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TOWARDS SUSTAINABLE AGRICULTURE: HARNESSING *AZOTOBACTER* SPECIES FOR ENHANCED CROP YIELD AND ENVIRONMENTAL RESILIENCE

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ABSTRACT

The symbiotic relationship between soil health, agricultural productivity, and environmental sustainability underscores the urgent need for innovative strategies to enhance crop yield while mitigating the adverse effects of modern agricultural practices. This study investigates the potential of Azotobacter species as biofertilizers to address these challenges. Azotobacter species, known for their nitrogen-fixing ability and other beneficial properties, were isolated from rhizosphere soil samples and characterized. Morphological, ecological, and biochemical analyses revealed diverse characteristics among the isolates, including stress tolerance, resistance to heavy metals, pesticides, and antibiotics, as well as enzymatic activities. Furthermore, the isolates exhibited plant growth-promoting properties such as the production of indole-3-acetic acid (IAA), siderophores, and hydrogen cyanide (HCN), as well as phosphate mineralization and solubilization. These findings highlight the potential of Azotobacter species as effective biofertilizers for sustainable agriculture.

Keywords: *Plant growth promotion, Azotobacter species, microbial isolates, innovative strategies.*

INTRODUCTION

The complex relationship among soil health, agricultural productivity, and environmental sustainability underscores the pressing need to develop innovative strategies for optimizing crop output while mitigating the adverse impacts of modern agricultural practices. Soil, as the fundamental medium for plant growth and ecosystem stability, holds a central position in the global food security discourse. However, the escalating challenges posed by climate change, including drought, flooding, heatwaves, and soil salinity, have magnified the urgency of addressing soil-related concerns in agricultural contexts.

Contemporary agricultural methods, predominantly reliant on agrochemicals to maximize yields, have inadvertently compromised soil health and fertility (Tilman, 1999; Gomiero, 2016; Nadarajah, 2019). Excessive fertilizer usage, in particular, has been implicated in diminished soil fertility, reduced crop yields and nutritional fruit quality (Pešaković, 2023), heightened environmental pollution, and associated human health risks. Recognizing the imperative to overcome these challenges, researchers have increasingly turned to environmentally sustainable alternatives in agricultural practices.

In recent years, the rapidly expanding field of microbiome engineering has provided new opportunities for modifying microbial communities to improve agricultural productivity and sustainability. The integration of beneficial microbes into agricultural

systems has emerged as a promising approach (Koskey et al., 2021; Lopes et al., 2021). Beneficial soil microorganisms, known as plant growth-promoting rhizobacteria (PGPR), play pivotal roles in enhancing soil fertility, supporting plant growth and development, and bolstering stress resilience. This has led to widespread recognition of PGPR as indispensable tools for sustainable agriculture, heralding a new paradigm in agricultural innovation.

Scientific investigations into the mechanisms underlying PGPR interactions with plants and soils encompass diverse disciplines, including microbiology, agronomy, and environmental science. Various bacterial genera, such as *Pseudomonas*, *Bacillus*, *Azospirillum*, and notably, *Azotobacter*, have been identified as key PGPR contributors, exerting significant influences on agricultural crop yields (Berg, 2009; Etesami and Beattie, 2017). Of particular interest is the *Azotobacter* genus, comprising free-living diazotrophic bacteria with widespread distribution in neutral to alkaline soils worldwide.

Azotobacter species, characterized by their aerobic, gram-negative, and pleomorphic nature, play pivotal roles in enhancing soil fertility and promoting plant health (Balow et al., 1979; Becking, 1981; Krishna Samal et al., 2020). According to the same authors, through mechanisms such as nitrogen fixation, hormone production, fungal inhibition, and phosphate solubilization, *Azotobacter* species contribute to soil enrichment and crop productivity. Recognizing their immense potential as biofertilizers, researchers have increasingly focused on exploring *Azotobacter*'s suitability for agricultural applications, particularly in challenging environmental conditions. By utilizing the potential of *Azotobacter* species as biofertilizers, researchers seek to strengthen crop resilience, alleviate environmental stressors, and contribute to a more sustainable agricultural future.

