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RESEARCH ARTICLE

Screening and assessment of PGP and biocontrol properties of Azotobacter species isolated from agriculture soils of North Karnataka

Lekhana SM[1], Sreenivasa MY[2], Hanumantharaju KN[1], Anusha MB[1], Lokesh AC[1], Rajadurai M[1] & Chennappa Gurikar[1]*

[1]Department of Food Technology, Ramaiah University of Applied Sciences, Bangalore 560 054, Karnataka, India

[2]Department of Studies in Microbiology, University of Mysore, Mysore 570 005, Karnataka, India

*Email: chinnagurikar@gmail.com

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Abstract

Plant growth-promoting rhizobacteria (PGPR) are recognized for their ability to produce phytohormones, root-stimulating compounds, anti-fungal compounds and other secondary metabolites, making them potential biocontrol agents in agriculture. In the present study, 85 *Azotobacter* isolates were isolated from the agricultural soils of Raichur and Chikkabalapura locations. The isolates were further accessed for morphological, biochemical and plant growth-promotion (PGP) properties. All the isolated strains showed brown to black colour colonies on the Waksmann 77 media plate. Similar biochemical results were obtained for all the *Azotobacter* isolates. The isolates such as Azt-85 recorded the highest N_2 fixation (33.36 µgN/mL/Day), Azt-69 produced IAA (24.67 µg/mL), and Azt-51 produced GA (23.7 µg/25mL). The anti-fungal efficacy studies were conducted using the dual culture technique using the efficient PGPR *Azotobacter* isolates against fungal species (*Fusarium oxysporum* f. sp. *lini*, *Fusarium oxysporum* f. sp. *ciceris* and *Aspergillus flavus*). After the incubation period, the Azt-41 isolate showed the highest zone of inhibition (18 mm) against *Fusarium oxysporum* f. sp. *lini*. Similarly, *Azotobacter* isolates viz., Azt-25, 38 and 41 showed maximum growth inhibition (9 mm) against *Fusarium oxysporum* f. sp. *ciceris*. Similarly, the Azt-31 isolate recorded a moderate (13 mm) zone of inhibition against *Aspergillus flavus*. Integrating sustainable biocontrol strategies by injecting beneficial microbes like *Azotobacter* can enhance resilient food production systems and reduce reliance on chemical inputs through PGPR properties.

Keywords

anti-fungal compounds; *Azotobacter*; bio-control agent; PGPR

Introduction

The rhizosphere is the soil region surrounding and influenced by plant roots, forming a dynamic ecosystem rich in bacteria, fungi and archaea (1,2). These microorganisms play pivotal roles within the rhizosphere, engaging in beneficial interactions with plants (3). Rhizosphere microorganisms play a significant role in nutrient cycling by decomposing organic matter and releasing essential nutrients, including nitrogen, phosphorus and potassium, to the root region into plant-accessible forms (4). Moreover, they augment the availability of critical micronutrients and synthesize growth-promoting compounds such as phytohormones, vitamins and enzymes, promoting plant growth and development (5). Additionally, rhizosphere microorganisms protect plants against phytopathogens through space competition, the synthesis of antibiotics and antimicrobial compounds and the induction of systemic resistance (6). This mutualistic association between plants and rhizosphere

microorganisms exerts their beneficial effects by producing anti-fungal compounds, which suppress the fungal pathogens in the rhizosphere and manage plant health (7, 8). Multiple bacterial genera, including *Bacillus*, *Azospirillum*, *Serratia*, *Enterobacter*, *Burkholderia*, *Pseudomonas*, *Rhizobium* and *Azotobacter*, have been recognized as predominant plant growth-promoting rhizosphere microorganisms (7).

These bacterial groups synthesize a spectrum of anti-fungal compounds, encompassing antibiotics, volatile organic compounds and siderophores, impeding pathogen growth (7). The antibiotics synthesized by PGPR exhibit broad-spectrum activity against diverse fungal pathogens, curbing their proliferation and pathogenicity (7, 8). Furthermore, volatile organic compounds disrupt fungal cellular processes or induce programmed cell death, augmenting plant defence responses. Moreover, siderophores sequester environmental iron, limiting its availability to fungal pathogens and supporting plant resilience against fungal infections (9). Among the PGPR group, the genus *Azotobacter* is a major nitrogen-fixing bacteria that can inhabit the rhizosphere region, interacting with soil microbes (10,11). *Azotobacter* species can improve plant growth through different mechanisms, including nitrogen fixation, hydrogen cyanide production, siderophore secretion, hormone production, etc (12). Nitrogen fixation is significant in plant development and helps uptake nutrients. *Azotobacter* is noted for its capacity to convert atmospheric nitrogen into readily available forms, which helps maintain nutrients (13). This process not only provides the plant with a readily available nitrogen source but also minimizes the need for synthetic nitrogen fertilizers, which will reduce the load of synthetic chemicals in the soil (14).

Siderophores are tiny compounds that can chelate iron, allowing plants to absorb it more efficiently and hydrogen cyanide has anti-fungal properties that inhibit the growth of other microorganisms (15, 16). *Azotobacter* secretes lytic enzymes such as chitinase and protease that target and break down fungal pathogen cell walls and proteins, killing the cell (17). *Azotobacter* and other plant growth-promoting rhizobacteria have various advantages that manage plant health and stimulate plant growth (18).

Materials and Methods

Collection of soil sample

Soil samples collected from the rhizosphere at a depth of 10-15 cm from different agricultural fields of Sultanpur, Miyapur, Doranahalli, Dadlapur, Devergud of Raichur district, Kadnur, Gejjigadahalli and Haniyur of Chikkabalapura district. The samples were collected and stored in sealed polythene bags at 4° C until further processing by using standard protocol (18).

Isolation and characterization of Azotobacter isolates

The collected soil samples were used to isolate *Azotobacter* isolates using the serial dilution method under aseptic conditions. The 10^{-5} dilutions were prepared and used to isolate *Azotobacter*. The serially diluted soil samples were spread onto the Waksmann 77 media plates under aseptic conditions. The inoculated plates were incubated at 30 ± 2°C under a BOD incubator for 3-5 days. After the incubation period, the plates were observed for the growth of *Azotobacter* species on Waksman 77 media plates (19). All tests were conducted in triplicate to ensure accuracy (20,21).

