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The Impact of Bio-fertilizers and Nitrogen Fertilizer on Barley Yield Productivity and Quality

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ABSTRACT

Biofertilizers present a viable strategy for decreasing dependence on chemical fertilizers in agriculture while mitigating their environmental and health impacts. These biofertilizers are composed of beneficial microorganisms that enhance plant growth through processes such as phosphate solubilization and nitrogen fixation. The objective of this study was to characterize bacterial isolates from the wheat rhizosphere and assess their potential as agents for promoting plant growth. The isolates were evaluated for their ability to solubilize phosphate, produce indole, and generate ammonia. Molecular identification through 16S rRNA gene sequencing revealed two effective strains: *Enterobacter cloacae* and *Pantoea agglomerans*. A field experiment was conducted at the Malawi Agricultural Research Station (Minya Governorate) over two agricultural seasons, 2022/2023 and 2023/2024, using the Egyptian barley cultivar Giza 138 to assess the effectiveness of these plant growth-promoting rhizobacteria (PGPR) when applied either individually or in combination, alongside three levels of nitrogen fertilizer (15, 30, and 45 kg N/fed^{-1}) on their agro-physiological characteristics and grain quality. The results indicated that the combined application of *Enterobacter cloacae* and *Pantoea agglomerans* at a nitrogen level of 30 kg N/fed^{-1} significantly enhanced barley performance, resulting in the highest grain yield, increased 1000-grain weight, longer spike length, a greater number of grains per spike, and improved chlorophyll a and b, as well as protein content. These findings illustrate that co-inoculation with these PGPR strains, coupled with a reduced quantity of chemical nitrogen fertilizer, can significantly enhance barley yield and quality, providing a sustainable alternative to traditional fertilization methods.

Keywords: Barley, biofertilizers, plant growth-promoting rhizobacteria (PGPR), microbial activity, chemical fertilizer.

1. Introduction

Barley (*Hordeum vulgare* L.) is one of the world's most important grain crops, cultivated across diverse environments due to its remarkable adaptability. It thrives in a wide range of climates, from the Middle East's arid deserts to the Himalayas' high altitudes, making it a cornerstone of global agriculture and food systems (Alotaibi *et al.,* 2024 a&b). Barley is a staple food in several North African countries and is recognized for its moderate stress tolerance, particularly in arid regions where it serves as a primary cereal crop. Its resilience not only ensures food security but also supports the livelihoods of countless farmers, providing both sustenance and economic stability (Masrahi *et al.,* 2023 and Mariey *et al.,* 2025 a&b). The barley plant yields grains, which are its most valuable product, widely used for food and fodder. Additionally, barley straw is commonly utilized as animal feed and can be incorporated into compost as an organic component. Barley grains also play a

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significant role in various industrial processes. Globally, barley ranks fourth in production after wheat, rice, and maize, and it remains a vital crop in many developing countries. However, barley plants often face drought ,salinity and heat stress at different growth stages, which can significantly impact yield and sustainability (Fatemi *et al.,* 2022; Zaib *et al.,* 2023 and Mariey *et al.,* 2025 a&b).

In recent years, the widespread use of chemical fertilizers has significantly boosted global food production and self-sufficiency. However, this progress has come at a substantial cost to ecosystems and the safety of living organisms (Delgado-Baquerizo *et al.,* 2020). Over the past few decades, the excessive application of chemical fertilizers has led to severe consequences, including degraded soil quality and fertility, increased soil salinity, and adverse effects on ecosystems and human health. To address these challenges, it is imperative to reduce dependence on chemical fertilizers in agriculture. Doing so would safeguard plant health, minimize environmental pollution, and lower production costs (Cardarelli *et al.,* 2023 and Mariey *et al.,* 2025 b).

Sustainable agricultural practices are essential to meet growing production demands without compromising environmental integrity. In this context, beneficial microorganisms offer a promising alternative to conventional farming methods. These microorganisms play a critical role in promoting plant growth and development by enhancing soil fertility, increasing soil biodiversity, and improving nutrient availability for plants. By integrating such biological solutions, agriculture can move toward a more sustainable and environmentally friendly future (Gebeyehu *et al.,* 2021).

Overuse of chemical fertilizers has damaged soil, ecosystems, and human health. Reducing their use is crucial for environmental protection and lowering farming costs (Cardarelli *et al.,* 2023). Biofertilizers, containing beneficial microorganisms like nitrogen-fixing bacteria and PGPR, offer a sustainable alternative by improving soil fertility, biodiversity, and nutrient availability, making them an increasingly important strategy for boosting agricultural productivity while safeguarding environmental health (Gebeyehu *et al.,* 2021).

The utilization of microorganisms, especially plant growth-promoting rhizobacteria (PGPR), offers environmentally friendly alternatives for agriculture (Chandran *et al.,* 2021). PGPR are free-living bacteria that colonize plant roots playing a vital role in enhancing nutrient mobility and supporting crop production (Chandler *et al.,* 2008). Their ability to fix nitrogen and solubilize nitrogen phosphate makes essential nutrients more available, enhancing plant growth. Additionally, PGPR regulates soil nutrient levels, synthesizes plant growth hormones, and protects plants from pathogens. Moreover, they improve soil structure and mitigate abiotic stressors such as salinity, drought, acidity, and humidity. Furthermore, PGPR is involved in the bioremediation of toxic heavy metals and the degradation of synthetic chemical compounds (Chauhan *et al.,* 2021). Bio-fertilizers differ from chemical fertilizers in several key aspects, making them a more preferable and sustainable choice. They are typically more cost-effective, cause minimal environmental harm, and exhibit higher activity levels, even when applied in smaller quantities. Additionally, bio-fertilizers are more efficient and have the unique ability to reproduce, regulated by plants and local microbial communities (Shahwar *et al.,* 2023).