This study aims to explore the effectiveness of Azotobacter species as promising tools for enhancing plant growth under diverse environmental stress conditions, with the overarching objective of advancing agricultural sustainability.

MATERIALS AND METHODS

Bacterial isolation

In laboratory settings, strains belonging to the *Azotobacter* genus were isolated from the rhizosphere soil. After sampling from the surface layer (0–30 cm) 25 samples in total were collected carefully under sterile conditions and placed into labeled plastic bags. These samples underwent air drying and prepared for bacterial isolation using the spread plate technique on L-agar medium. The plates were then incubated for 2–5 days at 28°C. Afterward, distinct and morphologically diverse bacterial colonies were selected from the plates and purified using the streaking method. The purity of the cultures was verified after each subculture by examining microscopic slides (Nikon Eclipse 50i, Japan). The isolated bacterial strains were transferred to MPA (meat peptone agar) slants and stored at 4°C for further analysis.

Morphological characteristics of isolates

Cell characteristics such as shape (form), Gram staining, cell motility, and cyst formation, as well as colony characteristics including shape (form), elevation, margin texture, color, and position on the medium, were assessed for each isolate in the collected samples. Microscopic and staining methods, microbiological culture techniques, motility assays followed with Bergey's Manual of Systematic Bacteriology (Holt et al., 1994) were employed to determine these characteristics.

For the purpose of cyst formation, isolates were grown on Burk's medium and incubated for 7 days. These isolates were stained with crystal violet to detect cysts and observed under oil immersion.

Stress tolerance and resistance profile of bacterial isolates

The stress tolerance capacity of bacterial isolates was evaluated using microbiological culture techniques. Ability of the isolates to grow under different ecological conditions. Ecological capacity of bacterial isolates was evaluated using microbiological culture techniques. The growth of isolates was examined at various temperatures (4°C, 17°C, 25°C, 35°C) and on media with different acidity levels (pH 0, 4, 7, 9), as well as on media with varying concentrations of NaCl (0%, 7%, 15%), using Fedorov's medium (Fedorov, 1975). After 48 hours of incubation, qualitative growth was observed and compared with the control. Growth observations were categorized as follows: complete absence (-), minimal growth (±), optimal growth (+), and abundant growth (++).

Resistance of isolates to heavy metals and pesticides were assessed using the diffusion method. This involved densely seeding a nutrient agar medium with microorganisms, followed by the placement of discs containing solutions of heavy metals and pesticides onto the surface of the solidified agar. Four heavy metals were tested at concentrations of 10^{-1}, 10^{-2}, and 10^{-3} (mol dm^{-3}): chromium ($KCr(SO_4)_2 \times 12\ H_2O$), copper [$Cu(NO_3)_2$], nickel ($NiSO_4 \times 6H_2O$), and lead [$Pb(CH3COO)_2 \times 3H_2O$].

Three commercial pesticides [two systemic fungicides under commercial names Luna (active ingredient: fluopyram 250 g L^{-1}, trifloxystrobin 250 g L^{-1}) and Sequence (active ingredient: difenoconazole) and one insecticide under the name Lamdex (active ingredient: lambda-cyhalothrin 50 g/L)] were selected for disease control in fruit growing. They were used at three doses: in accordance with the manufacturer's instructions (MI), concentrations 10 times higher than the MI (10× > MI), and concentrations 100 times higher than the MI (100× > MI) (Randhawa and Kullar, 2010).