Morphological and Biochemical characterization of Azotobacter isolates

All the isolates were screened based on colony characteristics, viz. shape, form, size, consistency, margin, elevation, colony appearance and pigment production, following the standard protocol described (22). All the *Azotobacter* isolates were characterized based on biochemical studies such as indole production test, methyl red test, vogues proskavu test, citrate utilization test, gelatin utilization test, starch hydrolysis test, glucose test, lactose test, catalase test using standard protocols (18).

Screening and selection of strains

Active and viable *Azotobacter* strains were used to evaluate plant growth-promoting properties and anti-fungal efficacy studies. To check the strains' viability and growth rate, the same strains are again re-inoculated onto Waksman 77 media plates by maintaining the same conditions as the previous ones (21).

Nitrogen fixation

All the *Azotobacter* isolates were evaluated for nitrogen fixation efficacy by inoculating the *Azotobacter* species in 50 mL of nitrogen-free Jenson's medium. The inoculated broth was subjected to continuous agitation on a rotary shaker at 32° C for seven days, along with the control sample (22). After 7 days of incubation period, the nitrogen fixation efficiency of *Azotobacter* has been evaluated by following the Kjeldahl method. The nitrogen content was quantified using the Kjeldahl method. The efficacy of nitrogen fixation was assessed by comparing the increase in total Kjeldahl nitrogen (µgN/mL/Day) in the inoculated sample against the control, utilizing an acid digestion method with a micro Kjeldahl apparatus (23,24).

Indole acetic acid (IAA) production

Azotobacter strains were grown on a Jensens' nitrogen-free medium supplemented with 1% L-tryptophan to promote growth and without the bacteria, the sample was maintained as a control. The inoculated and control media were incubated at 28°C for seven days. After the incubation period, the cultures were subjected to centrifugation at a speed of 5000 rpm for 15 minutes, the supernatant was collected into a clean flask. The solution was incubated without light for an hour at 28° C. The samples were subjected to various steps and the IAA production was estimated by measuring the optical density of the samples. The production of IAA has been determined for each *Azotobacter* strain by following the standard protocols described and maintaining triplicates throughout the study for better accuracy (25). The optical density of the processed sample was measured at a wavelength of 530 nanometres using a UV-spectrophotometer (26).

Gibberellic acid

Azotobacter strains were cultured into a sterilized malate broth medium and incubated at 28° C for 7 days under BOD. A control sample without a test organism has been maintained. In this assay, the gibberellic acid production of individual isolates was estimated by referring to the standard protocols described (27).

Fungal isolates used

The study obtained three fungal isolates from the Department of Microbiology, Mysore University, viz., *Aspergillus* and two *Fusarium* species. Previously, these isolates have been well characterized, identified and used as a standard strain for the present study (12). To check the viability, these fungal isolates (*Fusarium oxysporum* f. sp. *lini*, *Fusarium oxysporum* f. sp. *ciceris,* and *Aspergillus flavus*) have been reassessed by inoculating onto a freshly prepared potato dextrose agar media plate. The inoculated fungal culture plates were incubated at 30°C for three days under a BOD incubator.

Anti-fungal efficacy studies of Azotobacter species

A modified culture medium, Waksman 77 agar, was formulated to cultivate bacterial and fungal species on a single media. This medium has been formulated with Waksman broth and potato dextrose agar in equal proportions (1:1) (12,18). The antagonistic efficacy of selective *Azotobacter* species against *Fusarium oxysporum* f. sp. *lini*, *Fusarium oxysporum* f. sp. *ciceris* and *Aspergillus flavus* has been evaluated utilizing the dual culture method. *Azotobacter* at a cell concentration (10^9 CFU/mL) were streaked on the edge of the petriplate and the fungal pellet was placed in the centre of the petriplate using a cork borer (28). The inoculated petri plates were incubated at 28°C for 4 days. After incubation, the zone of inhibition was measured from the edge of the bacterial colonies to the periphery of the fungal colony (21,29).

Results

Isolation and characterization of *Azotobacter* isolates

85 *Azotobacter* isolates were isolated using serial dilution from different agricultural fields in Raichur and Chikkabalapura locations. After the incubation period, *Azotobacter* isolates were grown on Waksman 77 media and the colonies were dark brown to black in their pigmentation and a few strains showed milky white colonies on the media plates in (10^{-5} dilutions). *Azotobacter* isolates showed different colony characteristics such as colony sizes were small-medium, dense, smooth, glistening, raised-flat colonies, irregular, regular shaped, convex-umbonate, curled-undulate, opaque-translucent colonies. All isolates exhibited a rod-shaped morphology and gram-negative reaction (Table 1).

Biochemical characterization of *Azotobacter* isolates

All the *Azotobacter* isolates showed positive results for indole production, starch hydrolysis, catalase production and glucose utilization. The remaining tests, such as lactose utilization, citrate and gelatin, were negative in the reaction. A few of the 85 isolates showed positive responses to methyl red and vogue Proskauer tests (Table 2). The biochemical and morphological studies initially documented that these bacterial cultures isolated from soil samples belong to the group *Azotobacter*.

Plant growth promoting (PGP) attributes characterization of *Azotobacter* isolates

All 85 isolates were screened for nitrogen fixation, IAA and GA production activity. Among these, most of the *Azotobacter* isolates showed varied ranges of PGP activities. Among all, 45 isolates showed good activity for all three assays. The nitrogen fixation efficiency of the *Azotobacter* isolates was done using the Kjeldahl method. Among the 85 isolates, Azt-85 recorded the highest activity of 33.36 µgN/mL/Day and few isolates (Azt-41, 68, 49, 27, 84, 79, 71, 55, 67, 26, 48, 57, 36, 37, 82, 23, 80, 69, 77, 25, 54, 22 and 66) showed high N_2 fixation activity in a range of 32.2 to 25.7 µgN/mL/Day. Some of the *Azotobacter* isolates (Azt-38, 15, 56, 47, 20, 21, 35, 10, 24, 28, 39, 81, 16, 70, 40, 78, 29, 83, 8, 74, 60, 14, 34, 6, 43, 30, 11, 17, 65 and 9) showed moderate N_2 fixation activity in the varied range of 24.3 to 17.6 µgN/mL/Day. The remaining *Azotobacter* isolates showed moderate to low N_2 fixation activity ranging from 7 to 16 µgN/mL/Day. Azt-64 recorded the least N_2 fixation activity of 7.47 µgN/mL/Day (Fig. 1).