In addition, microbial inoculants in bio-fertilizers accelerate decomposition processes and do not contribute to the development of resistance to pathogens or pests. This highlights the significant environmental and agricultural benefits of bio-fertilizers compared to chemical alternatives (Maitra *et al.,* 2021). Certain strains of *Pantoea* spp. are classified as plant growth-promoting bacteria (PGPB) due to their ability to enhance plant growth through both direct and indirect mechanisms. Direct mechanisms include traits such as the production of phytohormones, nitrogen fixation, and the solubilization of ammonia and phosphorus (Cherif-Silini *et al.,* 2019). Furthermore, the genus *Enterobacter* is widely acknowledged for its biotechnological potential in improving soil health and boosting crop productivity (Wang *et al.,* 2023).

Nitrogen is a crucial nutrient for plants, as it is required at various stages of growth. It serves as an essential component of amino acids, proteins, enzymes, and protoplasm. Furthermore, nitrogen is integral to cofactors such as NAD and NADH, which play significant roles in amino acid formation reactions within cells (Tariq *et al.,* 2023). This study explores the potential of Enterobacter cloacae and Pantoea agglomerans, bacteria isolated from the wheat rhizosphere, as plant growth-promoting agents.

The research assesses the impact of these bacteria, used individually and in combination, on barley yield and quality while varying nitrogen fertilizer levels. The ultimate goal is to determine if

these biofertilizer treatments can reduce the need for nitrogen fertilizers, lower agricultural costs, improve sustainability, and encourage environmentally friendly farming.

2. Material and Methods

2.1. Isolation of strains and screening for PGP traits

These isolated strains were isolated from agricultural fields at the South El-Hossinia Research Farm Station in El-Sharkia Governorate, Egypt. The strains were identified, screened, and evaluated for various growth-promoting activities. Soluble phosphate content was measured using the method of Murphy and Riley (1962). IAA production was assessed using a modified protocol based on Khianngam *et al.* (2023). Ammonia production was evaluated following the Sagar *et al.* (2019) methodology and exopolysaccharide production was analyzed using a modified method by Paulo *et al.* (2012).

2.2. Molecular characterization of bacterial isolates

The selected bacterial strains were cultured overnight in 5 mL of LB broth. Genomic DNA was extracted using the Ezup column bacterial genomic DNA purification kit (Sangon Biotech, Shanghai Co., Ltd, China) following the manufacturer's instructions**.**

2.2.1. Phylogenetic and 16S rRNA sequence analysis

Bacterial isolates were identified through 16S rRNA gene sequencing. The 16S rRNA gene was amplified using the universal primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). PCR amplification was performed in a Gene Amp PCR System 9700 Thermal Cycler (Applied Biosystems, USA) under the following conditions: initial denaturation at 95°C for 10 min, followed by 30 cycles of denaturation (95°C for 10 s), annealing (56°C for 15 s), and elongation (72°C for 10 s), with a final extension at 72°C for 5 min. The PCR products were analyzed by electrophoresis on a 1.0% agarose gel prepared in 1×TAE buffer. The gel was stained with 4S Green Plus nucleic acid stain (8 µL) for DNA visualization. A 3-µL aliquot of each PCR product, along with 5 µL of DL-2000 DNA Marker, was loaded into the gel. Electrophoresis was conducted at 120 V for 30 min, and the gel was imaged using a UV transilluminator (Bio-Rad, Hercules, CA, USA). The partial 16S rRNA gene sequences were analyzed using MEGA-11 software and compared against the NCBI database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to obtain accession numbers for the isolated strains.

2.3. Physicochemical and biological properties of the experimental soil

The procedure of soil analysis followed by Blake, (1965).The properties of the study's clay soil (0–30 cm from the surface).

2.4. The experiment site experimental site, design, and treatments

The study was conducted at the Malawi Agricultural Research Station (Minya Governorate, Middle Egypt; coordinates: 27°43′53.04″N, 30°50′29.94″E) during two consecutive winter growing seasons (2022/2023 and 2023/2024). The experiments employed the barley (*Hordeum vulgare* L.) cultivar Giza 138 to evaluate the effects of nitrogen fertilization and biofertilizer application on the barley yield.

A split-plot design with three randomized replications was adopted: Main plots: Four nitrogen (N) fertilization levels: Control (0 kg N/fed); 15 kg N/fed; 30 kg N/fed; 45 kg N/fed. Furthermore, Subplots: Four biofertilizer treatments: Control (no biofertilizer); *Enterobacter cloacae* alone; *Pantoea agglomerans* alone; and Co-inoculation of *Enterobacter cloacae* + *Pantoea agglomerans.* The following treatments were deployed: C- (uninoculated and unfertilized), *Enterobacter cloacae*, *Pantoea agglomerans*, *Enterobacter cloacae* + *Pantoea agglomerans*, 15 kg N/fed, *Enterobacter cloacae* + 15 kg N/fed, *Pantoea agglomerans* + 15 kg N/fed, *Enterobacter cloacae* + *Pantoea agglomerans* + 15 kg N/fed, 30 kg N/fed, *Enterobacter cloacae* + 30 kg N/fed, *Pantoea agglomerans* + 30 kg N/fed, *Enterobacter cloacae* + *Pantoea agglomerans* + 30 kg N/fed, 45 kg N/fed, *Enterobacter cloacae* + 45 kg N/fed, *Pantoea agglomerans* + 45 kg N/fed, *Enterobacter cloacae* + *Pantoea agglomerans* + 45 kg N/fed.