Resistance of isolates to antibiotics was also assessed using the disk diffusion method following the antibiogram procedure. Five antibiotics [ampicillin (10 μg), neomycin (30 μg), erythromycin (15 μg), streptomycin (300 μg), and chloramphenicol (30 μg) (Biological LTD, England)] were employed. After incubation, the inhibitory effect was determined by observing translucent zones around the discs (Jarak and Đurić, 2004). The size of the inhibition zone indicated the sensitivity of bacteria to heavy metals, pesticides, or antibiotics, categorized as follows: absence of an inhibition zone (-), inhibition zone of 1-10 mm (+), and inhibition zone exceeding 10 mm (++).

Direct mechanisms of plant growth stimulation

Ammonia production: The ability of isolates to produce ammonia was assessed using the Nesslerization method. Initially, 10 mL of peptone water (Torlak, Serbia) was inoculated with the bacterial culture, followed by incubation at 30°C for 72 hours. Subsequently, 0.5 mL of Nessler's reagent (Alfapanon, Serbia) was added to each test tube. After a 5-minute incubation period, any appearance of yellow or brown coloration was recorded as a positive reaction, confirming the isolate's ability to produce ammonia.

Indole-3-acetic acid (IAA) production ability: The ability to produce indole-3-acetic acid (IAA) was determined by the colorimetric method (Patten and Glick, 2002). Incubation lasted for 24h at 30°C and 150 rpm (Biosan ES-20, Latvia), followed by an additional 72 h under the same conditions after enriching the medium with 100 μg mL^{-1} l-tryptophan (Sigma Aldrich, USA). Absorbance was measured at 540 nm (T70 UV/VIS

spectrometer, PG Instruments LTD). Obtained values were compared with the standard curve values of IAA, and the amount of produced IAA was expressed in μg mL^{-1}.

Siderophore production: The ability to produce siderophores was determined by the method of Milagres et al. (1999) using chrome azurol agar (CAS agar) and incubated at 30°C for 48–72h. Change in color from blue-green to orange-red at the line of separation between the nutrient medium and CAS agar [(±) < 1 mm, (+) 1–5 mm, (++) 5–15 mm, (+++) > 15mm)] indicates that the microorganism produces siderophores.

Phosphate solubilization: The ability to mineralize organic phosphorus compounds was tested on a modified Menkin's medium according to Rodina (Menkin, 1963). The ability of isolates to solubilize inorganic phosphates was tested on Pikovskaya's medium (Pikovskaya, 1948). After 5 days of incubation at 28°C, the appearance of transparent zones around the colonies indicates the microorganism's ability to dissolve phosphates.

Indirect mechanisms of plant growth stimulation

Biochemical characteristics of isolates: A total of 6 isolates were further examined for their biochemical properties, including catalase and oxidase production, lipase, protease, cellulase, pectinase, lecithinase, and urease activities, as well as starch, and gelatin hydrolysis. Nitrate and carbon utilization as energy sources were also evaluated. The catalase test was performed to detect the presence of catalase (Gill and Vickers, 1969). The absence of catalase was indicated by the lack of or weak bubble production.

Production of lipase: The production of lipase was examined on a medium (peptone 10 g L^{-1}, NaCl 5 g L^{-1}, $CaCl_2 \times H_2O$ 0,1 g L^{-1}, agar 15 g L^{-1}) with added Tween 80 (Lanui, 1987). The incubation period was seven days at 26°C. Cloudy zones around the colony indicated lipolytic activity.

Production of protease: To test bacteria for the ability to produce the gas hydrogen sulfide (H_2S), sulphur reduction test was used on SIM medium (Sulphide Indole Motility medium) according to MacFaddin (2000). A colony of a young (18- to 24-hour) culture was stabbed once in the middle of the tube and incubated at 28±2°C and examined daily for up to 7 days. Darkening of the medium (a black precipitate) or blackening of the line of inoculation indicates the presence of bacteria producing hydrogen sulfide.