The indole acetic acid production assay for the *Azotobacter* isolates was done using Solawaski's reagent, where the resultant pink colour sample was measured using a UV-vis spectrophotometer at an optical density of 530nm. Azt-69 recorded the highest indole acetic acid production activity of 24.67 µg/mL. High indole acetic acid production was recorded in a range of 20.2 to 24.5 µg/mL by a few *Azotobacter* isolates such as Azt-85, 56, 34, 22, 71, 60, 55, 5, 44, 70, 81, 20, 35, 11, 83, 45, 19, 43, 36, 41 and 68 (Fig. 2). High to moderate activity of indole acetic acid production was recorded in a varied range of 16.2 to 19.7 µg/mL by *Azotobacter* isolates (Azt-2, 24, 54, 59, 76, 32, 73, 49, 18, 40, 8, 61, 67, 78, 46, 82, 30, 38, 16, 64, 14, 25, 48, 79, 42, 50, 62, 27, 66, 84 and 10). The remaining *Azotobacter* isolates recorded IAA production activity in a moderate to low range of 10.43 to 15.7 µg/mL. Azt-7 recorded the least indole acetic acid production activity of 10.43 µg/mL.

Azt-51 demonstrated the highest activity at 23.7 µg/25 mL for gibberellic acid production (Fig. 3). Several isolates, including Azt-46, 41 and 40, exhibited significant GA production within the 20.2 to 23.6 µg/25 mL range. Conversely, isolates such as Azt-48 recorded the lowest GA production activity at 13.3 µg/25 mL.

Bio efficacy of Azotobacter species against fungal species

Out of 85 isolates, only 30 isolates have been selected and used for the bio-efficacy studies. The selection of 30 isolates is purely based on the growth rate and PGPR potentiality and efficient isolates have been employed for the study. Anti-fungal efficacy of *Azotobacter* has been conducted using the dual culture technique on modified Waksmann 77 agar media plates against *F. oxysporum* f. sp. *lini, F. oxysporum* f. sp. *ciceris,* and *A. flavus*.

After 4 days of incubation period, among the 30 *Azotobacter* isolates, the Azt-41 strain recorded the highest zone of inhibition against *F. oxysporum* f. sp. *lini* (18 mm) and Azt-54 recorded a 14 mm zone of inhibition against *F. oxysporum* f. sp. *ciceris*. Similarly, Azt-31 recorded a 13 mm zone of inhibition against *Aspergillus flavus*. The *Azotobacter* isolates exhibited a substantial zone of inhibition of an average of 14% zone of inhibition (Fig. 4-6) against fungal species. The zone of inhibition is evident that *Azotobacter* species have anti-fungal efficacy against major plant pathogens.

Discussion

The comprehensive study on the viability of *Azotobacter* strains isolated from different agro-climatic zones of Raichur and Chikkabalapura locations significantly contributes to understanding these bacteria's potential in sustainable agriculture. The soil samples were collected and *Azotobacter*

Table 1. Morphological and Cultural studies of *Azotobacter* isolates

Sl.no	Isolate	Shape	Elevation	Pigmentation	Margin	Consistency
1	Azt 1	Irregular	Convex	Dark brown	Undulate	Opaque
2	Azt 2	Irregular	Flat	Light brown	Curled	Translucent
3	Azt 3	Irregular	Flat	Brown	Undulate	Translucent
4	Azt 4	Irregular	Flat	Grey-brown	Undulate	Opaque
5	Azt 5	Irregular	Convex	Light brown	Undulate	Opaque
6	Azt 6	Irregular	Umbonate	Yellow-brown	Undulate	Translucent
7	Azt 7	Irregular	Umbonate	Yellow-brown	Curled	Translucent
8	Azt 8	Irregular	Flat	Light brown	Curled	Translucent
9	Azt 9	Irregular	Umbonate	White yellow	Curled	Translucent
10	Azt 10	Regular	Convex	Yellow-brown	Undulate	Opaque
11	Azt 11	Regular	Flat	Yellow-brown	Curled	Opaque
12	Azt 12	Irregular	Umbonate	Milky white	Curled	Opaque
13	Azt 13	Irregular	Raised	Brown	Curled	Opaque
14	Azt 14	Irregular	Umbonate	Light brown	Curled	Translucent
15	Azt 15	Irregular	Convex	Pale yellow	Undulate	Translucent
16	Azt 16	Irregular	Convex	Pale yellow	Undulate	Translucent
17	Azt 17	Irregular	Flat	Yellow-brown	Undulate	Translucent
18	Azt 18	Irregular	Convex	Grey black	Undulate	Opaque
19	Azt 19	Irregular	Umbonate	Pale brown	Curled	Opaque
20	Azt 20	Irregular	Convex	Light brown	Undulate	Opaque
21	Azt 21	Irregular	Umbonate	Yellow-brown	Undulate	Translucent
22	Azt 22	Irregular	Umbonate	Yellow-brown	Curled	Translucent
23	Azt 23	Irregular	Pulvinate	Pale yellow	Curled	Translucent
24	Azt 24	Irregular	Flat	Light brown	Curled	Translucent
25	Azt 25	Irregular	Flat	Brown	Undulate	Translucent
26	Azt 26	Irregular	Flat	Grey-brown	Undulate	Opaque
27	Azt 27	Irregular	Convex	Light brown	Undulate	Opaque
28	Azt 28	Irregular	Umbonate	Yellow-brown	Undulate	Translucent
29	Azt 29	Irregular	Umbonate	Yellow-brown	Curled	Translucent
30	Azt 30	Irregular	Flat	Light brown	Curled	Translucent
31	Azt 31	Irregular	Umbonate	White yellow	Curled	Translucent
32	Azt 32	Regular	Convex	Yellow-brown	Undulate	Opaque
33	Azt 33	Regular	Flat	Yellow-brown	Curled	Opaque
34	Azt 34	Irregular	Umbonate	Milky white	Curled	Opaque
35	Azt 35	Irregular	Raised	Brown	Curled	Opaque
36	Azt 36	Irregular	Umbonate	Light brown	Curled	Translucent
37	Azt 37	Irregular	Convex	Pale yellow	Undulate	Translucent
38	Azt 38	Regular	Convex	Yellow-brown	Undulate	Opaque
39	Azt 39	Regular	Flat	Yellow-brown	Curled	Opaque
40	Azt 40	Irregular	Umbonate	Light brown	Curled	Translucent
41	Azt 41	Irregular	Flat	Brown	Undulate	Translucent
42	Azt 42	Regular	Flat	Yellow-brown	Curled	Opaque
43	Azt 43	Regular	Flat	Yellow-brown	Curled	Opaque
44	Azt 44	Irregular	Umbonate	Milky white	Curled	Opaque
45	Azt 45	Irregular	Raised	Brown	Curled	Opaque
46	Azt 46	Irregular	Raised	Brown	Curled	Opaque
47	Azt 47	Irregular	Umbonate	Light brown	Curled	Translucent
48	Azt 48	Irregular	Convex	Dark brown	Undulate	Opaque
49	Azt 49	Irregular	Convex	Grey black	Undulate	Opaque
50	Azt 50	Regular	Convex	Yellow-brown	Undulate	Opaque
51	Azt 51	Irregular	Flat	Brown	Undulate	Translucent
52	Azt 52	Irregular	Flat	Grey-brown	Undulate	Opaque
53	Azt 53	Irregular	Convex	Pale yellow	Undulate	Translucent
54	Azt 54	Irregular	Umbonate	Light brown	Curled	Translucent
55	Azt 55	Irregular	Flat	Brown	Undulate	Translucent
56	Azt 56	Irregular	Flat	Grey-brown	Undulate	Opaque
57	Azt 57	Irregular	Flat	Grey-brown	Undulate	Opaque
58	Azt 58	Regular	Flat	Yellow-brown	Curled	Opaque
59	Azt 59	Irregular	Umbonate	Milky white	Curled	Opaque
60	Azt 60	Irregular	Raised	Brown	Curled	Opaque

