PLANT SCIENCE TODAY
Vol 12(sp4): 01–13
https://doi.org/10.14719/pst.10392

eISSN 2348-1900

RESEARCH ARTICLE

Analysis of plant growth promoting activities of *Azotobacter* isolates and study of their phenotypic effects on bio-primed rice seeds (*Oryza sativa* L.)

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Received: 01 July 2025; Accepted: 19 September 2025; Available online: Version 1.0: 27 November 2025

Cite this article: Aishwarya P, Lovkush S, Shweta M, Mariya A, Anirudha C, Jai P, Ankita S. Analysis of plant growth promoting activities of *Azotobacter* isolates and study of their phenotypic effects on bio-primed rice seeds (*Oryza sativa* L.). Plant Science Today. 2025;12(sp4):01–13. https://doi.org/10.14719/pst.10392

Abstract

Rising population demands increased agricultural productivity on limited arable land. Excessive use of chemical fertilizers is already affecting the environment so one must turn to environmental friendly solutions of using plant growth promoting and natural nitrogen fixing microbes and analyze their effects on plants. In this study, *Azotobacter* spp. was isolated from two different geographic areas and their properties were studied. This study investigates the plant growth-promoting (PGP) potential of three *Azotobacter* strains (M-1, UL-6, A-2.3) and their consortia on rice seeds through bio-priming under *in vitro* and pot trial conditions. Among 22 isolates tested for phosphate and zinc solubilization, ammonia and IAA production, siderophore formation and salinity tolerance, these strains (M-1, UL-6. A-2.3) performed best. Bio-priming treatments with these strains and a consortium revealed improved germination rates, vigor index (VI), root and shoot development along with chlorophyll content in rice plants. *In vitro* results showed a 69.06 % germination rate and 426.98 VI for the consortium, while pot trials highlighted UL-6's enhanced root architecture and shoot length with a germination rate of 56.08 % and VI of 375.32 under controlled environmental conditions. However, *in vitro* results was not always translate to pot trials, suggesting environmental influences, likely due to soil, microbial competition and abiotic factors in non-sterile conditions. The sequence of the best performing strain UL 6 was submitted to NCBI with the accession number PV864958. This study highlights *Azotobacter* spp. as potential bio-fertilizers for sustainable agriculture and emphasizes the need for optimized bio-priming protocols to ensure consistent plant growth across varying conditions.

Keywords: *Azotobacter*; bio-priming; plant growth-promoting traits; rice (*Oryza sativa* L.)

Introduction

Azotobacter, a genus of Gram-negative and diazotrophic bacteria, has garnered significant attention for its potential to promote plant growth and enhance agricultural productivity. *Azotobacter* cells are typically oval-shaped but have also been reported to be rods and cocci (single or in chains). Cells may be dispersed or form irregular clusters or chains of varying lengths in microscopic preparations (1). This genus is also known to form thick-walled cysts under upon exposure to adverse environmental conditions. *Azotobacter* is resistant to oxygen during nitrogen fixation due to respiratory protection of the nitrogenase enzyme, as well as other protective mechanisms such as hydrogenase uptake and switch-on-off mechanisms (2). In recent years, bio-priming techniques utilizing *Azotobacter* strains have emerged as promising strategies for improving seed germination, nutrient uptake and overall plant vigor. As a promising adjunct to conventional chemical nitrogen fertilizers, especially for rice and other N_2-dependent crops, it mitigates environmental pollution and promotes sustainable production.

Rice has been a staple food for half of the world's population since ancient times (3). It is produced in over 100 countries, Asia alone accounts for 90 % of global rice production (3). In India, rice cultivated primarily as a *kharif* crop, but also grown in *rabi*/winter season in some regions, with an annual production of 104.32 million tons (4). Rice cultivation requires a significant amount of nitrogen fertilizers (25 kg urea/acre), particularly from transplanting to panicle initiation, contributing demands high nitrogen input (~25 kg urea/acre), leading to environmental pollution. Moreover, excessive use of fertilizers during the flowering stage can lead to sterility. Hence in light of sustainable agriculture practice, the use of bio-fertilizers with PGP traits has drawn the attention of the research community. *Azotobacter* capable of fixing approximately 20 kg ha^{-1} $year^{1}$ of nitrogen into usable forms like nitrates (NO_3^-) and ammonium ions

(NH_4^+), solubilize phosphate and zinc like soil minerals and production of phytohormones like Indole Acetic Acid (bioauxin) and Gibberellins, has proven to be an efficient bio-fertilizer (5, 6). These free-living bacteria are known to form thick-walled cysts providing them with resilience in adverse environmental conditions (7). Some *Azotobacter* spp. have been found to have salinity tolerance of up to 8 % (8). These traits make them a potential bio-fertilizer candidate for diverse environmental conditions. The application of *Azotobacter* -based bio-fertilizers can substantially reduce the dependence on chemical nitrogen inputs, thereby promoting green and organic agricultural practices.

In this study, we investigate the efficacy of bio-priming treatments utilizing three distinct *Azotobacter* strains, namely M-1, UL-6 and A-2.3, as well as a consortium treatment combining these strains. The experiment compares the germination and growth performance of bio-primed rice seeds under both *in vitro* laboratory settings and pot trials within a controlled polyhouse environment. The findings from this study shed light on the potential of *Azotobacter*-based bio-priming treatments to enhance plant growth and development, while also highlighting the influence of environmental factors on the efficacy of these treatments. Specifically, we analyze the germination rates, VI, root and shoot development, chlorophyll content of bio-primed rice seeds treated with M-1, UL-6, A-2.3 and their consortium, providing insights into their comparative effectiveness and adaptability across different growing conditions.

This research seeks to optimize sustainable agricultural practices by elucidating the performance of these bio-priming treatments, with implications for enhancing crop yield and resilience across diverse farming ecosystems.

Materials and Methods

Collection of samples

The soil samples were collected from two different locations (Suppl. Table 1); Banaras Hindu University (BHU), South Campus, Barkachhakalan, Varanasi, Mirzapur, Uttar Pradesh and from the riverbanks of Ganges (Anta Ghat), Patna, Bihar. Soil was excavated from 15 cm; sample was brought to laboratory and processed within one day of sampling.

Isolation of *Azotobacter*

Azotobacter spp. were isolated using two methods: (A) the single soil grain sowing technique, where 1 mm soil grains were air-dried and placed on mannitol medium agar plates and incubated at 28±2 °C for 5-6 days (9, 10). (B) The soil grain sprinkle method involved grinding air-dried soil in an autoclaved mortar pestle and sprinkling the fine dust (15-16 particles) onto mannitol agar plates, followed by incubation at 28±2 °C for 5-6 days. After incubation, slimy, translucent colonies with various morphologies appeared, which were then transferred to fresh mannitol plates (11).

Among the total isolates obtained, a subset was selected for further evaluation based on their consistent performance in preliminary *in vitro* screening assays, including germination enhancement, seedling VI and root growth promotion. These criteria ensured that only the most promising isolates, representing the desired PGP traits, were advanced to pot trials and subsequent evaluations.

Characterization of *Azotobacter* isolate

All the isolates were diluted in HEPES buffer. The 2 µL sample was evenly spread over a 1 mm microscopic slide, heat fixed, gram stained and observed under 100x magnification (immersion oil). Morphological traits of the isolates were assessed through Gram staining, shape, colony morphology, cyst formation and pigment production (5, 9). PGP activities including phosphate, zinc, potassium solubilization, siderophore, amylase, IAA, ammonia and HCN production were tested in triplicates and controls were used for all *Azotobacter* isolates.