Production of cellulase: The production of cellulase was examined on CMC agar (carboxymethyl-cellulose agar) (MacFaddin, 2000) . The incubation period was 7 days at 28°C. After incubation, petri dishes were flooded with a Congo red solution (mgcm-3 water). After 15 minutes, Congo red was poured off, and petri dishes were flooded with a 1M NaCl solution. Decolorized zones around colonies indicated the cellulase activity of microorganisms.

Production of pectinase: The ability to produce pectinase was investigated by the agar plate method on pectin agar (MacFaddin, 2000). Incubation lasted 24 hours at 37°C, after which colonies were flooded with iodine solution. The appearance of uncolored zones around the colony indicated pectinase activity (Soares et al., 2001).

Production of lechitinase: The test was performed on Egg Yolk Agar (Galanos et al., 1985). The incubation period lasted 24–72 h at 28±2°C. Appearance of a white, opaque, diffuse zone that extends into the medium surrounding the colonies was the indicator bacterial capability for lechitinase production.

Ability of urea hydrolyzes: To differentiate bacteria based on their ability to hydrolyze urea with the enzyme urease, Christensen's urea agar was used (Christensen, 1946). Incubation was carried out at 35°–37°C in ambient air for 48 hours to 7 days. Development of an intense magenta to bright pink color in 15 min to 24 h indicate positive reaction.

Starch hydrolysis tests: The ability of microorganisms to hydrolyze starch was determined using the agar plate method on starch agar (Edwards and Ewing, 1939). Incubation was carried out at 28°C for 48 hours. Colonies were then flooded with iodine solution. If the microorganism hydrolyzes starch, an uncolored zone (hydrolysis zone) appears around its colonies.

Gelatin hydrolysis test: For this test, the nutrient gelatin stab method was used (Erdos and Tully, 1986). Incubation was at 22–23°C for 7 days. Partial or total liquefaction of the inoculated tube after 30 minutes cooling in the refrigerator at 4°C is indicated gelatinase-positive colonies.

Energy source utilization: Nitrate reduction: To determine the ability of a bacteria to reduce nitrate to nitrite, nitrate agar was used (Huddleson and Sneath, 1933). Development of a cherry red coloration on addition of reagent A and B indicated capability of strain for producing the nitrate reductase enzyme.

Ability of citrate utilization: Simons Citrate agar was used to test a strain's ability to utilize citrate as a source of energy (Simons, 1923). Growth with color change from green to intense blue along the slant indicated the capability of bacteria to grow on this medium and produce an enzyme, citrate-permease, capable of converting citrate to pyruvate.

Ability of glucose, fructose, sucrose, galactose, lactose, and xylose utilization was tested as described in MacFaddin's biochemical tests for identification of medical bacteria (MacFaddin, 2000).

RESULTS AND DISCUSSION

Out of a total of 25 soil samples collected from the rhizosphere of different agricultural crops, Azotobacter sp. was isolated from six samples (AZC1, AZC2, AZC3, AZC4, AZC5, AZC6), and a collection of cultures consisting of them was formed. Colonies were isolated based on morphological characteristics and subcultured several times on appropriate media to obtain pure cultures.

Morphological characteristics of isolates

The findings concerning the morphological characteristics of cell and colony formations of the selected isolates are outlined in Table 1.

Microscopic examination of cells revealed that the majority of the collection comprised non-sporogenic isolates. Gram staining of bacteria showed that all six isolates were Gram-negative. Additionally, all six isolates were observed to be motile, and all exhibited cyst formation, which is a means of asexual reproduction in *Azotobacter* species under favorable conditions (Salhia, 2013).

Regarding colony appearance, all six isolates exhibited a circular shape, with four isolates having a convex elevation, while 2 showed a raised elevation. Except for isolate AZC5, which had an undulate margin, all other isolates had a smooth margin. The colony color varied from yellowish (AZC1), through brown (AZC2), and beige (AZC5) to white (AZC3, AZC4 AZC6). All colonies had a moist texture.