2.5. Seed inoculation

For bio-fertilizer inoculation, the barley seeds underwent specific preparations. Initially, the seeds were sterilized with 70% ethanol for 2 minutes, thoroughly rinsed five times with distilled water, and then coated with a sugar solution to serve as a carrier. Individual inoculations of *Enterobacter cloacae* and *Pantoea agglomerans* strains were performed the recipe for Luria-Bertani broth is as follows. Combine 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, and 1 liter of distilled water; adjust the pH to 7.0 with 1 N NaOH; and autoclave the mixture for 15 min at 121° C, followed by incubation at 30°C with shaking at 180 rpm for 72 hours to yield a mixed bacterial stock solution (approximately 1 × 10^10 CFU/mL). This stock solution was diluted 100-fold to create a liquid bacterial fertilizer with a concentration of roughly 10^8 CFU/mL. The seeds were inoculated with either *Enterobacter cloacae* or *Pantoea agglomerans* separately for specific treatments, or combined in others before being sown in the experimental plots. Sowing was conducted at a rate of 50 kg per fed using the broadcasting method, specifically the Afir technique, on December 5 and 8 in the years 2023 and 2024, respectively, for both seasons. Standard cultural practices for barley cultivation were applied as per the recommendations. Additionally, representative soil samples were collected from each site at a depth of 0-30 cm from the soil surface. This sampling was carried out to ensure accurate assessments of soil conditions and fertility before planting.

Barley seeds were inoculated with bacterial suspensions (10^8 CFU/mL) before sowing. Nitrogen was applied as urea (46% N) in two split doses: 50% at sowing and 50% at the tillering stage. Field management practices, including irrigation and pest control, followed standard protocols for barley cultivation.

2.6. Crop management

Soil preparation for barley planting commenced in November. To encourage robust growth, the seeds were treated with a water-sugar solution enriched with beneficial bacterial strains. The Afir method of hand cultivation was carefully implemented, with gloves used for each plot to minimize contamination. In plots designated for chemical fertilizers, nitrogen was applied in two phases: half at sowing and the remainder during the branching period. Manual weed control was performed during the branching stage to limit resource competition. Irrigation was conducted three times, coinciding with sowing, branching, and flowering, to maintain optimal soil saturation. Once adequately saturated, irrigation ceased to inhibit bacterial formation, and each plot was meticulously cleaned to prevent contamination. Harvesting took place upon full maturity of the plants. To optimize yield measurements, the side rows and the top and bottom 30 cm of each plot were excluded from harvesting, concentrating efforts on the central area. After harvesting, the plants were left to dry in the field for three days before being threshed using a threshing machine.

2.7. Data recorded

2.7.1. Agronomic traits

At harvest, ten plants were randomly selected from each plot to evaluate plant height (cm), spike length (cm), spike weight (g), number of grains spike^{-1}, number of spikes/m^2, 1000 grain weight, while the biological yield, grain yield, and straw yield were evaluated per plot and then converted to grain yield (kg fed^{-1}), straw yield (kg fed^{-1}), and biological yield (kg fed^{-1}).

2.7.2. Chemical Composition

According to AOAC (AC, 1990), the micro-Kjeldahl method was followed and used to evaluate, total N in grains. The protein content of grain was estimated by multiplying the total nitrogen by 5.83 (Jones, 1931).To determine the concentrations of chlorophylls, a 1 g fresh plant leaf sample was accurately weighed, macerated, and extracted. Three extraction solvents were used for each sample: 95% aqueous diethyl ether (petroleum ether), 90% aqueous methanol, and 100% acetone, with an extraction ratio of 1:50. The homogenized mixture was then separated by centrifugation at 3000 rpm for 10 minutes. Analytical measurements were conducted using a Helios α spectrophotometer at the following wavelengths: 645, 653, 666, and 664 nm for chlorophyll a and b (depending on the extraction solvent) by(Lichtenthaler & Wellburn, 1983) as follows:

Chlorophyll a = 15.65 A666 – 7.340 A653……. (1)
Chlorophyll b = 27.05 A653 – 11.21 A666……..(2)

2.8. Soil Bio-Activity

Soil samples were collected from the upper layer at a depth of 30 cm from three distinct patches. A drill was employed to ensure random sampling across various replicates for each treatment. Following collection, the samples were meticulously mixed to achieve a homogeneous blend and subsequently air-dried. Subsamples were obtained from this prepared material to calculate microbial counts (Allen, 1949).

2.9. Statistical Analyses

Statistical analysis was conducted on the data collected over two seasons, utilizing the analysis of variance (ANOVA) technique for two factors in a split-plot design. The mean values were compared using Duncan's Multiple Range Test (Duncan, 1955). All calculations were performed following the methodologies outlined by (Gomez & Gomez, 1984). The statistical analyses were executed using the "MSTAT-C" software Package (1990) to facilitate the ANOVA process. Additionally, Pearson's correlation test was applied using SPSS version 22.0 (SPSS Inc., Chicago, IL) to explore the relationships between each pair of studied traits.

3. Results

3.1. Physicochemical and biological properties of the experimental soils

Table 1 presents the physicochemical and biological properties of experimental soil over two seasons, 2022-2023 and 2023-2024. The analysis reveals that the sand percentage increased from 53.01% to 55.81%, while the silt percentage decreased slightly from 24.55% to 23.5%. The clay content also showed a decline from 22.44% to 20.69%, indicating a consistent textural class of clay across both seasons.

