up to flowering. In both the season (dry and wet) root weight, 1000 grain weight, grain yield per panicle, panicle length, panicle weight, was maximum in T3 whereas the number of chaps were more in T0.

3.4. Determination of population dynamics of Azotobacter sp. strain Avi2

The root endophytic population dynamics were determined from all the treatments and were found to be in the range of 1.8×10^5 to 6.96×10^6 cfu. g^{-1} of dry root. The identity of re-isolated bacteria (present inside the roots) were confirmed as *Azotobacter* sp. strain Avi2 (sequence similarity 100%) by 16S rRNA gene amplification, antibiotic sensitivity test, cyst and pigment formation on Ashby's nitrogen-free medium to support Koch's postulates.

4. Discussion

The present work focused on the field application and evaluation of a potent polyvalent root endophytic bacteria of rice, which may reduce half of the nitrogen fertilizer used in the field condition. From the observation, it was quite evident that different plant growth parameters in fertilizer (RDF; 120:60:60 mg N:P:K kg^{-1} soil) and bacteria treated (60:60:60 mg N:P:K kg^{-1} soil + 1.2×10^8 cells mL^{-1} of *AzA*) plants were quite similar. Considering the current scenario of salinity and metal contamination in agriculturally available soil and concern regarding different stress due to global warming, the efficiency of rhizosphere microbial community is hampered (Dary et al., 2010; Rajkumar et al., 2013; Banik et al., 2018). The upshot of the study will be of great interest for rice cultivation by inoculation in different agricultural system as the studied bacterial strain is an endophyte and

physical factors (temperature, rainfall, UV radiation, soil quality etc.) are less pronounced than those on the rhizospheric one (de Santi Ferrara et al., 2012).

The phylogenetic analysis showed that the strain is a member of *Azotobacter* sp. with closest 16S rDNA sequence similarity with *Azotobacter chroococcum*^{T} ATCC 9043 (99%), followed by *Azotobacter beijerinckii*^{T} ATCC 19360 (99%). However, in absence of overall genome relatedness and fatty acid methyl ester (FAME) data, the species status of the strain remained undetermined (Fig. 1).

From the result (Fig. 2A, B and Table S1) it can be suggested that the concentration of *AzA* and seedling age effects the overall physiological development of rice seedlings. The possible reasons for physiological improvement could be a luxurious production of IAA, NH_3, nitrite, and siderophore by the native *AzA* (Banik et al., 2016a). Previous works conducted by Ambreetha et al., (2018) demonstrated that plant-associated microbes can modulate the expression of auxin-responsive genes of rice roots to modify its architecture. Similarly, it was observed that seedlings developed more adventitious roots as well as root hairs when treated with *AzA* cultures (Fig. 2A and B).

Azotobacter sp. strain Avi2 had differential effects on growth of rice i.e. it assisted overall growth and physiological aspects (plant height, tiller number, effective tiller, root length, root fresh weight, root dry weight, root volume, flag leaf area, second leaf area, third leaf area, panicle length, panicle weight, grain weight, grain yield, filled grain, fewer chaps, 1000 grain weight, chlorophyll content) than the *AzC* and RDF treatments (Table 3). Performance of the *AzA* in the field was quite similar and broadly corroborated the effects of the pot experiments (Table 1). Perhaps all the PGP characters of the native *AzA* guilds supported and improved plant growth over other treatments. The results indicated that *AzA* could enhance growth and yield of the rice var. Swarna by dominating the interaction with other endophytic and rhizospheric microbial population which might not as superior in the field condition (Choudhury and Kennedy, 2004; Bashan et al., 2014). Field application studies reported by de Salamone et al. (2010), demonstrated that a rhizospheric *Azospirillum* inoculation increased the rice production by 4%, compared to the uninoculated treatment. Whereas the native endophytic *AzA* strain increased the yield by 6% in *Rabi* and 10% in *Kharif* season, perhaps demonstrates the dominance of endophytes over rhizosphere strains.

Interestingly, the number of chaps were higher in those treatments when compared with treatment with nitrogenous fertilizer. This might be a good indication of sink–source relationships and requirement of a higher amount of nitrogen during grain filling stages (Wei et al., 2018). However, treatments (T4) with half doses of nitrogen supplemented with *AzA* inoculation displayed higher number of filled grains and lower number of chaps, indicating the native *AzA* was able to fulfil the additional requirement of plant nutrition with different plant growth promoting features. During pot experiment in green house (treatment T6) and field experiment (treatment T6), treatments cultivated without nitrogenous fertilizer produces almost similar number of grains per panicle.

No significant variation in F_0 was observed between treatments in flowering and grain filling stage. But the significant variation in maximal fluorescence (Fm) was observed. T0 had lower Fm value compared to other treatments which indicated that low photochemical efficiency in T0 was due to increase in non-photochemical quenching and not due to destruction in PSII reaction center. Increase in F_0 or the minimal fluorescence level represents the oxidized state of quinone Q_A. A rise in F_0 value indicates destruction of PS II reaction center as energy is dissipated as fluorescence when all reaction center is open and photochemical quenching is minimum. The decrease in maximal fluorescence (Fm) indicates dissipation of excitation energy in the form of heat (nonphotochemical quenching) as photoprotection measure (Araus et al., 1998). Fm in all the treatments was higher compared to T0 but T1, T2, T3, and T6 maintained higher Fm compared to other treatments at flowering stage. But such trend was not observed at grain filling stage. However, treatment with *Azotobacter* had higher Fm compared to recommended fertilizer dose treatment (T3).

A similar trend was observed in Fv which was higher in the treatments with *Azotobacter* than recommended fertilizer dose. This indicates, treatment with *Azotobacter* can replace recommended fertilizer application and can maintain higher photosynthetic rate at grain filling stage. Though Fv/Fm which is an indicator of photochemical efficiency was high in all treatments compared to T0. But much difference in Fv/Fm among the treatment was not observed. According to Moffat et al. (1990), Fv/Fm is the least reliable trait in discriminating photochemical efficiency. Performance index (PI) which estimates plant vitality was also high in T4 and T5 during both flowering and grain filling stage. The decrease in PI occurred mainly due to a decrease of photochemical efficiency of photosynthetic electron transport (Oukarroum et al., 2007). High PI in T4 and T5 compared to other treatments signifies higher vitality and photochemical efficiency even at grain filling stage. Higher photochemical efficiency at grain filling stage represents a delay in leaf senescence process and the slower rate of grain filling that can give better productivity in comparison to the recommended dose of fertilizer.

5. Conclusion

Overall, all the treatments of *AzA* exhibited better vegetative and reproductive growth of rice in pot and field experiment compared to the other treatments. The study was unique and encouraging in the field of rice endophytic research as recent past research was focused only on rhizopheric *Azotobacter* (Sahoo et al., 2014; Yadav et al., 2014; Chen et al., 2018). In addition to endophytic behaviour, *AzA* formed a biofilm in the root associated areas to form a strong association with the host plant (Fig. 2C). Formation of biofilm may also help in enhancement of nitrogen fixation (as biofilm reduces the oxygen tension from nitrogenase system which is very sensitive to oxygen), trapping of nutrition, quorum sensing between microbes etc (Wang et al., 2017). The PGP characters of *Azotobacter* has been well studied in other crops like cotton too, where it increases the yield by 15–28% and plant N concentration due to N_2 fixation (Iruthayaraj, 1981). Nevertheless, better performance of indigenous rice root endophytic *Azotobacter* strain suggested that they should be preferred over the rhizobacteria, for inoculation and further development of a successful inoculant.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2018.11.004.

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