Biochemical assays

Catalase test

The catalase test was performed using the slide method (9). A small sample from an 18 hr-24 hr colony was placed on a microscope slide and 2 mL of 3 % H_2O_2 was added. Immediate effervescence indicated a positive result; weak bubbling was noted as weakly positive and the absence of bubbles was recorded as negative.

Phosphate solubilizing assay

Azotobacter isolates were spot inoculated on NBRI-BPB media containing tricalcium phosphate and incubated at 28±2 °C for 5-6 days (12). The appearance of clear zones around colonies indicated phosphate solubilization.

Amylase assay

Azotobacter are amylase positive and hence can hydrolyse starch (13). The starch hydrolysis test was conducted (14). Isolates were inoculated onto starch agar plates and incubated at 28±2 °C for 4-5 days. After staining with Gram's Iodine, a halo zone around colonies was considered a positive result for amylase production.

Urease test

Christensen's urea agar slants were inoculated with the test isolates. Colour change from orange to fuchsia pink indicated urease activity (15).

Ammonia production assay

Isolates were inoculated into 10 mL peptone water and incubated for 4–5 days at 28±2 °C (16). After adding 0.5 mL of Nessler's reagent, colour change to orange or brown indicated ammonia production.

Hydrogen cyanide (HCN) production assay

HCN production in the test isolates was assessed (17). Isolates were streaked onto 4.4 g/L glycine-modified nutrient agar plates and plates were incubated for 24 hr. After sealing with filter paper soaked in 2 % sodium carbonate (Na_2CO_3) and 0.5 % picric acid, colour change from yellow to brown indicated HCN production and plates were incubated for 3-5 days.

Zinc solubilization assay

Zinc oxide (ZnO) enriched zinc solubilizing media was spot inoculated with test isolates followed by incubation (18). The Halo zone around the colony signifies positive test results.

Siderophore production test

Siderophore-producing microbes could address zinc and other deficiencies in soil. , Chrome Azurol S (CAS) media was prepared to assay the iron sequestering capabilities of the test isolates (19).

Nutrient agar (20 mL) was poured into Petri plates and allowed to solidify. The agar was then cut into two halves and one half was replaced with CAS media by pouring slowly to avoid foaming. Split plate method allowed us to view the change in colour of CAS medium when isolates produced siderophore. After solidification, test isolates were streaked onto the nutrient agar and incubated at 28±2 °C for 24 hr-48 hr. A pink colour in the CAS media indicated a positive result.

Indole-3 acetic acid (IAA) production assay

IAA secretion by test isolates was assessed (20). Isolates were inoculated in 20 mL LB broth containing 5 mmol L-tryptophan and incubated at 28±2 °C for 4 days on a rotary shaker (100 rpm). After centrifugation at 10000 rpm for 10 min, 2 -3 drops of O-phosphoric acid and 2 mL of Salkowski reagent were added to the supernatant. A pink tint indicated a positive result. The change was visually observed without any special equipment.

Potassium solubilization assay

Even though necessary for plant growth, K-solubilization is the least studied property of a PGPB. To study K-solubilization, test isolates were spot inoculated on prepared Aleksandrow medium plates. Incubated at 28±2 °C for 7 days and the diameter of halo zone and colony was measured to quantify solubilization. The appearance of a halo zone around the colony marked positive results (21). Halo zone was visually observed after 3–5 days of incubation.

Salinity tolerance

To test the salinity tolerance of the three best-performing isolates (M-1, UL-6 and A-2.3), isolates were subjected to different salt concentrations of 2 %, 4 %, 6 % and 8 % of Ashby's Mannitol (ASM) Agar (8). Inoculation was done in duplicates and growth in different salt concentrations was noted after 4-7 days of incubation. Growth was observed through visual scoring as it was observed on solid media.

Preparation of the bacterial consortium (M-1+UL-6+A-2.3)

A compatibility test was conducted by streaking 24 hr-old M-1, UL-6 and A-2.3 cultures on the ASM agar plates. The plates were prepared in triplets and incubated at 30 °C for 2-3 days.

After the compatibility testing, the bacterial consortium was prepared by mixing the bacterial cultures M-1, A-2.3 and UL-6 (OD_{600nm}=1.0) in equal amounts (1:1:1 ratio) and making the final volume up to 5 mL (22). The prepared consortium was tested again on ASM plates for growth.

Testing the effects of *Azotobacter* isolates on germination of bio-primed rice seeds (*O. sativa* L.) *in vitro.*

Three best-performing strains M-1, UL-6 and A-2.3 were selected for bio-priming the rice seeds (NDR-97 variety). Four flasks containing 100 mL ASM broth were inoculated with *Azotobacter* isolates; M-1, A-2.3, UL-6 and the bacterial consortium and incubated in the shaker incubator at 30 °C (6-7 days). Cultures of 10^6CFU were centrifuged at 5000 rpm for 5 min and the pellets were suspended in HEPES buffer. With the help of the UV-Vis spectrophotometer, the OD of cell suspensions was taken at 600 nm. OD was recorded as 1.0 for cell suspensions.

Overnight-soaked rice seeds were surface sterilised with 4 % $HOCl_4$ and 70 % ethanol and approx. 80 seeds were placed in each of five sterile 100 mL beakers labelled M-1, A-2.3, UL-6, consortium and control (without bacteria). 1 mL of cell suspensions was added in labelled beakers respectively. Simultaneously, moist chambers inside Petri dishes (90 x 18 mm) were prepared with Whatman filter paper no. 1. Soaked seeds were placed inside and sealed with parafilm. After 5 days, seed germination percentage, growth, root and shoot development were evaluated.

Testing the effects of *Azotobacter* isolates on bio-primed rice seeds in pot trials

Pot trials were conducted to assess the effectiveness of PGP traits of the three best-performing *Azotobacter* isolates M-1, UL-6, A-2.3 and consortium on NDR-97 variety *in vivo*. Ten big-sized earthen pots were prepared with fumigated soil ($KMnO_4$ crystals mixed with formaldehyde) and in replicates bio-primed rice seeds (up to 4 hr) with the cell suspension sown in each respectively labelled pot (two control pots). Each pot contained five rice plants, arranged in a completely randomized design to minimize bias. Seedlings were examined on the 5th day (on germination), 15th day, 30th day and 50th day after the germination based on leaf chlorophyll content, length of the plants, root length, shoot length, thickness of the root and the development of the root system. No chemical fertilizers were added to any of the bio-primed and control plants to study the length of crop sustainability by N_2 fixing *Azotobacter* spp. without the intervention of urea and other nitrogen chemicals. Molecular identification of strain UL 6 was done by 16s RNA primers.

Results and Discussion

Following isolation, pure bacterial isolates were obtained by sub-culturing the colonies twice on ASM agar medium. For identification, colony morphologies were studied under a microscope and are described below. Isolates were reported as Morphologies. The isolates appeared clear, translucent and mucoid in consistency on the culture plates. They were viscous, milky, glistening, mucoid and sticky (difficult to transfer using an inoculating loop), displaying colony colours from cream-white to pale yellow and brown., consistent with previous descriptions (1, 13). Colony characteristics such as shape, size and appearance of each strain of *Azotobacter* were recorded (Table 1).

All *Azotobacter* isolates are normally brown, black and yellow pigment-producing cells (13). *Azotobacter chroococcum* produces characteristic brown pigments which can also be used in their characterisation. These are water-soluble melanin produced during the high metabolism of N_2 fixation (23).