Similarly, Balow (1979) and Becking (1981) characterized *Azotobacter* as a genus of free-living, diazotrophic, nitrogen-fixing bacteria. They noted that *Azotobacter* species are aerobic, gram-negative, pleomorphic bacteria that can exist singly, in chains, or in clumps. Additionally, during their resting stage, they form thick-walled cysts that protect them from harsh environmental conditions. According to Krishna Samal (2019), *Azotobacter* species play a pivotal role in maintaining soil fertility due to their various beneficial effects on plants.

Table 1. Morphological characteristics of isolates

Cell					Colony				
Isolates	**Shape**	**Gram staining**	**Motility**	**Cyst forma**	**Shape**	**Elevation**	**Margin**	**Colour**	**Texture**
AZC1	rod-shaped to oval	-	+	+	circular	convex	entire	yellowish	moist
AZC2	rod-shaped to oval	-	+	+	circular	convex	entire	brown	moist
AZC3	rod-shaped	-	+	+	circular	raised	entire	white	moist
AZC4	rod-shaped to oval	-	+	+	circular	convex	entire	white	moist
AZC5	rod-shaped	-	+	+	circular	convex	undulate	beige	moist
AZC6	oval	-	+	+	circular	raised	entire	white	moist

Table 2a. Ecological resilience of bacterial isolates

Isolates	**Temperature (°C)**				**pH**			**NaCl (%)**		
	4	**17**	**25**	**35**	**4**	**7**	**9**	**0**	**7**	**15**
AZC1	-	++	++	++	-	++	-	++	±	-
AZC2	-	++	++	+	±	++	-	++	±	-
AZC3	-	++	++	+	-	++	-	++	+	-
AZC4	-	+	++	+	±	++	-	++	±	-
AZC5	-	++	++	+	-	++	-	++	+	-
AZC6	-	+	++	++	-	++	-	++	++	-

complete absence of growth (-); minimal growth (±); optimal (+); abundant growth (++)

The results of the examination of the ecological resilience of isolates are presented in Table 2a. It was found that all isolates exhibited abundant growth at a temperature of 25°C. However, at 4°C, the complete absence of growth was observed. At 35°C, optimal growth was observed for all isolates except for isolate AZC1 and AZC6, where abundant colony growth was observed. Additionally, optimal growth was recorded at 17°C for isolates AZC4 and AZC6.

On media with pH values of 4, 7, and 9 optimal growth was observed for all isolates only at pH 7. It was observed in this study that minimal colony growth occurred for isolates AZC2 and AZC4 on media with a pH of 4, while no growth was observed for the rest of the studied isolates on media with pH values of 4 and 9. When 7% NaCl was added to the media, 50% of the studied isolates (AZC1, AZC2, and AZC4) exhibited minimal growth. In the same media, isolates AZC3 and AZC5 exhibited optimal growth, while AZC6 exhibited abundant growth. However, with an increase in the concentration of salt, the growth decreased, and there was negligible growth in the presence of 15% NaCl (Table 2a). Conversely, abundant growth was observed on media without the addition of NaCl. In line with our findings, Tchan and New (1989) noted that *Azotobacter* species exhibit sensitivity to acidic pH, high salt concentrations, and temperature variations. Several other researchers have also documented the limited prevalence of the Azotobacter genus in acidic soil, often making its presence challenging to confirm (Milicic, 2009). Conditions conducive to the robust growth of *Azotobacter* typically include a neutral pH, adequate moisture levels, the presence of organic matter, and sufficient quantities of biologically active substances, particularly phosphorus (Aquilanti et al., 2004).