61	Azt 61	Irregular	Umbonate	Light brown	Curled	Translucent
62	Azt 62	Regular	Convex	Yellow-brown	Undulate	Opaque
63	Azt 63	Irregular	Flat	Brown	Undulate	Translucent
64	Azt 64	Irregular	Flat	Grey-brown	Undulate	Opaque
65	Azt 65	Irregular	Convex	Pale yellow	Undulate	Translucent
66	Azt 66	Irregular	Umbonate	Light brown	Curled	Translucent
67	Azt 67	Irregular	Umbonate	Light brown	Curled	Translucent
68	Azt 68	Irregular	Flat	Brown	Undulate	Translucent
69	Azt 69	Regular	Flat	Yellow-brown	Curled	Opaque
70	Azt 70	Irregular	Umbonate	Milky white	Curled	Opaque
71	Azt 71	Regular	Flat	Yellow-brown	Curled	Opaque
72	Azt 72	Irregular	Umbonate	Milky white	Curled	Opaque
73	Azt 73	Irregular	Raised	Brown	Curled	Opaque
74	Azt 74	Irregular	Umbonate	Light brown	Curled	Translucent
75	Azt 75	Irregular	Flat	Grey-brown	Undulate	Opaque
76	Azt 76	Irregular	Convex	Pale yellow	Undulate	Translucent
77	Azt 77	Irregular	Umbonate	Light brown	Curled	Translucent
78	Azt 78	Regular	Flat	Yellow-brown	Undulate	Translucent
79	Azt 79	Irregular	Umbonate	Yellow-brown	Curled	Translucent
80	Azt 80	Irregular	Flat	Light brown	Curled	Translucent
81	Azt 81	Irregular	Umbonate	White yellow	Curled	Translucent
82	Azt 82	Irregular	Flat	Grey-brown	Undulate	Opaque
83	Azt 83	Regular	Raised	Brown black	Undulate	Opaque
84	Azt 84	Regular	Flat	Yellow-brown	Undulate	Translucent
85	Azt 85	Irregular	Convex	Dark brown	Undulate	Opaque

Table 2. Biochemical studies of *Azotobacter* isolates

Sl.no.	Isolate	IND*	GLU	LAC	CAT	SH	MR	CIT	VP	GEL
1	Azt 1	+	+	-	+	+	-	-	+	-
2	Azt 2	+	+	-	+	+	+	-	-	-
3	Azt 3	+	+	-	+	+	+	-	-	-
4	Azt 4	+	+	-	+	+	-	-	+	-
5	Azt 5	+	+	-	+	+	+	-	-	-
6	Azt 6	+	+	-	+	+	+	-	-	-
7	Azt 7	+	+	-	+	+	+	-	-	-
8	Azt 8	+	+	-	+	+	-	-	+	-
9	Azt 9	+	+	-	+	+	+	-	-	-
10	Azt 10	+	+	-	+	+	+	-	-	-
11	Azt 11	+	+	-	+	+	-	-	+	-
12	Azt 12	+	+	-	+	+	-	-	+	-
13	Azt 13	+	+	-	+	+	+	-	-	-
14	Azt 14	+	+	-	+	+	-	-	+	-
15	Azt 15	+	+	-	+	+	+	-	-	-
16	Azt 16	+	+	-	+	+	-	-	+	-
17	Azt 17	+	+	-	+	+	+	-	-	-
18	Azt 18	+	+	-	+	+	-	-	+	-
19	Azt 19	+	+	-	+	+	+	-	-	-
20	Azt 20	+	+	-	+	+	-	-	+	-
21	Azt 21	+	+	-	+	+	+	-	-	-
22	Azt 22	+	+	-	+	+	-	-	+	-
23	Azt 23	+	+	-	+	+	+	-	-	-
24	Azt 24	+	+	-	+	+	-	-	+	-
25	Azt 25	+	+	-	+	+	+	-	-	-
26	Azt 26	+	+	-	+	+	-	-	+	-
27	Azt 27	+	+	-	+	+	+	-	-	-
28	Azt 28	+	+	-	+	+	-	-	+	-
29	Azt 29	+	+	-	+	+	+	-	-	-
30	Azt 30	+	+	-	+	+	-	-	+	-
31	Azt 31	+	+	-	+	+	-	-	+	-
32	Azt 32	+	+	-	+	+	-	-	+	-
33	Azt 33	+	+	-	+	+	+	-	-	-
34	Azt 34	+	+	-	+	+	-	-	+	-
35	Azt 35	+	+	-	+	+	+	-	-	-
36	Azt 36	+	+	-	+	+	-	-	+	-
37	Azt 37	+	+	-	+	+	+	-	-	-
38	Azt 38	+	+	-	+	+	+	-	-	-
39	Azt 39	+	+	-	+	+	-	-	+	-
40	Azt 40	+	+	-	+	+	+	-	-	-