Under 100 × oil immersion magnification, Gram-stained *Azotobacter* strains appeared Gram-negative (24). Strains such as A-3.1, A-4.1, A-4.2, UL-6 and UL-2 were rod-shaped, while strains like M, M-1, A-1.2, A-2.3, A-2.2 and A-2.4 exhibited cocci-shaped cells (1, 25). Some cocci-shaped cells were in pairs, clumps or chains. Isolates with rod-shaped cells were found to be more in number than isolates with cocci or oval-shaped cells (Fig. 1).

Some cells seemed to have thick-walled cysts with contracted oval cells covered with a two-layer capsule which is the prominent feature of *Azotobacter* characterisation. Cysts did not take up the staining. Test isolates showed a broad range of responses in the different PGP tests performed summarized in Table 2. A few of the strains like M-1, M, A-2.3, UL-6, etc. performed well in almost every test which was later selected for bio-priming of NDR-97 variety seeds for the pot trials.

Catalase test results were positive for almost all strains (Fig. 2A), in agreement with previous findings (1, 13, 24). Strains UL-2 (+++), M (+++), M-1 (+++), A-1.3 (++) and A-2.1 (++) demonstrated strong catalase activity, indicated by vigorous effervescence.

All 22 isolates were catalase positive as, all *Azotobacter* spp. have the capacity to produce catalase for the protection of their nitrogenase enzyme confirms the previous findings (10, 25, 14).

Table 1. Morphological and cultural characteristics of isolated *Azotobacter* strains (Colony characteristics 5-6 days after streaking)

Strains	Consistency	Colour	Colony shape & margin	Pigmentation	Growth	Shape
M	Viscous	Whitish/ milky	Punctiform, undulate	-	Moderate	Cocci in clusters
M-1	Viscous	Whitish/ milky	Punctiform, undulate	-	Moderate	Cocci in clumps
M-2	Viscous	Whitish/ milky	Punctiform, undulate	-	Extremely slow	Cocci
U	Slimy	Transparent	Punctiform	-	Slow	Small rods
UL-1	Slimy	Transparent	Punctiform	-	Slow	Small rods
UL-2	Gummy	Transparent	Spherical, elevated	-	Moderate	Rods
UL-3	Gummy	Transparent	Small, round	-	Extremely slow	Cocci
UL-5	Slimy	Transparent	Small, irregular	-	Slow	Rods
UL-6	Gummy	Transparent	Round, small	-	Slow	Small rods
A-1.1	Slimy, viscous	Translucent	Merged, round	Brown (after 7 days)	Dense	Cocci
A-1.2	Slimy, viscous	Opaque	Irregular, undulate	Brownish	Dense	Oval in chains
A-1.3	Slimy, viscous	Translucent	Round, small, elevated	Slight brown (after 7 days)	Moderate	Oval
A-1.4	Slimy, viscous	Opaque	Irregular, undulate	Slight brown	Moderate	Cocci in clusters
A-2.1	Slimy, viscous	Opaque	Irregular, uneven	Brown (after 7 days)	Dense	Cocci in clumps
A-2.2	Slimy, viscous	Opaque	Round, large, even	Brownish (after 7 days)	Dense	Oval in pairs
A-2.3	Slimy, viscous	Opaque	Merged, undulate	Brownish (after 18 days)	Dense	Cocci singly/ pairs
A-2.4	Slimy, viscous	Translucent	Merged, undulate	Brown (after 7 days)	Dense	Cocci singly/ pairs
A-3.1	Slimy, viscous	Translucent	Irregular slightly elevated	-	Moderate	Rods in clumps
A-3.3	Slimy,viscous	Translucent	Punctiform, slightly elevated	-	Moderate	Small rods
A-4.2	Slimy,viscous	Translucent	Flat, round	-	Moderate	Long rods
A-4.B	Slimy,viscous	Translucent	Merged	-	Moderate	Rods singly
A-5	Slimy	Translucent	Irregular	-	Moderate	Small ovals (singly)

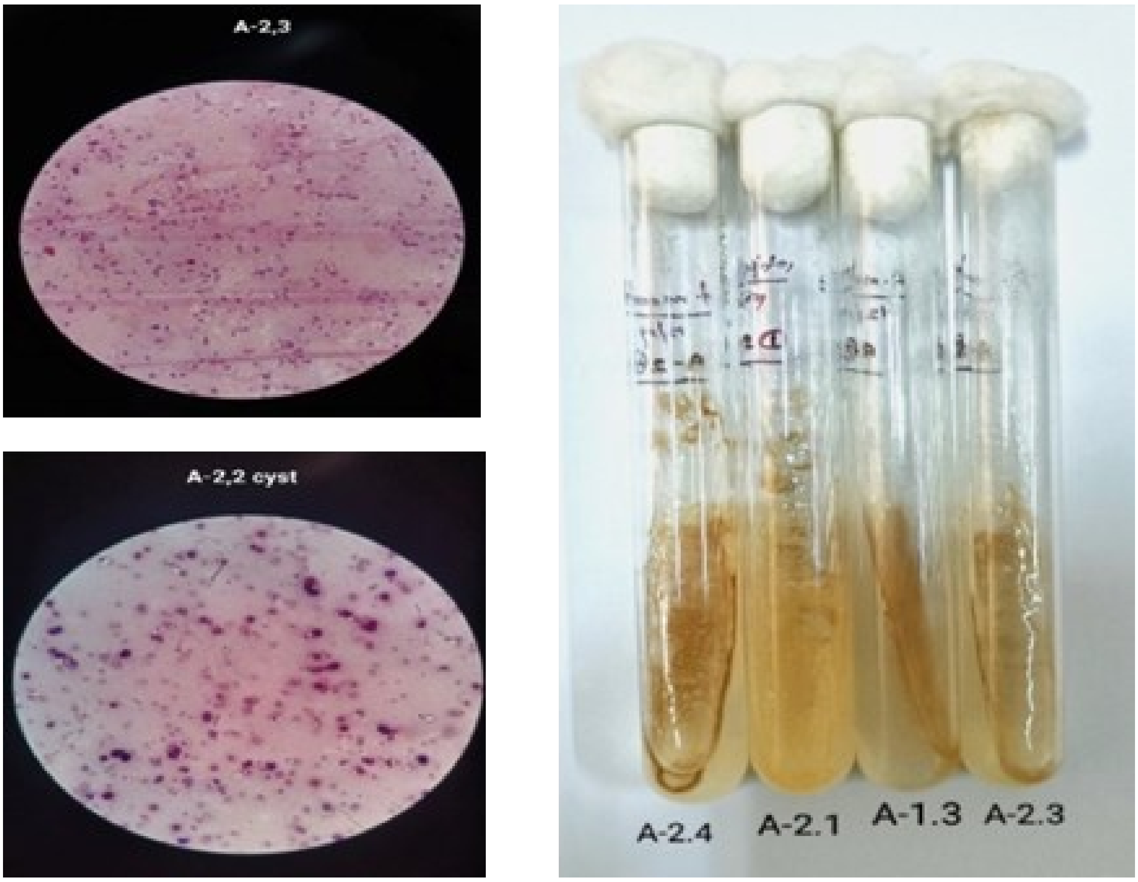
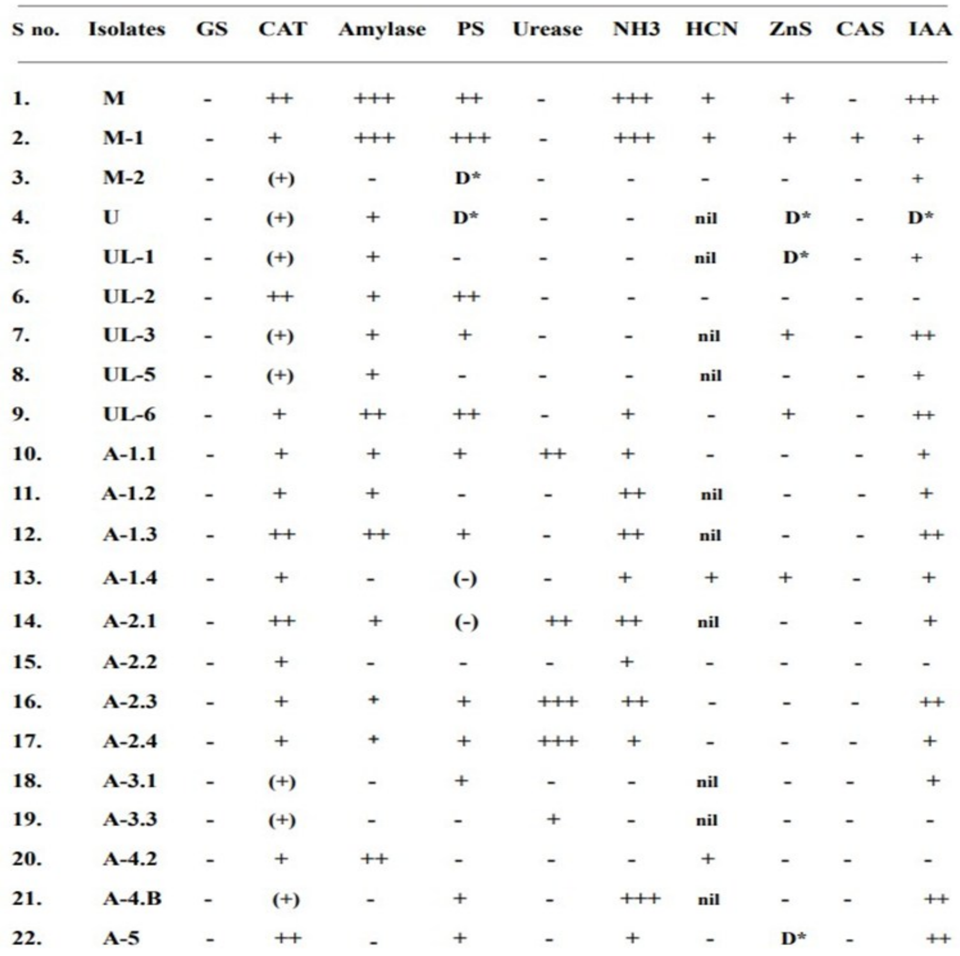