Cooper did not impact the growth of *Azotobacter* isolates at any of the applied concentrations (Table 2b). Similarly, according to the results presented in Table 2b the growth of *Azotobacter* isolates was not affected at any of the applied concentrations of chromium, nickel, and lead, except for isolate AZC4. Inhibition zones of 1-10 mm were observed for AZC4 at concentrations of 10^{-2}, 10^{-3}, and 10^{-4}, and even >10 mm at 10^{-1} chromium solutions. The application of lead at concentrations of 10^{-1} and 10^{-2} also resulted in growth inhibition for *Azotobacter* isolate AZC4, with inhibition zones ranging from 1–10 mm. Additionally, nickel also led to the inhibition of isolate growth, as indicated by the appearance of inhibition zones up to 10 mm at all concentrations.

The impact of pesticides on soil microorganisms can be inhibitory, stimulatory, or without effect (Bending and Turner, 1999). When used as directed, most pesticides have little effect on soil microorganisms. The effect of the recommended dose, doses 10 and 100 times higher than recommended, was examined. It was found that the recommended doses of the tested pesticides did not have an inhibitory effect on the growth of most isolates (Table 2c). An exception is the impact of the Sequence preparation on the growth of isolates AZC 5 and AZC 6, where inhibition zones of up to 5 mm in diameter were observed. Pesticides Luna and Sequence had inhibitory effects on the growth of all isolates at concentrations ten and 100 times higher than recommended. In contrast, Lamdex did not have an inhibitory effect on the growth of any isolates at the same doses.

Among the isolates of the genus *Azotobacter*, resistance to ampicillin at a concentration of 10 μg ml^{-1} was observed in all tested isolates (Table 2d). On the other hand, all isolates showed high sensitivity to the presence of streptomycin at a concentration of 300 μg (zone of inhibition > 10 mm), which can also be said for the effect of chloramphenicol at a concentration of 30 μg. Weaker growth of all isolates (zone of inhibition 1–10 mm) was recorded under the influence of erythromycin at a concentration of 15 μg and neomycin at a concentration of 30 μg, which can also be said for the effect of chloramphenicol at a concentration of 30 μg in isolates AZC 4 and AZC5.

Table 2b. Resistance of isolates to heavy metals

Isolates	Heavy metals (mol dm^{-3})															
	Chromium (Cr)				Copper (Cu)				Nickel (Ni)				Lead (Pb)			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
AZC1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AZC2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AZC3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AZC4	++	+	+	+	-	-	-	-	++	++	++	++	+	+	-	-
AZC5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AZC6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

without zone of inhibition (-); zone of inhibition 1-10 mm (+); zone of inhibition > 10 mm (++)

Table 2c. Resistance of isolates to pesticides

Isolates	Pesticides (mol dm^{-3})								
	Luna			Lamdex			Sekvence		
	MI	(10x>MI)	(100x>MI)	MI	(10x>MI)	(100x>MI)	MI	(10x>MI)	(100x>MI)
AZC1	-	+	+	-	-	-	-	+	+
AZC2	-	+	+	-	-	-	-	+	+
AZC3	-	+	+	-	-	-	-	+	+
AZC4	-	+	+	-	-	-	-	+	+
AZC5	-	+	+	-	-	-	+	+	+
AZC6	-	+	+	-	-	-	+	+	+

without zone of inhibition (-); zone of inhibition 1-10 mm zone (+); zone of inhibition > 10 mm (++)

The results of the examination of biochemical characteristics of isolates are detailed in Table 3. It was observed that all isolates exhibited positive catalase and oxidase reactions. Moreover, enzymatic analyses revealed the production of extracellular lecithinases and proteases in all isolates except for AZC1, while lipase production was absent in isolate AZC6. Additionally, urease activity was absent in isolates AZC2 and AZC5. Cellulolytic activity was identified in isolates AZC3, AZC5, and AZC6, whereas pectinolytic activity was detected only in isolates AZC1 and AZC4. Contrary to expectations, none of the tested isolates showed capability for starch hydrolysis. However, gelatin hydrolysis was confirmed in 50% of the tested isolates (AZC1, AZC5, and AZC6).