41	Azt 41	+	+	-	+	+	-	-	+	-
42	Azt 42	+	+	-	+	+	+	-	-	-
43	Azt 43	+	+	-	+	+	+	-	-	-
44	Azt 44	+	+	-	+	+	+	-	-	-
45	Azt 45	+	+	-	+	+	-	-	+	-
46	Azt 46	+	+	-	+	+	+	-	-	-
47	Azt 47	+	+	-	+	+	+	-	-	-
48	Azt 48	+	+	-	+	+	+	-	-	-
49	Azt 49	+	+	-	+	+	-	-	+	-
50	Azt 50	+	+	-	+	+	+	-	-	-
51	Azt 51	+	+	-	+	+	-	-	+	-
52	Azt 52	+	+	-	+	+	+	-	-	-
53	Azt 53	+	+	-	+	+	-	-	+	-
54	Azt 54	+	+	-	+	+	+	-	-	-
55	Azt 55	+	+	-	+	+	-	-	+	-
56	Azt 56	+	+	-	+	+	+	-	-	-
57	Azt 57	+	+	-	+	+	-	-	+	-
58	Azt 58	+	+	-	+	+	+	-	-	-
59	Azt 59	+	+	-	+	+	-	-	+	-
60	Azt 60	+	+	-	+	+	+	-	-	-
61	Azt 61	+	+	-	+	+	-	-	+	-
62	Azt 62	+	+	-	+	+	+	-	-	-
63	Azt 63	+	+	-	+	+	-	-	+	-
64	Azt 64	+	+	-	+	+	-	-	+	-
65	Azt 65	+	+	-	+	+	+	-	-	-
66	Azt 66	+	+	-	+	+	-	-	+	-
67	Azt 67	+	+	-	+	+	+	-	-	-
68	Azt 68	+	+	-	+	+	-	-	+	-
69	Azt 69	+	+	-	+	+	+	-	-	-
70	Azt 70	+	+	-	+	+	-	-	+	-
71	Azt 71	+	+	-	+	+	-	-	+	-
72	Azt 72	+	+	-	+	+	+	-	-	-
73	Azt 73	+	+	-	+	+	+	-	-	-
74	Azt 74	+	+	-	+	+	+	-	-	-
75	Azt 75	+	+	-	+	+	-	-	+	-
76	Azt 76	+	+	-	+	+	+	-	-	-
77	Azt 77	+	+	-	+	+	-	-	+	-
78	Azt 78	+	+	-	+	+	+	-	-	-
79	Azt 79	+	+	-	+	+	-	-	+	-
80	Azt 80	+	+	-	+	+	+	-	-	-
81	Azt 81	+	+	-	+	+	-	-	+	-
82	Azt 82	+	+	-	+	+	+	-	-	-
83	Azt 83	+	+	-	+	+	-	-	+	-
84	Azt 84	+	+	-	+	+	+	-	-	-
85	Azt 85	+	+	-	+	+	+	-	-	-

IND* - Indole production, GLU- Glucose, LAC- Lactose, CAT- Catalase, SH- Starch Hydrolysis, MR- Methyl Red test, CIT- Citrate Utilisation, VP- Vogues Proskavur test, GEL- Gelatin Utilisation "+" = Indicate positive, "-" = indicative negative