Fig. 1. (A) Oval-shaped, Gram-negative cells under the microscope (100x); (B) Cyst formation by the cells; (C) Brown pigment production by some strains.

Table 2. A conclusive table of plant growth promoting analysis of the test isolates



S no.	Isolates	GS	CAT	Amylase	PS	Urease	NH3	HCN	ZnS	CAS	IAA
1.	M	-	++	+++	++	-	+++	+	+	-	+++
2.	M-1	-	+	+++	+++	-	+++	+	+	+	+
3.	M-2	-	(+)	-	D*	-	-	-	-	-	+
4.	U	-	(+)	+	D*	-	-	nil	D*	-	D*
5.	UL-1	-	(+)	+	-	-	-	nil	D*	-	+
6.	UL-2	-	++	+	++	-	-	-	-	-	-
7.	UL-3	-	(+)	+	+	-	-	nil	+	-	++
8.	UL-5	-	(+)	+	-	-	-	nil	-	-	+
9.	UL-6	-	+	++	++	-	+	-	+	-	++
10.	A-1.1	-	+	+	+	++	+	-	-	-	+
11.	A-1.2	-	+	+	-	-	++	nil	-	-	+
12.	A-1.3	-	++	++	+	-	++	nil	-	-	++
13.	A-1.4	-	+	-	(-)	-	+	+	+	-	+
14.	A-2.1	-	++	+	(-)	++	++	nil	-	-	+
15.	A-2.2	-	+	-	-	-	+	-	-	-	-
16.	A-2.3	-	+	+	+	+++	++	-	-	-	++
17.	A-2.4	-	+	+	+	+++	+	-	-	-	+
18.	A-3.1	-	(+)	-	+	-	-	nil	-	-	+
19.	A-3.3	-	(+)	-	-	+	-	nil	-	-	-
20.	A-4.2	-	+	++	-	-	-	+	-	-	-
21.	A-4.B	-	(+)	-	+	-	+++	nil	-	-	++
22.	A-5	-	++	-	+	-	+	-	D*	-	++

Phosphate solubilizing assay

Phosphate solubilizing bacteria secretes organic acids and acid phosphatases which play an important role in the mineralization of insoluble soil phosphates. Five strains produced halo zones around their colonies (Fig. 2B). Among these, isolates M-1 and A-5 exhibited the highest solubilizing activity. Strains A-2.3, A-3.1 and M formed smaller halo zones. Meanwhile, strains A-2.3, A-3.1 and M formed small halo zones around the colonies.

These observations confirm that *Azotobacter* possesses the ability to solubilize both inorganic and organic phosphate compounds, consistent with earlier reports across diverse crops (25-28).

Amylase assay

Bacteria secrete exo-enzymes (such as α-amylase and oligo-1, 6-glucosidase) which can hydrolyze starch into subunits (dextrin, maltose or glucose) (29). Amylase test is used to identify the ability of the organism to hydrolyze starch as a source of carbon (Fig. 2C). Fifteen out of 22 strains tested positive. Strains M and M-1 showed the strongest amylase activity (+++), followed by A-1.3, A-4.2 and UL-6 (++).

These results indicate that a considerable proportion of the isolates possess strong starch-degrading ability, which is advantageous for their survival in the rhizosphere where starch is a common plant-derived polysaccharide. The high activity observed in strains M and M-1 highlights their potential role in nutrient mobilization and PGP. Similar observations on amylase production in plant-associated bacteria have also been reported, supporting the consistency of our findings (13, 14).

Urease test

In 22 test isolates, 6 test isolates viz. A-2.3, A-2.4, A1.1, A-2.1, A-3.3 and A-3.1 were marked as positive when the colour of the media changed from orange (pH 6.8) to fuchsia pink (pH 8.2) after 24 hr of incubation (Fig. 2D).

The test is used to identify the organisms that are capable of hydrolysing urea. Urease is a constitutively expressed enzyme that hydrolyses urea to produce NH_3 and CO_2. Urease production by *Azotobacter* has been previously reported, which is consistent with our findings (15, 30, 31).

Ammonia production test

Ammonia production was assessed in all 22 test strains, with 13 showing positive results. Strains M, M-1 and A-4. B exhibited the highest activity (+++), followed by A-2.3, A-1.2, A-1.3 and A-2.1 (++). An intense brown colour indicated strong ammonia production (Fig. 2E). These results indicate considerable variation in ammonia production ability among isolates, which may reflect differences in nitrogen metabolism efficiency. Previous studies have also reported high frequency of ammonia production by *Azotobacter* isolates, with some works showing up to 100 % positivity for both IAA and ammonia production, which supports our observations. HCN production assay: Microbes produce HCN as a secondary metabolite, often as part of their defence mechanisms or when interacting with the environment (20, 32-34). Out of 22 test strains 12 best performing strains were selected for the HCN production test. Isolates M, M-1, A-1.4 and A-4.2 were found capable of changing the colour of sterile Whatman filter paper no.1 impregnated with 2 % of Na_2CO_3 and 0.5 % picric acid from yellow to reddish hue (Fig. 2F). The reaction occurs due to the formation of alkaline picrate, which gives a light-yellow background that changes upon HCN release.