Table 1: Physicochemical and biological properties of the experimental soil

Type of analysis	Experimental seasons	
	2022-2023	2023-2024
Sand %	53.01	55.81
Silt %	24.55	23.5
Clay %	22.44	20.69
Textural class	Clay	Clay
Bulk density (g/cm^{-3})	1.28	1.32
Field capacity % (V/V^{-1})	42.01	46.62
Wilting point % (V/V^{-1})	30.44	32.27
Chemical analysis		
pH of soil–water suspension (1:2.5)	7.95	7.90
E.C of soil – water extract (dS/m) (1:5)	1.46	1.52
Organic matter %	1.32	1.41
$CaCO_3$ %	2.26	2.13
Available nutrients (mg/1 kg soil)		
N	78	82
P	15	19
K	492	512
Microbiological analysis		
Total bacterial (cfu × 10^5)	10	8
Total actinomycetes (cfu ×10^3)	11	14
Total fungi (cfu × 10^3)	8	10

In terms of bulk density, there was a minor increase from 1.28 g/cm³ to 1.32 g/cm³, which suggests a slight compaction of the soil over the period. Additionally, field capacity improved from 42.01% to 46.62%, indicating better water retention capabilities in the 2023-2024 season. The wilting point also rose from 30.44% to 32.27%, reflecting an increase in the moisture content required for plants to extract water.

The chemical analysis showed that the pH of the soil-water suspension remained relatively stable, decreasing slightly from 7.95 to 7.90. The electrical conductivity (E.C) of the soil-water extract increased from 1.46 dS/m to 1.52 dS/m, suggesting a potential rise in salinity levels. Organic matter

content also showed a modest increase from 1.32% to 1.41%, while calcium carbonate (CaCO3) decreased from 2.26% to 2.13%.

Regarding available nutrients, nitrogen (N) levels rose from 78 mg/kg to 82 mg/kg, phosphorus (P) increased from 15 mg/kg to 19 mg/kg, and potassium (K) also showed a notable rise from 492 mg/kg to 512 mg/kg. Lastly, the microbiological analysis indicated a decrease in total bacterial counts from 10 to 8 cfu × 10^5, while total actinomycetes increased from 11 to 14 cfu × 10^3, and total fungi counts rose from 8 to 10 cfu × 10^3.

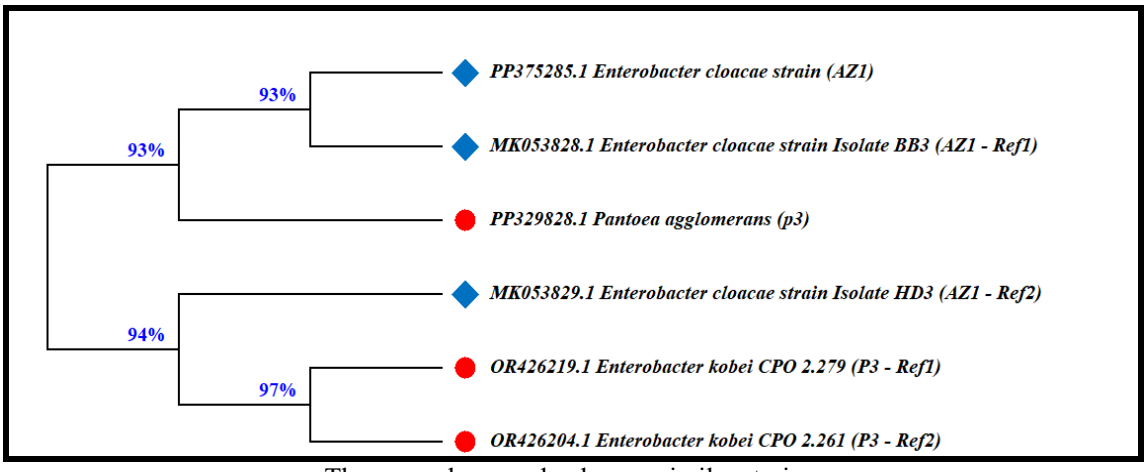
3.2. Molecular identification of *Enterobacter cloacae* and *Pantoea agglomerans*

The isolated strains were identified via 16S rRNA gene sequencing, using the NCBI BLAST tool (Basic Local Alignment Search Tool). Accession numbers for each strain are provided in Table 2.

Table 2: Phylogenetic and 16S rRNA sequence analysis.

Isolated strains	Accession version	Reference strains	Accession version	Similarity % (Per. identity)
***Enterobacter cloacae* (AZ1)**	PP375285.1	*Enterobacter cloacae* BB3	MK053828.1	93
		Enterobacter cloacae HD3	MK053829.1	94
***Pantoea agglomerans* (P3)**	PP329828.1	*Enterobacter kobei* CPO 2.279	OR426219.1	97
		Enterobacter kobei CPO 2.261	OR426204.1	97

The phylogenetic analysis of the isolated strains is illustrated in Fig. 1. The phylogenetic tree highlights the relationships between the two reference strains, *Enterobacter cloacae,* and *Pantoea agglomerans*, with sequence similarity ranging from 93% to 97%. This analysis provides insights into the genetic relatedness and evolutionary connections of the isolates.



The same shape and color are similar strains

3.3. Plant growth-promoting characteristics of isolated strains

The isolated strains did not exhibit antagonistic interactions with each other. Furthermore, they are capable of producing indole-3-acetic acid (IAA) in nutrient broth (NB) supplemented with 0.5 g L^{-1} L-tryptophan., Both *Enterobacter cloacae* and *Pantoea agglomerans* demonstrated the ability to produce ammonia. Additionally, the isolated strains exhibited phosphorus-solubilizing properties, as detailed in Table 3.