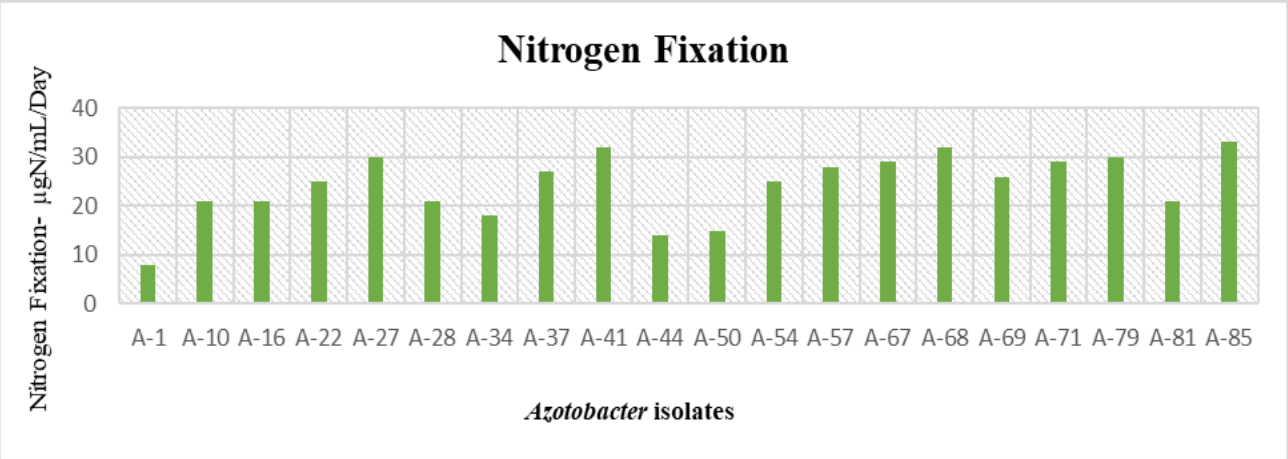

Fig. 1. Nitrogen fixation of *Azotobacter* isolates

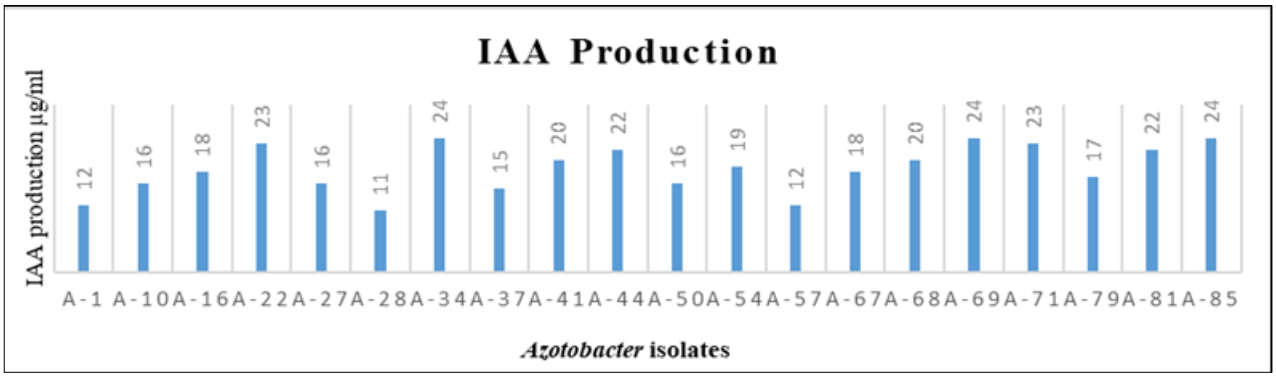

Fig. 2. IAA production of *Azotobacter* isolates

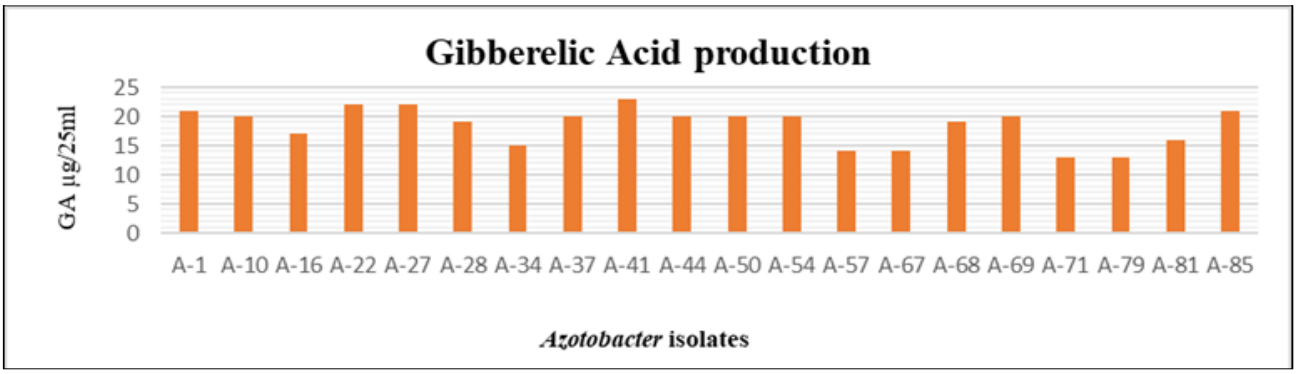

Fig. 3. Gibberellic acid production of *Azotobacter* isolates

Fig. 4. Bio efficacy studies of *Azotobacter against* ***Fusarium oxysporum*** f. sp. *lini* using dual culture assay-Quadrant streak method.

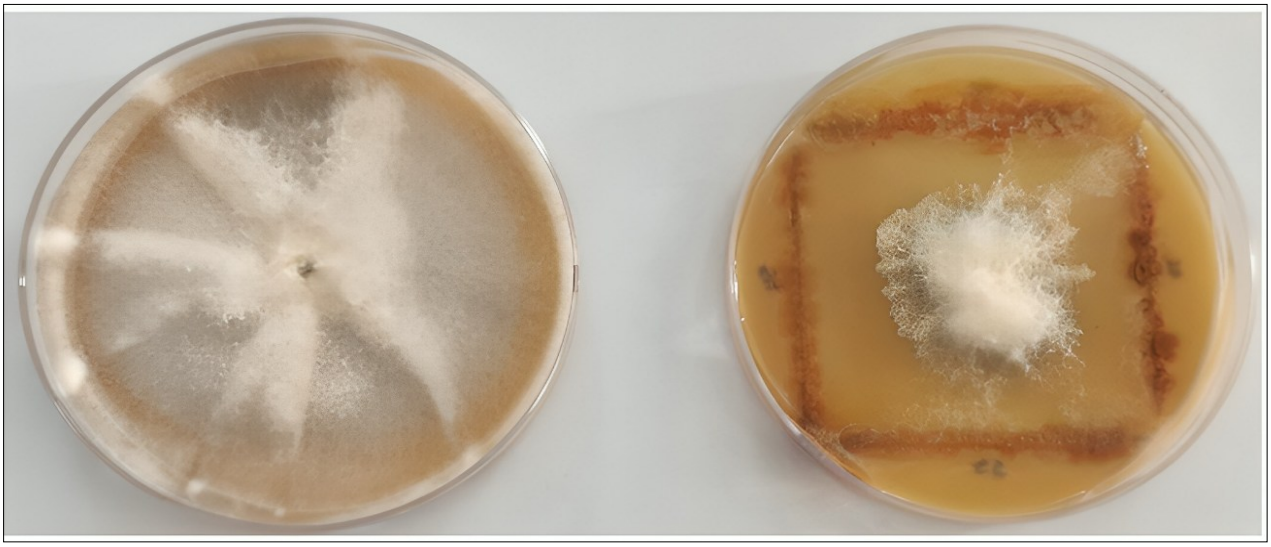

Fig. 5. Bio efficacy studies of *Azotobacter* against ***Fusarium oxysporum*** f. sp. *ciceris* using dual culture assay - Quadrant streak method.