These findings suggest limited but detectable HCN production in the tested strains. Previous studies have reported higher frequencies of HCN-positive isolates, where all 24 *Azotobacter* isolates tested were positive, with 10 showing strong activity (33). Previous studies have documented the consistent production of HCN among *Azotobacter* isolates (20, 35, 36). The comparatively weaker responses in the present study indicate possible strain-specific variability in HCN biosynthesis.

Zinc solubilization test

In the present study, four out of 22 test isolates viz. UL-6, UL-3, UL-2 and M were able to form halo zones in the media (Fig. 2G). Among these, isolates UL-3, UL-2 and M exhibited the highest solubilization activity (+++), while UL-6 showed comparatively lower activity (+).

Acidification is one of the common mechanisms employed by zinc-solubilizing bacteria. These microorganisms secrete organic acids into the soil, which bind zinc cations and subsequently lower the pH of the surrounding environment (37). The anions produced during this process have the ability to chelate zinc, thereby increasing its solubility (38). Previous studies have also reported that abiotic stress-tolerant *Azotobacter* spp. possess zinc solubilization potential along with other PGP traits (39).

Siderophore production test

Out of 22 strains, only M-1 was found to be positive for this test (Fig. 2H). Bacteria produce siderophores to sequester the iron from their surroundings and help solubilization of insoluble soil minerals (40). Production of new siderophores by *A. chroococcum*, such as vibrioferrin, amphibactins and crochelins, which can bind iron in a hexadentate form using a new iron-chelating γ-amino acid, is another process that may be implicated in mineral solubilization (41). These siderophores not only assist bacteria in gaining access to iron resources but also aid in the management of soil-borne plant diseases (42). This dual role emphasizes the potential of siderophore -producing isolates like strain M-1 in integrated and sustainable crop management.

IAA production test

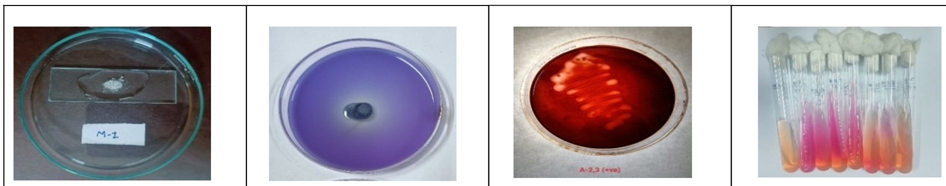
Sixteen of the 22 strains tested positive for IAA production. Strain M exhibited the highest activity (+++), followed by A-2.3, A-1.3, A-4. B, UL -3 and UL-6 (all ++), as evidenced by the medium changing from yellow to orange/pink (Fig. 2I).

IAA is also called 'bioauxin' because it is one of the auxins with the highest physiological activity. Many microorganisms, particularly plant growth-promoting rhizobacteria (PGPR) synthesize IAA as a typical by-product of L-tryptophan metabolism (43). IAA production is an important PGP characteristic as it aids plant growth and development. Seed germination improves when *Azotobacter* is applied to seeds due to its ability to produce IAA when tryptophan is added to the medium (35, 44).

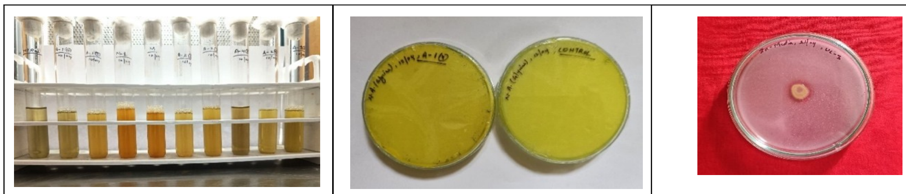
Potassium solubilization assay

To study K-solubilization, three selected strains (M-1, UL-6 and A-2.3) were tested for this activity out of which M-1 (Fig. 2J) and UL-6 (++) showed a comparatively large clear zone than A-2.3 (+).

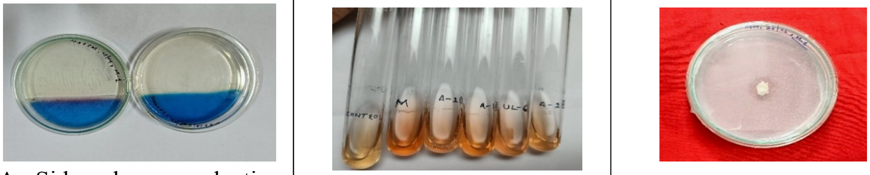
Minerals such as orthoclase, biotite, feldspar and micabear potassium that remains immobilized and is not readily accessible to plants (45). Through the secretion of organic acids and enzymes, *Azotobacter* spp. facilitates the breakdown of mineral complexes containing potassium, releasing it into the soil solution where plant roots can readily absorb it, led the study on potassium solubilization by rhizosphere bacteria in 2016 has reported K-solubilizing



A. Catalase test B. P-solubilization C. Amylase assay D. Urease test



A. Ammonia assay B. HCN production test C. Zn-solubilizing test



A. Siderophore production test B. IAA production assay K-solubilization

Fig. 2. (A) Catalase positive response of strain M-1; (B) Phosphate solubilization clear zone around the colony; (C) Clear zone formation in starch agar media (amylase positive); (D) Pink test tubes are positives for urease test; (E) NH_3 production in test tubes; (F) Weakly positive (brownish) results for HCN production test - right: control, left: positive test; (G) positive Zn-solubilizing strain; (H) Weakly positive strain M-1 for a siderophore production test on CAS media (reddish tint on the blue media) along with the control; (I) Positive results for IAA production assay (development of pink tint in the supernatant); (J) Halo zone formation in K-solubilizing media.

Azotobacter spp. Az70 and Az63 strains are reported to be highly efficient in k-solubilization earlier (46). Previous studies have indicated that *Azotobacter* spp. possess the ability to solubilize potassium, thereby playing a crucial role in enhancing potassium uptake by plants (47, 48). Potassium solubilizing *Azotobacter* spp. thus recognized as a potent biofertilizer (49).

Salinity tolerance

Three selected strains (M-1, UL-6 and A-2.3) were tested for salinity tolerance at different salt concentrations. Results from the test after 4-7 days of incubation are mentioned in the Table 3 below, where treatments A-2.3 and M-1 showed tolerance up to 4 %.

Table 3. Salt tolerance of isolates M-1, UL-6 and A-2.3 on different NaCl concentrations (mg/mL)

Isolates	NaCl (2 %)	NaCl (4 %)	NaCl (6 %)	NaCl (8 %)
A-2.3	Growth	Growth (7th day of incubation)	No growth	No growth
M-1	Growth	Growth (7th day of incubation)	No growth	No growth
UL-6	No growth	No growth	No growth	No growth

Soil salinity poses a significant challenge across the globe, necessitating urgent solutions to enable plant growth in affected regions. Therefore, in the present study, attention was given to testing the salinity tolerance of the isolates. Salinity tolerance in *Azotobacter* spp. has also been previously reported where up to 4 % NaCl concentration, most of the test isolates showed growth (14). Similarly, salt-tolerant strains of *Azotobacter* have been used for growth stimulation in wheat plants (8). *Azotobacter* spp. is tolerant to higher NaCl concentrations i.e., 6 %-8 % (21). Moreover, biopriming with *A. chroococcum* has been shown to enhance plant growth under saline conditions (50), supporting the present findings that *Azotobacter* strains possess effective salt tolerance.