Table 3: Plant growth-promoting (PGP) traits of isolated strains

Traits	*Enterobacter cloacae*	*Pantoea agglomerans*
P solubilization (µg/mL)	878	949
IAA production (µg/mL)	23.410	16.145
Ammonia production	+	+

3.4. The effect of Bio-fertilizers and nitrogen fertilizer rates on barley yield

3.4.1. Grain yield of barley

Table 4. details the grain yield (GY), measured in kilograms per feddan (kg/fed^{-1}), resulting from different treatments applied over two years. These treatments included varying levels of inorganic nitrogen (15N, 30N, and 45N kg/fed^{-1}) and the application of growth-promoting microorganisms (*Enterobacter cloacae* and *Pantoea agglomerans*), both individually and in combination. The negative control, which received no treatment, exhibited the lowest grain yield in both years, recording 1297.00 kg/fed^{-1} in Year 1 and 1257.24 kg/fed^{-1} in Year 2. Conversely, the combined application of both microorganisms (*Enterobacter cloacae* and *Pantoea agglomerans*) led to an increased yield, reaching 1487.67 kg/fed^{-1} in Year 1 and 1467.67 kg/fed^{-1} in Year 2. The application of inorganic nitrogen significantly enhanced grain yield across all tested levels (15N, 30N, and 45N kg/fed^{-1}). Notably, the highest yield in both years was observed with the 30N kg/fed^{-1} treatment, producing 2190.05 kg/fed^{-1} in Year 1 and 2085.71 kg/fed^{-1} in Year The most effective treatment proved to be the combination of *Enterobacter cloacae*, *Pantoea agglomerans*, and 30N kg/fed^{-1}, which yielded the highest grain production in both years 2285.57 kg/fed^{-1} in Year 1 and 2176.19 kg/fed^{-1} in Year 2. This outcome suggests a synergistic relationship between these microorganisms and an optimal level of inorganic nitrogen, leading to a substantial improvement in grain production.

3.4.2. Straw yield of barley

Table 4 presents the straw yield (SY) in kilograms per feddan (kg/fed^{-1}) for various treatments over two years. These treatments include the application of inorganic nitrogen (15N, 30N, and 45N kg/fed^{-1}) and growth-promoting microorganisms (*Enterobacter cloacae* and *Pantoea agglomerans*), applied individually and in combination.

Table 4: Impact of Bio-fertilizers and nitrogen fertilizers on grain, straw, and biological yield of barley variety Giza 138 (2022/2023 and 2023/2024).

Treatments (kg/fed^{-1})	Year 1			Year 2		
	GY	SY	BY	GY	SY	BY
Ctrl (negative control)	1297.00	3350.62	4647.62	1257.24	3543.01	4800.25
Ent. cloacae	1342.86	3361.90	4704.76	1352.38	3780.95	5133.33
Pan. agglomerans	1452.38	3576.19	5028.57	1415.21	4365.38	5780.59
Ent+Pan	1487.67	3731.38	5219.05	1467.67	4570.43	6038.10
15 N	1485.71	3923.79	5409.50	1477.66	4922.34	6400.00
Ent+15 N	1619.50	3904.31	5523.81	1600.45	4818.60	6419.05
Pan+15 N	1728.75	4023.63	5752.38	1728.51	4766.73	6495.24
Ent+Pan+15 N	1876.19	4219.05	6095.24	1828.57	5390.48	7219.05
30 N	1785.71	4252.39	6038.10	1771.43	5504.76	7276.19
Ent+30 N	1847.62	4780.95	6628.57	1885.71	5771.43	7657.14
Pan+30 N	2190.05	5219.47	7409.52	2085.71	5590.48	7676.19
Ent+Pan+30 N	2285.57	5467.19	7752.76	2176.19	5603.81	7780.00
45 N	2228.05	5409.67	7637.72	2097.19	5693.29	7790.48
Ent+45 N	1980.95	5752.38	7733.33	1942.82	5942.89	7885.71
Pan+45 N	1904.46	5905.06	7809.52	1891.43	6032.38	7923.81
Ent+Pan+45 N	1830.65	6683.64	8514.29	1714.29	7066.66	8780.95
Mean	1771.445	4597.601	6369.046	1730.779	5210.226	6941.00
F. Test						
Bio-fertilizers (B)	**	**	**	**	**	**
Nitrogen fertilizers (N)	**	**	**	**	**	**
LSD 0.05						
Bio-fertilizers (B)	6.836	6.241	6.423	6.130	5.791	6.324
Nitrogen fertilizers (N)	2.763	1.216	2.700	2.170	1290	2.780
Interaction NXB	**	**	**	**	**	**

Grain Yield (GY), Straw Yield (SY), Biological Yield (BY); and (**) are significantly different (P<0.05).

The negative control (no treatment) resulted in the lowest straw yield in both years, with 3350.62 kg/fed^{-1} in Year 1 and 3543.01 kg/fed^{-1} in Year 2. The combination of both microorganisms increased the yield further, reaching 3731.38 kg/fed^{-1} in Year 1 and 4570.43 kg/fed^{-1} in Year 2,

Inorganic nitrogen significantly increased straw yield across all levels (15N, 30N, and 45N kg/fed^{-1})., and the highest yield in both years was observed with 45N kg/fed^{-1}, producing 5409.67 kg/fed^{-1} in Year 1 and 5693.29 kg/fed^{-1} in Year The combination of *Enterobacter cloacae*, *Pantoea agglomerans*, and 45N kg/fed^{-1} produced the highest straw yield in both years, with 6683.64 kg/fed^{-1} in Year 1 and 7066.66 kg/fed^{-1} in Year 2