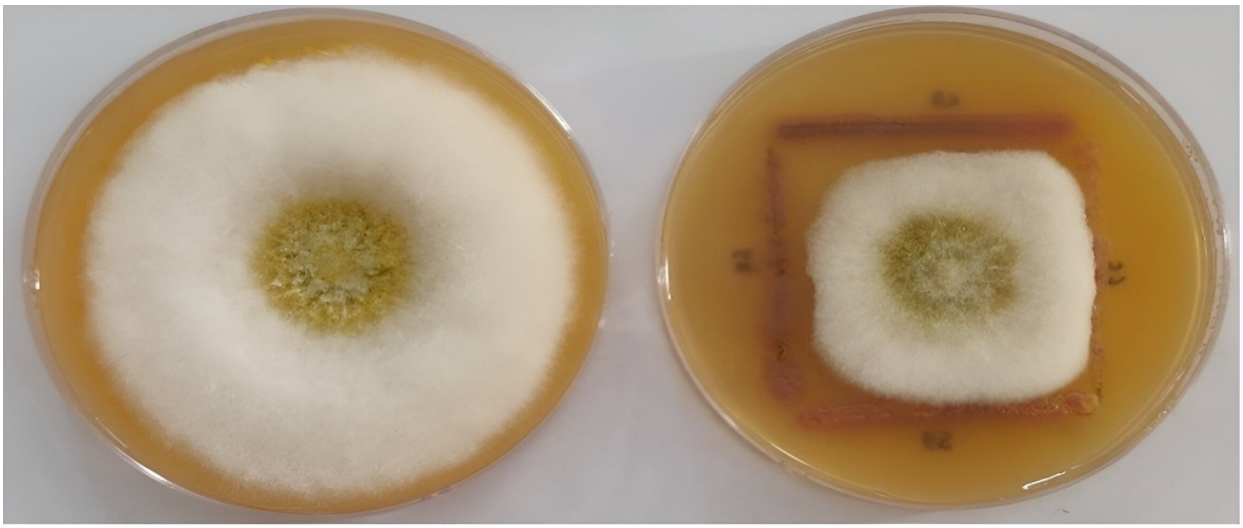

Fig. 6. Bio-efficacy studies of *Azotobacter* against *Aspergillus flavus* using dual culture assay - Quadrant streak method.

species were isolated using the serial dilution method on Waksmann 77 agar plates. Waksmann 77 media is a specific media devoid of nitrogen and *Azotobacter* species belongs to the diazotrophic group. From the soil samples, 85 *Azotobacter* cultures were isolated and the *Azotobacter* species required a minimum of 3 to 4 days to grow on N-free media.

After 3-4 days of incubation, *Azotobacter* cultures showed different coloured colonies such as pale white, white, brown, light brown, blackish brown and dark brown colonies on plates. These *Azotobacter* isolates showed different colony characters such as dense, smooth, glistening, raised-flat colonies, irregular, regular shaped, convex-umbonate, curled-undulate, opaque-translucent colonies on Waksmann 77 plates. This gram-negative bacterium is catalase and oxidase-positive and does not form spores (31). Several strains of *Azotobacter* have been recognized, including *Azotobacter vinelandii*, *A. paspali, A. insignis, A. salinestris, A. armeniacus, A. brasilense, A. beijerinckii, A. tropicalis, A. macrocytogenes* and *A. nigricans*. Among these, *A. chroococcum* and *A. vinelandii* are the most prevalent species found in the rhizosphere (30-33).

After identifying the species through morphological, cultural studies and staining studies, all 85 isolates have been tested for biochemical analysis. Similar morphological diversity was observed among the *Azotobacter* isolates and the adaptability of these bacteria to various environmental conditions was also noticed (34). The biochemical characterization, revealing universal indole production and the ability of some isolates to utilize glucose and lactose, suggests a broad metabolic capacity. These findings align with the role of *Azotobacter* in promoting plant growth through nitrogen fixation and phytohormone production (25).

Azotobacter, a nitrogen-fixing prokaryote, plays a pivotal role by converting atmospheric nitrogen into ammonia, which is readily assimilated by plants (34-37). In this study, various *Azotobacter* strains were evaluated for their PGP activities, such as nitrogen fixation, indole acetic acid production, gibberellic acid production, siderophore production and hydrogen cyanide production and efficacy in controlling fungal species, contributing to the understanding of their biocontrol potential.

These biochemical tests also recorded results similar to the previous results demonstrated (18). All 85 isolates have been used to evaluate PGPR activities such as N_2 fixation, IAA and GA. Nitrogen fixation efficacy of *Azotobacter* species has been determined by using the Kjeldahl method and by following the standard protocols (18,37). Among 85 isolates, 20 isolates (Azt-1, 10, 16, 22, 27, 28, 34, 37, 41, 44, 50, 54, 57, 67, 68, 69, 71, 79, 81 and 85) recorded varied ranges of N_2 fixation (7.47-33.36 µgN/mL/Day). Azt-41 isolate showed the highest (33.36 µgN/mL/Day) N_2 fixation efficiency among the isolates. Azt-1 showed the least nitrogen fixation efficiency among the 20 isolates. The remaining 35 isolates recorded the least nitrogen fixation efficiency compared to Azt-1. Earlier reports concurred with the present study and reported the highest N_2 fixation efficiency of *Azotobacter* (GVT-1) of 34.50 µgN/mL/Day isolated from paddy field soil samples (18).

Similarly, Kizilkaya., (38) reported that the *A. chroococcum* fixed nitrogen in the range of 3.5 to 29.35 µgN/mL/Day, which is in concurrence with the present results and all the other species will not fix nitrogen freely compared to the *Azotobacter* group. Kadam and Gangvani., (39) reported a similar kind of nitrogen fixation efficacy of *Azotobacter* species. This nitrogen fixation efficiency may vary from species to species, depending on the location of the samples and conditions of the soils. This demonstrates Azotobacters' ability to fix nitrogen under diverse agroclimatic conditions, enhancing soil fertility and crop productivity (40).

Indole acetic acid production has been estimated per the standard protocols using Jenson's N-free media and 1% tryptophan. Adding tryptophan helps the *Azotobacter* species grow on a particular media and supports the improvement of indole acetic acid (IAA) production. After completing Solawaskis' reagent reaction, the pink colour was analyzed using a UV-visible spectrophotometer at 530 nm. The production of IAA has been estimated using the standard indole acetic acid. A total of 47 isolates have been selected based on growth rate and morphological and cultural characteristics. Among the 47 isolates, Azt-34 produced the highest indole acetic acid (24 µg/mL) and Azt-28 recorded the least indole acetic acid production under *in-vitro* conditions. The IAA production and growth conditions may vary from species to species. Similarly, a similar trend of IAA production by *Azotobacter* species was isolated from pesticide-flooded paddy field soil samples of the Karnataka region (18). Further research shows the IAA production by different *Azotobacter* species (18).