Effects of test isolates on germination of bio-primed rice seeds (*in vitro*)

Concerning all the PGP tests performed in this study, three strains M-1, UL-6 and A-2.3 were selected for the further study of their effects on rice seeds (NDR-97 variety) based on the overall best performance in the tests. To test the effects of these three best-performing strains and their consortium on the rice seeds, seeds were soaked, surface sterilized and bio-primed for 4 hours and then incubated in the moist chamber prepared inside a Petri plate (45). Under controlled conditions after 5 days, bio-primed germinated seeds were observed along with control seeds based on their growth indices and germination percentages (Fig. 3). Seed VIs were calculated (51). Root/shoot lengths were estimated on average of the germinated seeds.

The growth parameters of *invitro* germination of all the isolates are diverse. Each strain shows a different parameter compared to the others and the control. ANOVA results (Table 4), indicate that the bio-priming treatments significantly influenced various early growth parameters of rice seeds, as evident from the critical difference (CD) values. For shoot length, UL-6 showed the highest mean (3.893 cm), significantly exceeding A-2.3, with differences greater than the CD (0.278) and indicating statistical significance. In contrast, A-2.3 recorded the longest root length (3.423 cm), followed by UL-6 and consortium, all significantly better than the control, as the CD (0.149) confirms. Germination percentage was highest in the consortium treatment (69.06 %), significantly surpassing the control and A-2.3, with the CD at 6.215. The VI was also maximum in consortium, significantly higher than the control and all other treatments, as the CD of 28.540 confirms. Low CV values across all traits reflect high precision and consistency of the experiment. Overall, consortium and UL-6 emerged as the most promising bio-priming treatments for enhancing seedling vigor and early growth in rice.

By the 5th day, the consortium treatment showed the most balanced growth with good germination, thick shoots, strong roots and intense branching. M-1 also performed well in shoot thickness and root branching. A-2.3 promoted root elongation but had poor germination and shoot growth. UL-6 showed long shoots with moderate root growth and minimal branching. The control had good germination but weak overall development. Overall, the consortium proved most effective for early seedling vigor.

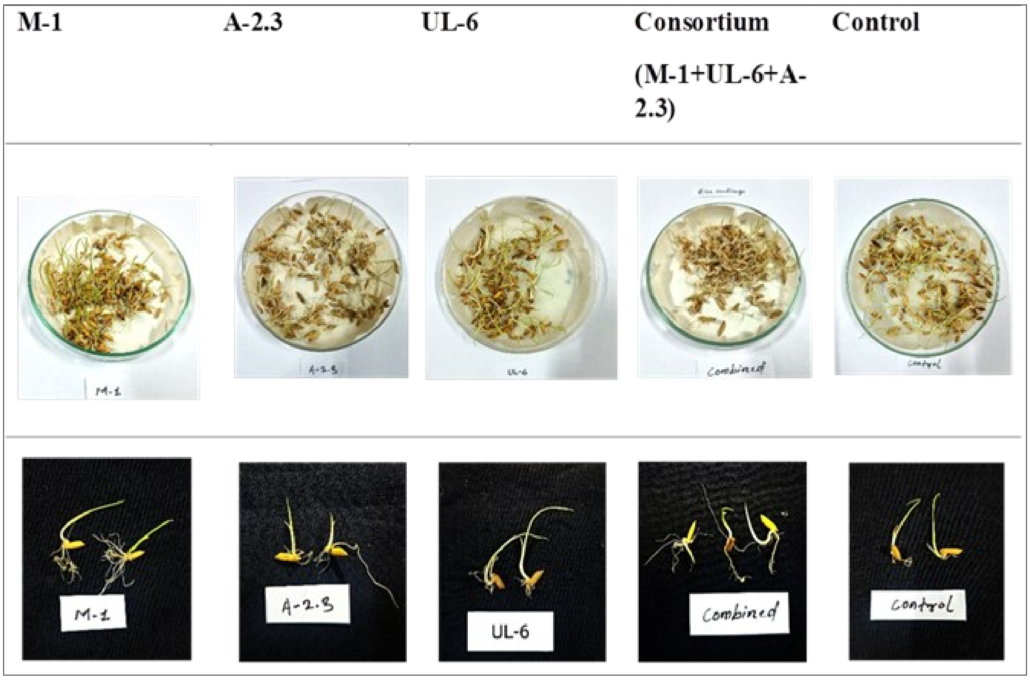


Fig. 3. Bio-primed germinated rice seeds with four treatments + control (*in vitro*). Comparative growth parameters of root and shoot developments in rice seeds after bio-priming with test isolates.

Table 4. Different growth parameters of the germinated bio-primed rice seeds (*O. Sativa* L.) including germination percentage, VI, root/shoot development and root/shoot lengths

Treatments	**Growth parameters of rice seeds on the 5th day of germination (*in vitro*)**							
	Germination	**Root development**	**Shoot development**	**Root branching**	**Mean shoot length (cm)**	**Mean root length (cm)**	**Germination percentage (%)**	**VI**
M-1	Good	Moderate	Thick	Intense (fine hairy network like)	3.253	2.037	64.697	324.39
A-2.3	Poor	Good (few long roots)	Poor (short shoot)	Present	1.887	3.423	55.713	302.660
UL-6	Moderate	Moderate	Thin (long shoot)	Less	3.893	2.977	56.083	375.317
Consortium (M-1+UL-6+A-2.3)	Good	Good	Thick	Intense (fine hair like)	3.323	2.543	69.060	426.98
Control	3.437	Poor	Thin	Less	3.5	1.5	60.390	301.330
SEm					0.088	0.047	1.972	9.057
CV					4.844	3.284	5.583	4.532
CD					0.278	0.149	6.215	28.540

Effects of *Azotobacter* isolates on bio-primed rice (*O. sativa* L.) seeds in pot trial

Bio-primed seeds may exhibit different responses *in vitro* (controlled laboratory conditions) compared to *in vivo* (field conditions). *In vitro* studies typically provide controlled environments with precise conditions to study specific variables. In these conditions, bio-primed seeds may show promising results, such as improved germination rates, enhanced root development or increased resistance to stress factors. However, *in vivo* conditions involve complex interactions within the natural environment and can present different challenges and influences (52). Field trials are essential for validating the effectiveness of bio-priming techniques and assessing their practical applicability in agricultural and horticultural settings.

To validate the *in vitro* findings, replicated pot trials were conducted under polyhouse conditions at IAS, BHU. Treated seeds were potted in July when there is high humidity and precipitation due to monsoon. Pots were filled with water levels up to 2-3 cm thrice a week. Because the study was centred on phenotypical parameters of root/shoot development induced by PGP *Azotobacter* spp. majorly, no nitrogen-containing fertilizers were used in this pot trial. Growth parameters in pot trials were noted as shoot length, root length, plant height and plant chlorophyll content measured with a SPAD meter between 9 -10 AM. Inferences of the pot trials are mentioned below.

From the table 5, it can be highlighted that germination of the bio-primed rice seeds in the pot trials in polyhouse showed significantly different results than *in vitro* conditions of the bio-primed seeds. The treatment consortium (M-1+UL-6+A-2.3) exhibited good growth parameters with a germination percentage of 69.060 and VI of 426.98 *in vitro* but pot trials of all treatments did not show exceptional outcomes as plant's growth parameters did not completely align according to the *in vitro* growth expectations.

The ANOVA results (Table 5) indicate a significant difference among treatments for shoot length, root length and overall plant height, as confirmed by the critical difference (CD) values. The treatment UL-6 showed the highest increase in shoot length (10.65 cm), root length (8.32 cm) and plant height (15.68 cm), significantly outperforming other treatments. The consortium treatment also performed well, followed by M-1. A-2.3 recorded the lowest values across all parameters. Since the observed differences exceed the respective CD values (0.183 for shoot length, 0.168 for root length and 0.268 cm for plant height), the effects of the treatments are statistically significant. This suggests that UL-6 and the consortium have a strong positive impact on plant growth.