The role of hydrolytic enzymes in maintaining soil fertility is paramount, as they facilitate the breakdown of complex compounds such as polysaccharides, proteins, and urea into simpler forms, thereby enhancing soil fertility. Furthermore, hydrolytic enzymes have been implicated in the paralysis and demise of pathogenic microorganisms, particularly fungi (Beneduzi et al., 2012). Consequently, microorganisms capable of producing hydrolytic enzymes may hold promise in combating various plant pathogenic fungi and bacteria, as well as promoting plant growth (Gomes et al., 2001). Notably, isolates AZC1 and AZC3 exhibited the highest enzyme production levels, suggesting their potential as biocontrol agents against phytopathogens. All isolates demonstrated the ability to utilize nitrate as an energy source. In terms of carbon utilization capability, only isolates AZC1 and AZC4 were proficient in citrate utilization. Additionally, all tested isolates, except for AZC4, exhibited the ability to utilize galactose, lactose, and xylose as energy sources. Similar trends were observed with sucrose, with the exception of isolates AZC4 and AZC5. Isolates AZC1, AZC2, and AZC3 displayed the ability to utilize fructose as an energy source, while only isolates AZC2 and AZC3 demonstrated glucose utilization capability. These findings corroborate the classification of these isolates within the genus *Azotobacter* (Upadhyay et al., 2015).

The proportion of plant growth-promoting bacteria (PGPR) in the total population of rhizospheric bacteria is usually low, estimated at 2-5%. However, their significance for plant growth and development is extremely important due to specific mechanisms of functioning (Mrkovački and Milić, 2001; Gusain and Bhandari, 2019; Nadarajah and Abdul Rahman, 2023). *Azotobacter* species are considered members of plant growth-promoting rhizobacteria (Jnawali et al., 2015), as many of their strains have been shown to produce phytohormones such as thiamine, riboflavin, niacin, IAA, and gibberellin, which can stimulate root and shoot development (Althaf and Srinivas, 2013). PGP properties of the studied isolates, such as the production of indole-3-acetic acid (IAA), siderophore, and HCN, as well as phosphate mineralization and phosphate solubilization of six isolates, were studied and presented in Table 4.

An essential trait among PGP characteristics of microorganisms is the capacity to synthesize IAA (indole-3-acetic acid), a hormone belonging to the auxin group, responsible for regulating various physiological functions in plants, such as cell elongation, tissue specialization, and responses to light, gravity, and environmental stress factors (Gupta et al., 2015). In this study, IAA production was observed in 50% of the examined isolates (Table 4). Some authors suggest that *Azotobacter* generates indole-3-acetic acid (IAA) when tryptophan is added to the culture media (Brakel and Hilger, 1965). Another crucial PGP characteristic is the production of siderophores, a process through which plants and bacteria acquire iron by synthesizing low molecular weight molecules that exhibit a strong affinity for Fe+3 ions (Souza et al., 2015). Among the tested isolates, siderophore production was not observed only in the case of isolate AZC5. In contrast to these results, in the study by Minut et al. (2022), all studied *Azotobacter* isolates did not show the ability to produce siderophores.

Table 2d. Resistance of isolates to antibiotics

Isolates	Antibiotics				
	Ampicilin	**Erithromicin**	**Neomicin**	**Streptomycine**	**Chloramphenicol**
	10 µg	**15 µg**	**30 µg**	**300 µg**	**30 µg**
AZC1	-	+	+	++	++
AZC2	-	+	+	++	++
AZC3	-	+	+	++	++
AZC4	-	+	+	++	+
AZC5	-	+	+	++	+
AZC6	-	+	+	++	++

without zone of inhibition (-); zone of inhibition 1-10 mm zone (+); zone of inhibition > 10 mm (++)

Table 3. Enzymatic, hydrolysis, energy and carbon source utilization properties of the bacterial isolates