The previous studies also reported various IAA production by different PGPR bacterial groups. Many PGPR bacteria, such as *Agrobacterium*, *Bacillus*, *Pseudomonas, and Rhizobium*, can produce indole acetic acid, a significant compound during metabolic activities (41). The IAA-producing activities of *Azotobacter chroococcum* concluded that it could increase seed germination, root length and shoot length, respectively (22). IAA production of *Azotobacter* isolate was recorded in the range of 1.47 to 32.8 µg/mL supplemented with 1 to 5 mg of tryptophan, which concurs with the present results (26). Forty-seven isolates have been used for Gibberellic acid production; among them, the Azt-41 isolate produced the highest amount of GA under *in-vitro* conditions, and Azt-79 produced a negligible amount of GA. This data shows that *Azotobacter* can also produce varied amounts of GA, which helps plant growth under abiotic stress conditions similar to N_2 fixation and IAA production mechanisms.

All the PGPR activities such as N_2 fixation, IAA production and GA production of *Azotobacter* have been recorded and proved that all the *Azotobacter* species are capable of fixing atmospheric nitrogen, production of IAA and production of GA at a varied concentration under *in-vitro* conditions. A similar PGPR efficacy of *Azotobacter* and other species has been documented (18, 37). The *Azotobacter* species such as *Azotobacter vinelandii*, *Azotobacter chroococcum*, *Azotobacter salinestris*, *A. tropicalis*, *A. nigricans* and *A. armeniacus* have proved their PGPR activities under varied agro climatic conditions. Similarly, *Azotobacter* and *Pseudomonas* fluorescence have produced IAA in the absence of tryptophan, but the production of IAA is comparatively lower (26). Isolated endophytic N_2 fixing bacteria affected the growth and development of the Casava plant. Similarly, the influence of *Azotobacter chroococcum* strains on the growth and development of biomass of the *Adathoda vasica Nees* crop plant was also noticed (12). The present study results are evident and confirm the relevance and efficacy of *Azotobacter* species (42). The screening for nitrogen fixation, IAA production and GA production activities among the *Azotobacter* isolates highlights their potential as biofertilizers (35). The variability in these PGP attributes among the isolates suggests the possibility of selecting specific strains for targeted agricultural applications (12). All these previously reported results show that the *Azotobacter* species is a better PGPR bacteria that supports the growth and development of different types of crops through various mechanisms such as N_2 fixation, IAA production and GA production (41,42). This proves *Azotobacter* is a better PGPR bacteria for sustainable plant growth and development (43,44).

Anti-fungal efficacy

The modified medium supported the growth of *Azotobacter* and fungal pathogens under a single medium. The modified Waksmann 77 media consists of a 1:1 ratio of Waksmann agar and potato dextrose agar media, facilitating the growth of *Azotobacter* and the fungal species. This media consists of nutrients required for the development of both organisms (*Azotobacter* and fungal species), and this organism requires different nutrient compositions for its growth and metabolic activities under varied conditions.

After incubation, among 85 isolates, the Azt-41 isolate showed a maximum zone of inhibition against *F. oxysporum* f. sp. *lini* (18mm). Similarly, Azt-54 and Azt-31 isolates recorded around 13mm zone of inhibition against *F. oxysporum* f. sp. *ciceris. Aspergillus flavus* under *in-vitro* conditions. The *Azotobacter* isolate showed good anti-fungal efficacy against three fungal pathogens, which are common plant pathogens that can affect various crops. The average percentage zone of inhibition against these pathogens is around 14%, indicating that *Azotobacter* has good anti-fungal properties.

The bio-efficacy studies against fungal pathogens, including *F. oxysporum* and *A. flavus*, revealed that *Azotobacter* isolates showed an average of 14% zone of inhibition. This finding is particularly relevant in the context of increasing concerns over fungal diseases in crops and the overuse of chemical fungicides (33). The ability of *Azotobacter* to inhibit fungal growth through the production of anti-fungal compounds or competition for nutrients and space offers a sustainable alternative for disease management in agriculture (38). The anti-fungal potential of *Azotobacter* species against various Fusarium species (12,34). Characterizing these *Azotobacter* isolates underscores their potential as biofertilizers and biocontrol agents, offering a sustainable alternative to chemical fertilizers and pesticides (41,45,46). The ability of *Azotobacter* to enhance soil fertility, promote plant growth and control fungal pathogens aligns with the goals of sustainable agriculture, which are to reduce chemical inputs, enhance crop productivity and sustainability and improve soil health (47). Further research highlighted the pesticide tolerance and biocontrol PGPR properties of *Azotobacter* isolates (18,28,36).

The use of *Azotobacter* holds significant potential in agriculture as PGPR and an effective biocontrol agent. Still, several limitations hinder its widespread application. It cannot grow in nutrient-less mediums or soils and requires nearly a week to establish growth, making its application in specific environments challenging. Additionally, under stress conditions, *Azotobacter* forms cysts, which may affect its ability to fix nitrogen efficiently. The bacterium also faces competition with native microorganisms and large-scale inoculum production can be difficult. Despite these challenges, *Azotobacter* offers benefits for sustainable farming by improving soil health and reducing dependence on synthetic fertilizers, provided appropriate management practices are implemented and further research addresses these issues. Using sustainable, safe and eco-friendly antagonistic bacteria to control *Fusarium* spp. Biological interventions have emerged as a key area of study (48,33). Various microorganisms, including *Pseudomonas*, *Bacillus* and *Azotobacter*, exhibit anti-fungal properties against foodborne pathogens. All the tests proved that *Azotobacter* species are better PGPR microorganisms for sustainable agriculture and to maintain soil fertility (46,49,50).

Conclusion

The study demonstrated that the *Azotobacter* strains have significant potential as plant growth-promoting properties for plant health management. These results suggested that *Azotobacter* strains could be promising for enhancing plant growth and controlling fungal diseases in agricultural systems. Overall, the study provides valuable insights into the significance of *Azotobacter* strains as a sustainable and effective solution for promoting plant growth and health.

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Authors' contributions

LSM conducted research experiments, and prepared, edited and reviewed manuscripts. HR edited the manuscript. AMB performed critical review of the manuscript. ACL reviewed and edited the manuscript. MYS performed a critical review and conceptualized the biocontrol efficacy studies. RD performed a critical review of the manuscript. CG conceptualized and critically reviewed the manuscript. The authors have read the article and approved it for publication.

Compliance with ethical standards

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

Ethical issues: Explicitly state that ethical guidelines were adhered to for the use of fungal isolates.

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