The outcomes of the *in vitro* plant growth experiments align with the previous findings, demonstrating that all maize varieties treated with the PR19 strain of *Azotobacter* exhibited superior growth performance across various parameters compared to the control plants under *in vitro* conditions (46).

In a study conducted on *Azotobacter* bio-primed rice seeds, the PGP activities were highly variable with differential effects on growth and yield parameters (52). Hence all PGPRs may not be equally effective and not possess polyvalent functions to support plant growth (53). This might be due to various factors like competition for nutrients, availability of nutrients and minerals, biotic and abiotic conditions etc. among the consortium which compromised the plant growth. Whereas the treatment UL-6 in lab conditions showed longer root and shoot development compared to the other treatments but root system development in the pot trial was more promising with dense and well-developed lateral roots (Fig. 4) (54).

Previous studies have reported that plants grown under laboratory conditions often exhibit different growth parameters compared to those in field trials. There was less growth of oats plants in native soil as compared to *in vitro* and pot tests when bio-primed

Table 5. Growth parameters of four treatments (M-1, A-2.3, UL-6 and Consortium) and control observed on 30 days after germination of rice seeds

Treatment	**Shoot length (cm)**	**Root length (cm)**	**Plant height (cm)**
M-1	9.28	7.583	14.383
A-2.3	6.183	5.483	9.900
UL-6	10.650	8.317	15.683
Consortium	9.717	8.00	14.967
Control	9.017	7.300	14.067
SEm	0.058	0.053	0.085
CV	1.124	1.257	1.067
CD	0.183	0.168	0.268

with PGP bacilli (52). This is because a higher abundance of nitrogen-fixing organisms does not necessarily translate to better field performance, as native microbial community interactions may modulate inoculant efficacy (52, 55-57).

It could be inferred from Tables 5, Suppl. Table 2 and Fig. 4 that after bio priming root systems showed prominent development patterns across different treatments; the root systems of M-1 and A-2.3 were morphologically similar both having thick lateral roots with FLRs. Similar enhanced root growth was reported in maize treated with *Azotobacter nigricans PR19* strain em, consortium treated plants showed slower growth rate and stunted growth (45). The control plant as indicated in Fig. 4, exhibited significant improvement (plant height) but the root system was not as developed as the treated plants. Other plant growth parameters such as shoot length, plant height and chlorophyll content (noted on the 50th day with SPAD meter) were in close range to each other. However, the UL-6 bio-primed plant showed significant drift from the range with a plant height of 74.0 cm along with well-developed roots. *Azotobacter* SRIAz3 promoted greater plant growth compared to other formulations under field conditions (52). This suggests that the effectiveness of biofertilizers is influenced by factors such as the environment, soil properties and interactions with native soil microbial communities.

M-1 plants: Rice seeds bio-primed with M-1 showed good results in both pot trials and in lab conditions. Seeds showed the fastest germination, an intense fine hairy network of roots and thick shoot development. In pot trials, germination was fast though plant height was moderate. But the shoot was healthy (number of leaves more than the control) and the root comprised thick nodal and lateral roots with root hair.

A-2.3 plants, isolate A-2.3 bio-primed rice seeds showed good results in both. *in vitro* and the pot trials. *In vitro* germination of bio-primed seeds showed G.P. of 56.52 % and VI of 310.86 where shoot development was reported as poor. In pot trials, the results were somewhat similar to the M-1 plants in plant height, root/shoot length and root development. Roots mostly showed TLRs and moderate FLRs though roots were thick and long.

UL-6 plants, amongst all the treatments UL-6 bio-primed seeds showed the best results. In lab conditions, germinated seeds developed exceptionally long shoots but small and less developed roots. In the pot trial at the polyhouse, the bio-primed plants attained a height of 74.0 cm (tallest among all the treatments and the control) with a dense and well-developed root system comprising a network of numerous lateral roots and root hairs that strongly held up the soil. Though early growth was moderately paced gradually the rice plants grew taller.

Consortium plant

Consortium (M-1+UL-6+A-2.3) bio-primed rice seeds showed good germination and growth parameters *in vitro* and the seedlings showed intense fine roots and thick shoot development. However, in the pot trial, the results were very contradictory as the plant growth was stunted and a moderately developed root system with few FLRs and germination of the seedlings were also reported to be slower growth rate.

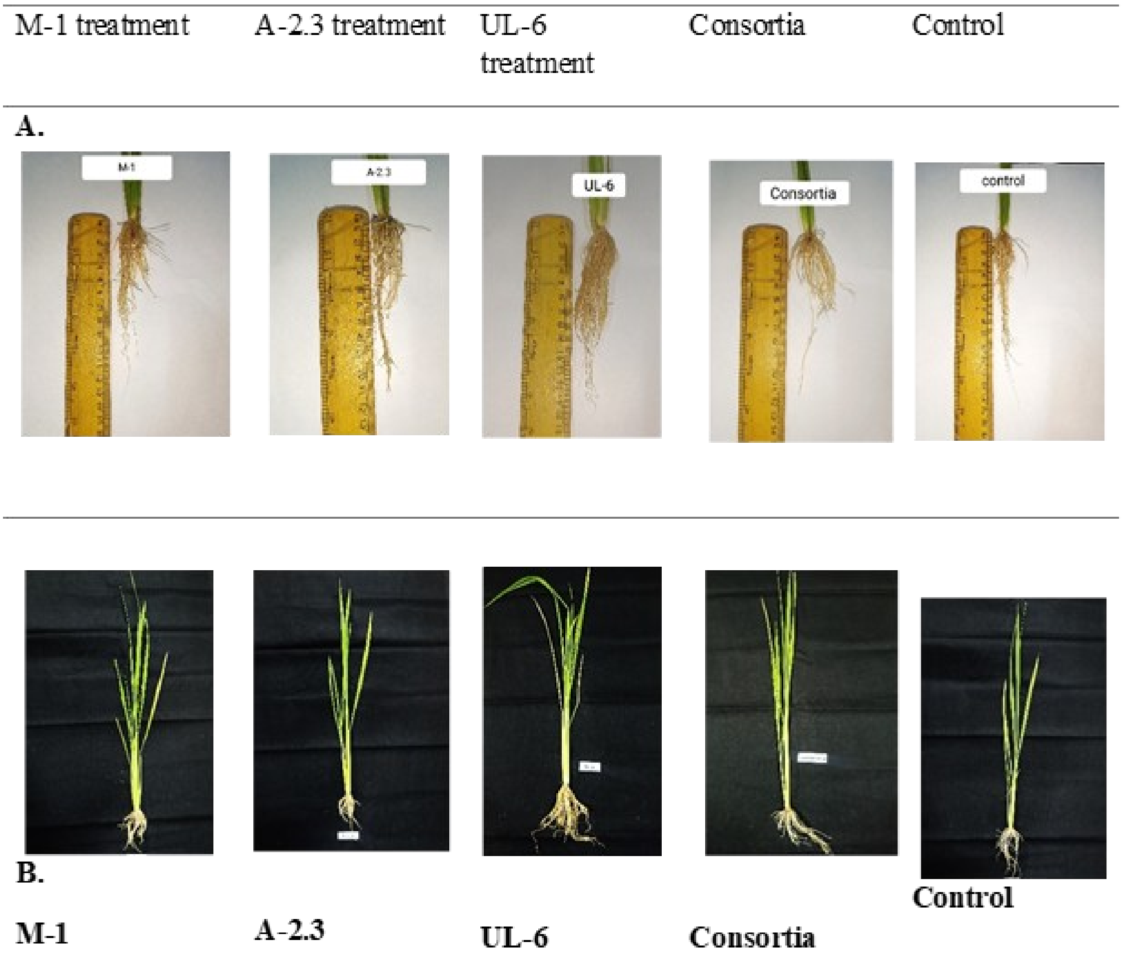


Fig. 4. (A) Root development of bio-primed rice plants (day 50th) along with the control plant. (B) Whole uprooted plant with root and shoot of all the four treatments along with control plant.