3.4.3. Biological yield of barley

The biological yield (BY) was present in Table 4 as kilograms per feddan (kg/fed^{-1}) for various treatments over two years. The treatments include the application of inorganic nitrogen (15N, 30N, and 45N kg/fed^{-1}) and growth-promoting microorganisms (*Enterobacter cloacae* and *Pantoea agglomerans*), both individually and in combination. The negative control (no treatment) yielded the lowest biological yield in both years, with 4647.62 kg/fed^{-1} in Year 1 and 4800.25 kg/fed^{-1} in Year 2, The combination of both microorganisms further increased the yield, with 5219.05 kg/fed^{-1} in Year 1 and 6038.10 kg/fed^{-1} in Year 2, The application of inorganic nitrogen significantly boosted biological yield across all levels (15N, 30N, and 45N kg/fed^{-1}). The highest yield in both years was observed with 45N kg/fed^{-1}, which produced 7637.72 kg/fed^{-1} in Year 1 and 7790.48 kg/fed^{-1} in Year 2 and Ent cloacae + Pan agglomerans + 45N kg/fed^{-1}: This combination produced the highest yield in both years, with 8514.29 kg/fed^{-1} in Year 1 and 8780.95 kg/fed^{-1} in Year 2.

3.4.4. Plant height

Table 5 presents the effects of various treatments on the plant height of barley over two years. The negative control, which received no additional treatments, exhibited the lowest plant heights in both years (83.33 cm in Year 1 and 78.00 cm in Year 2). In contrast, the combination of both microbes (*Ent cloacae* + *Pan agglomerans*) significantly enhanced plant growth, resulting in heights of 97.67 cm in Year 1 and 100.00 cm in Year When the nitrogen level was increased to 30N kg/fed^{-1}, plant heights were further boosted. The combination of both microbes with 30N kg/fed^{-1} achieved the highest heights in this group, reaching 116.67 cm in both years. At the highest nitrogen level (45N kg/fed^{-1}), plant heights continued to rise, with the combination of both microbes and 45N kg/fed^{-1} yielding the greatest overall heights (125.00 cm in Year 1 and 121.67 cm in Year 2).

3.4.5. 1000-grain weight

Results in Table 5 presents the effects of different treatments on the 1000-grain weight (g) of barley over two years. The negative control, which received no additional treatments, exhibited the lowest 1000-grain weights in both years (34.00 g in Year 1 and 35.00 g in Year 2), serving as a baseline for comparing the effects of other treatments. The combination of both microbes (*Ent cloacae* + *Pan agglomerans*) further enhanced grain weight, reaching 40.00 g in Year 1 and 39.00 g in Year 2. Increasing the nitrogen level to 30N kg/fed^{-1} further boosted grain weights, with the combination of both microbes and 30N kg/fed^{-1} resulting in the highest weights in this group (70.00 g in Year 1 and 66.00 g in Year 2). At the highest nitrogen level (45N kg/fed^{-1}), grain weights showed a slight decline compared to the 30N treatment. However, the combination of both microbes with 45N kg/fed^{-1} still yielded higher weights (48.00 g in Year 1 and 50.00 g in Year 2) compared to the control and lower nitrogen treatments.

3.4.6. Spike length

The impact of various treatments on barley spike length (cm) over two years were illustrated in (Table 5), the negative control, without any additional treatments, exhibited the shortest spike lengths in both Year 1 (6.10 cm) and Year 2 (6.40 cm). The application of combined microbes (*Ent cloacae* + *Pan agglomerans*) enhanced spike length, achieving 6.80 cm in Year 1 and 7.00 cm in Year Increasing the nitrogen level to 30N kg/fed^{-1} further improved spike lengths. The combination of both microbes with 30N kg/fed^{-1} resulted in the longest spikes within this group, measuring 9.50 cm in Year 1 and 9.70 cm in Year 2. At the highest nitrogen level (45N kg/fed^{-1}), spike lengths were marginally shorter compared to the 30N treatment. Nevertheless, the combination of both microbes with 45N kg/fed^{-1} still produced longer spikes (8.50 cm in Year 1 and 8.30 cm in Year 2) compared to the control and lower nitrogen treatments.

Table 5: Comparative analysis of Bio-fertilizers and nitrogen fertilizers on plant height, 1000-grain weight, spike length, and number of spikes in barley variety Giza 138 (2022/2023 and 2023/2024).

Treatments (kg/fed^{-1})	Year 1				Year 2			
	Plant Height (cm)	1000-Grain Weight (g)	Spike Length (cm)	No. of Spikes/m^2	Plant Height (cm)	1000-Grain Weight (g)	Spike Length (cm)	No. of Spikes/m^2
C*trl* (negative control)	83.33	34.00	6.10	303.60	78.00	35.00	6.40	309.80
Ent. cloacae	91.67	36.00	6.30	309.40	86.00	35.00	6.50	317.00
Pan. agglomerans	93.67	38.00	6.40	327.10	90.00	37.00	6.70	320.10
Ent+Pan	97.67	40.00	6.80	318.90	100.00	39.00	7.00	323.50
15 N	100.20	46.00	7.10	326.40	98.00	43.00	6.90	333.20
Ent+15 N	101.00	50.00	7.50	341.90	104.00	51.00	7.30	349.40
Pan+15 N	108.00	55.00	8.00	356.90	106.00	53.00	7.80	366.50
Ent+Pan+15 N	111.00	56.00	8.40	368.90	108.00	58.00	8.20	376.10
30 N	105.00	48.00	7.60	399.40	102.00	50.00	7.80	418.60
Ent+30 N	108.00	60.00	8.90	451.00	105.00	62.00	8.60	443.70
Pan+30 N	111.67	66.00	9.20	468.30	109.00	64.00	9.40	478.70
Ent+Pan+30 N	116.67	70.00	9.50	499.20	116.67	66.00	9.70	501.60
45 N	115.00	43.00	7.10	359.40	113.00	41.00	7.50	339.50
Ent+45 N	118.00	45.00	7.60	353.00	116.00	43.00	7.90	363.80
Pan+45 N	123.00	47.00	8.10	389.30	119.00	46.00	8.30	372.40
Ent+Pan+45 N	125.00	48.00	8.50	414.40	121.67	50.00	8.30	396.70
Mean	106.805	48.875	7.694	374.194	106.805	48.313	7.769	375.66
F test								
Bio-fertilizers (B)	**	**	**	**	**	**	**	**
Nitrogen fertilizers (N)	**	**	**	**	**	**	**	**
LSD 0.05								
Bio-fertilizers (B)	3.670	2.874	5.655	2.070	6.002	2.230	4.000	2.120
Nitrogen fertilizers (N)	2.751	6.000	5.403	1.057	2.512	6.422	5.342	1.004
Interaction BxN	**	**	**	**	**	**	**	**