Biochemical characterization	**Type of test/Isolates**		**AZC1**	**AZC2**	**AZC3**	**AZC4**	**AZC5**	**AZC6**
Enzymatic activity	Catalase production		+	+	+	+	+	+
	Oxidase production		+	+	+	+	+	+
	Lipase production		+	+	+	+	+	-
	Protease production		-	+	+	+	+	+
	Cellulase production		-	-	+	-	+	+
	Pectinase production		+	-	-	+	-	-
	Lechitinase production		+	+	+	+	+	+
	Urease production		+	-	+	+	-	+
Complex organic substrates degradation	Starch hydrolysis		-	-	-	-	-	-
	Gelatin hydrolysis		+	-	-	-	+	+
Energy source utilization	Nitrogen	nitrate reduction	+	+	+	+	+	+
	Carbon	citrate utilization	+	-	-	+	-	-
		glucose utilization	-	+	+	-	-	-
		fructose utilization	+	+	+	-	-	-
		sucrose utilization	+	+	+	-	-	+
		galactose utilization	+	+	+	-	+	+
		lactose utilization	+	+	+	-	+	+
		xylose utilization	+	+	+	-	+	+

(+) positive reaction /produce, hydrolyze, reduce, utilize/; (-) negative reaction /does not produce, hydrolyze, reduce, utilize/

Table 4. Plant growth promoting properties of the isolates

Isolates	IAA	Siderophores	HCN	Mineralization of phosphorus	Solubilize phosphates
AZC1	+	+	+	+	-
AZC2	-	+	+	+	-
AZC3	-	+	+	-	-
AZC4	+	+	+	-	-
AZC5	-	-	+	+	+
AZC6	+	+	+	-	+
Percentage (%) of positive isolates	50.00	83.33	100.00	50.00	33.33

(+) positive reaction / produces/ performs decomposition; (-) negative reaction / does not produce/ does not performs decomposition

HCN production is another important mechanism of PGP properties of microorganisms. It inhibits ATP synthesis and leads to the death of pathogenic microorganisms. According to Datta et al. (2011), the production of HCN by microorganisms has a favorable impact on plants. It was observed in all isolates examined in this study.

Phosphorus serves as a vital nutrient for plants, playing a role in nucleic acids, phospholipids, ATP, and various metabolic and biochemical pathways such as biological nitrogen fixation and photosynthesis (Khan et al., 2007). In our study, the capacity to mineralize organic phosphorus compounds, was found in 50% of the tested isolates, specifically AZC1, AZC2, and AZC5, while the remaining isolates lacked this capability.

The ability of isolates to solubilize inorganic phosphates was observed in only 33.33% of the tested isolates, specifically AZC5 and AZC6. Azzawi and Kamal (2022) reported similar findings, demonstrating the ability of *Azotobacter* genus isolates to mineralize and solubilize phosphorus compounds. Study of Krishna et al. (2020) also confirm the phosphate solubilization ability of *Azotobacter* bacteria. Specifically, all six isolates studied from this genus demonstrated the capacity to solubilize phosphates. Althaf and Srinivas (2013) further supported these results, indicating that representatives of the *Azotobacter* genus exhibited not only higher gibberellic acid production but also an enhanced ability to solubilize phosphates.

CONCLUSION

In conclusion, this study provides valuable insights into the utilization of *Azotobacter* species as biofertilizers to enhance soil fertility and promote plant growth in agricultural systems. The isolation and characterization of *Azotobacter* isolates from rhizosphere soil demonstrate their diverse morphological, ecological, and biochemical characteristics. Moreover, the observed stress tolerance, heavy metal resistance, and plant growth-promoting properties underscore the suitability of *Azotobacter* species for application in diverse environmental conditions. The findings suggest that Azotobacter-based biofertilizers could serve as environmentally sustainable alternatives to conventional agrochemicals, thereby contributing to agricultural sustainability and food security. Further research is warranted to explore the efficacy of *Azotobacter* species in field trials and their potential integration into existing agricultural practices.

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