Control plant

Control plants that were only treated with buffer solution exhibited significant improvement with a G.P of 60.390 % and the lowest VI of 301.330 but the root development was very poor. The same findings were reported in pot trials as well where the plant height was good i.e. 72.6 cm (closest to UL-6) but the roots developed poorly.

Most of the studies using *Azotobacter* bio-fertilizers focus on an integrated approach of bio-fertilizers with chemical fertilizers to enhance crop productivity. The synergistic effects of *A. nigricans* with N, P, K fertilizer on maize and the effect of *A. Chroococcum* on cotton plants with a 50 % reduced recommended dose of N-fertilizers has been reported previously. Combining 50 % chemical fertilizers with manure alongside seed inoculation with *Rhizobium* bacteria and PSB significantly enhanced the grain yield and biomass of sorghum and chickpea. The present study is primarily focused on analysing the PGP effects of *Azotobacter* isolates solely i.e. without applying N-fertilizers. On observing the plants, it was noted that bio-primed plants were green and healthy up to 70 days from germination, bearing flowers and seeds. However, seed production was not healthy and plants gradually entered senescence after ~70 days. It might be due to the scarcity of essential macro and micronutrients in the soil. This observation led to the conclusion that the application of intermediate doses of biofertilizers with a few essential synthetic fertilizers in a balanced proportion is necessary to sustain healthy crops.

Most studies carried out on PGP micro-organisms are in controlled lab conditions which hinder plants from environmental variables, stresses and allow more targeted expression of PGP effects than field experiments (59). This produces biased results with missing field trials and microbial effects which are usually not documented in the literature (60). In the current study, the pot trial results confirmed significant treatment effects, with UL-6 showing the highest enhancement in shoot and root growth under polyhouse conditions. However, the divergence between *in vitro* and pot trial outcomes underscores the complexity of soil-plant-microbe interactions under more realistic conditions. Overall, UL-6 consistently outperformed other treatments in pot trials, particularly in root development and shoot height. In contrast, while the consortium performed best under controlled *in vitro* conditions, its effects were less pronounced in pots, suggesting strong environmental influence. These findings align with earlier reports that laboratory successes may not always translate to field-level efficacy (61–65). The sequence of strain UL 6 was submitted in NCBI with the accession number PV864958. A phylogenetic tree was constructed where AZTUL 6 was in a clad with *Azotobacter chroococcum* OR294177.1 (Fig. 5).

Conclusion

In this study, 22 *Azotobacter* spp. were isolated from soil samples and evaluated for their PGP traits viz. phosphate solubilization, zinc solubilization, ammonia production, IAA production, siderophore production, etc. Three strains namely, M-1, A-2.3 and UL-6, were found to perform comparatively best in all the PGP assays conducted in the study hence for further investigation of their PGP efficacy, rice (*O. sativa* L.) seeds of NDR-97 variety were bio-primed. Overall, bio-priming enhanced seed germination, VI and seedling growth, though outcomes varied between *in vitro* and pot trial conditions. While consortium treatments showed superior performance under controlled settings, individual strains, particularly UL-6 exhibited more consistent effects in soil-based trials. These discrepancies highlight the strong influence of environmental factors and microbial interactions on the success of bio-priming.

The findings reaffirm the potential of *Azotobacter* spp. as effective biofertilizers, yet also stress that single-strain or consortium applications may not universally guarantee optimal performance. Sustainable agricultural strategies must therefore account for nutrient dynamics, soil conditions and microbial competition to achieve reliable benefits.

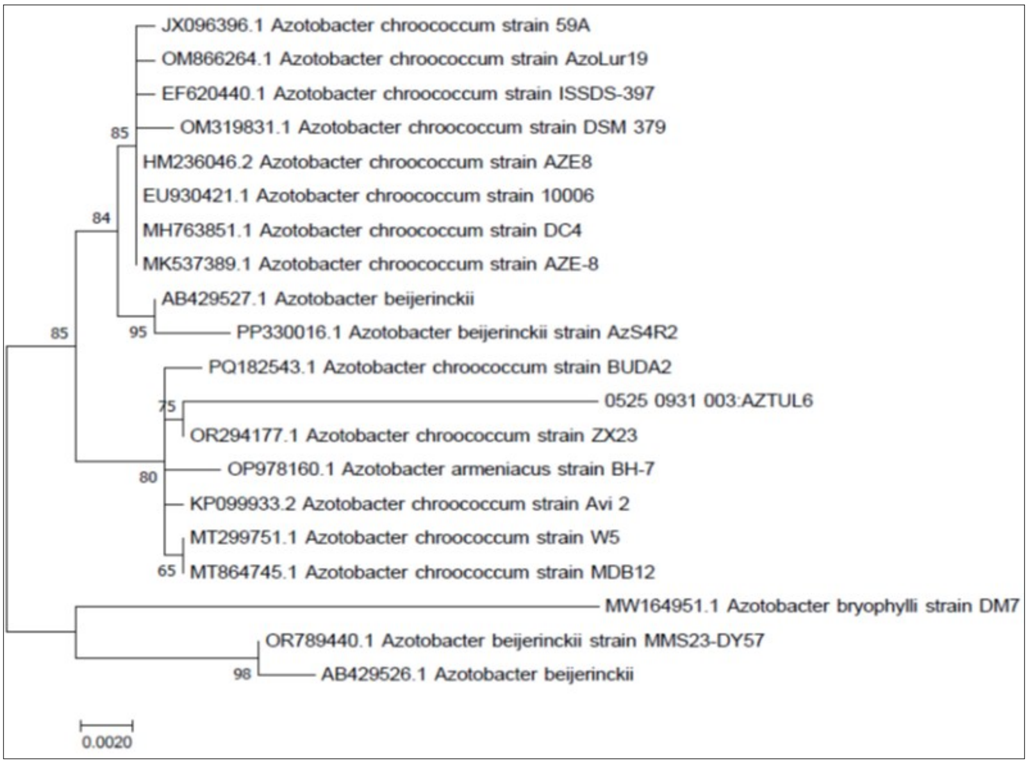


Fig. 5. Submitted 16s rRNA sequence of UL 6 in NCBI AZTUL 6 (Accession number PV864958).

Future work should focus on unraveling the mechanisms underlying strain-specific responses, refining bio-priming protocols and testing under diverse field conditions. Such efforts will be crucial for translating the promise of *Azotobacter*-based biofertilizers into practical, scalable solutions for enhancing crop productivity in an environmentally sustainable manner.

Acknowledgements

All the authors are thankful to Dr. Ankita Sarkar for providing lab facilities and funds for successful conduction of the experimentation.

Authors' contributions

AP executed the experimentation and drafting of the manuscript. SM contributed to the manuscript's drafting and editing. LS performed experimentation and MA undertook statistical analysis. AC conducted the bioinformatical analysis and JP did the editing of manuscript. AS participated in conceptualization, providing laboratory and necessary chemicals for experiment conduction and contributed to the manuscript editing. All authors have read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interests to declare.

Ethical issues: None

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Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

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