(**) are significantly different (P<0.05).

3.4.7. Number of spikes

Results in Table 5 details the effects of various treatments on the number of barley spikes per square meter (spikes/m²) over two years. The negative control, receiving no additional treatments, exhibited the lowest spike density in both Year 1 (303.60 spikes/m²) and Year 2 (309.80 spikes/m²). The application of combined microbes (*Ent cloacae* + *Pan agglomerans*) further increased spike density, reaching 318.90 spikes/m² in Year 1 and 323.50 spikes/m² in Year 2. Increasing the nitrogen level to 30N kg/fed⁻¹ further enhanced spike numbers. The combination of both microbes with 30N kg/fed⁻¹ resulted in the highest spike densities overall, with 499.20 spikes/m² in Year 1 and 501.60 spikes/m² in Year 2. At the highest nitrogen level (45N kg/fed⁻¹), spike numbers were slightly lower compared to the 30N treatment. However, the combination of both microbes with 45N kg/fed⁻¹ still yielded higher spike densities (414.40 spikes/m² in Year 1 and 396.70 spikes/m² in Year 2).

3.5. The effect of Bio-fertilizers and nitrogen fertilizer rates on barley protein content %

Results in Table 6 presents the effects of different treatments on the protein content (%) of barley over two years. The negative control, which received no additional treatments, showed the lowest protein content in both years (7.50% in Year 1 and 7.38% in Year 2). The combination of both microbes (*Ent cloacae* + *Pan agglomerans*) further enhanced protein content, reaching 8.56% in Year 1 and 8.31% in Year 2. The combination of both microbes with 30N kg/fed⁻¹ resulted in the highest protein content overall (11.50% in Year 1 and 11.63% in Year 2). At the highest nitrogen level (45N kg/fed⁻¹), protein content was slightly lower compared to the 30N treatment. However, the combination of both microbes with 45N kg/fed⁻¹ still yielded higher protein content (10.31% in Year 1 and 10.56% in Year 2) compared to the control and lower nitrogen treatments.

Table 6: Bio-fertilizers and nitrogen fertilizers influence protein content % in barley variety Giza 138 (2022/2023 and 2023/2024).

Treatments (kg/fed^{-1})	Year 1		Year 2	
	Total nitrogen (%)	Protein content (%)	Total nitrogen (%)	Protein content (%)
Ctrl (negative control)	1.20	7.50	1.18	7.38
Ent. cloacae	1.22	7.63	1.24	7.75
Pan. agglomerans	1.27	7.94	1.24	7.75
Ent+Pan	1.37	8.56	1.33	8.31
15 N	1.27	7.94	1.26	7.88
Ent+15 N	1.37	8.56	1.34	8.38
Pan+15 N	1.42	8.88	1.39	8.69
Ent+Pan+15 N	1.51	9.44	1.54	9.63
30 N	1.39	8.69	1.41	8.81
Ent+30 N	1.56	9.75	1.52	9.50
Pan+30 N	1.60	10.00	1.55	9.69
Ent+Pan+30 N	1.84	11.50	1.86	11.63
45 N	1.32	8.25	1.29	8.06
Ent+45 N	1.46	9.13	1.39	8.69
Pan+45 N	1.56	9.75	1.51	9.44
Ent+Pan+45 N	1.65	10.31	1.69	10.56
Mean	1.438	8.989	1.421	8.881
F. Test				
Bio-fertilizers (B)	**	**	**	**
Nitrogen fertilizers (N)	**	**	**	**
LSD 0.05				
Bio-fertilizers (B)	4.720	3.136	6.790	3.136
Nitrogen fertilizers (N)	4.115	7.471	1.679	2.652
Interaction BxN	**	**	**	**

(**) are significantly different (P<0.05).

3.6. The effect of Bio-fertilizers and nitrogen fertilizer rates on barley Chlorophyll A& B content

The effect of Bio-fertilizers and nitrogen fertilizer rates on barley chlorophyll content (Chlorophyll A and Chlorophyll B) in response to various treatments over two years were showed in Table 7. The control group had stable Chlorophyll a (23.22–23.25) and Chlorophyll b (13.85–13.86) levels across both years. The Ent+Pan treatment increased Chlorophyll b to 16.950 in Year 1 and 16.943 in Year 2. The most significant increases were seen with the Ent+Pan+30 N treatment, which had Chlorophyll b levels of 29.240 in Year 1 and 29.223 in Year 2. While 45 N also increased Chlorophyll b, its effect was less pronounced than that of 30 N, with the highest levels observed in the Ent+Pan+45 N treatment (25.073 in Year 1 and 25.083 in Year 2).

3.7. Biological activity in the rhizosphere of barley

The effects of various treatments on biological activity within the barley rhizosphere, specifically focusing on the counts of *Pantoea agglomerans* (measured in CFU/g of dry soil) over two years were illustrated in (Figure 2-a). The negative control, which received no additional treatments, exhibited the lowest counts of *Pantoea agglomerans*: 6.9×10^6 CFU/g in Year 1 and 8.06×10^6 CFU/g in Year The combination of *Enterobacter cloacae* and *Pantoea agglomerans* resulted in increased counts, reaching 10.56×10^6 CFU/g in Year 1 and 11.16×10^6 CFU/g in Year 2. Integrating both microbes with 30 N kg/fed^{-1} led to the highest overall counts, peaking at 20.0×10^6 CFU/g in Year 1 and 22.0×10^6 CFU/g in Year 2 and at the 45 N kg/fed^{-1} nitrogen level, *Pantoea agglomerans* counts showed a slight reduction compared to the 30 N treatment. Nevertheless, the combination of both microbes with 45 N kg/fed^{-1} still yielded higher counts (19.0×10^6 CFU/g in Year 1 and 20.1×10^6 CFU/g in Year 2) than the control and lower nitrogen treatments.

Table 7: The effect of Bio-fertilizers and nitrogen fertilizers on Chlorophyll A and Chlorophyll B content in barley variety Giza 138 (2022/2023 and 2023/2024).

Treatments (kg/fed^{-1})	Year 1		Year 2	
	Chl-A	Chl-B	Chl-A	Chl-B
Ctrl (negative control)	23.22	13.850	23.250	13.860
Ent. cloacae	24.93	15.663	24.893	15.650
Pan. agglomerans	24.05	16.560	24.040	16.573
Ent+Pan	23.94	16.950	23.927	16.943
15 N	24.45	17.357	24.440	17.353
Ent+15 N	23.16	17.563	24.380	17.580
Pan+15 N	24.43	19.960	24.430	19.973
Ent+Pan+15 N	24.28	22.160	24.273	22.173
30 N	24.31	19.150	24.340	19.153
Ent+30 N	23.82	26.073	23.820	26.077
Pan+30 N	24.03	27.057	24.030	27.040
Ent+Pan+30 N	24.36	29.240	23.193	29.223
45 N	23.92	19.070	23.920	19.090
Ent+45 N	23.67	23.567	24.073	23.570
Pan+45 N	23.91	24.637	23.927	24.657
Ent+Pan+45 N	24.04	25.073	23.670	25.083
Mean	24.044	24.038	20.871	20.875
F. Test				
Bio-fertilizers (B)	**	**	**	**
Nitrogen fertilizers (N)	**	**	**	**
LSD 0.05				
Bio-fertilizers (B)	0.222	0.032	0.021	0.017
Nitrogen fertilizers (N)	0.281	0.029	0.020	0.022
Interaction BXN	**	**	**	**

Chlorophyll A (Chl A), Chlorophyll B (Chl B), (**) are significantly different (P<0.05).

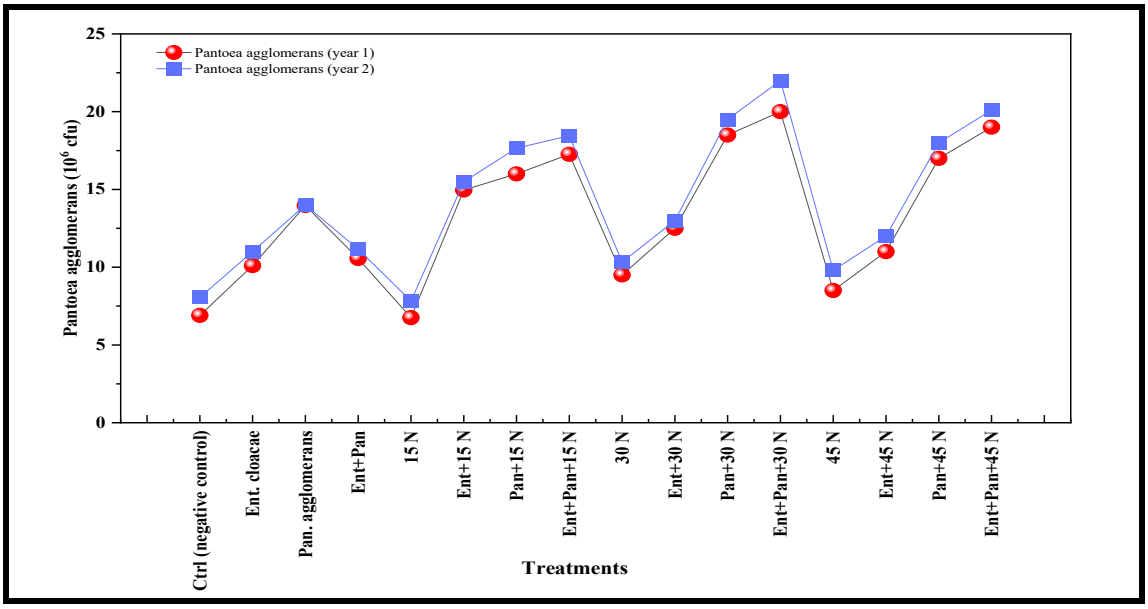


Fig. 2a: Biological activity in the rhizosphere in barley, a) *Pantoea agglomerans* counts (CFU/gm of dry soil).

Figure 2-b presented the effects of different treatments on the biological activity in the rhizosphere of barley, specifically the count of *Enterobacter cloacae* (measured in CFU/gm of dry soil) over two years. The negative control, which received no additional treatments, showed the lowest *Enterobacter cloacae* counts in both years (4.9 × 10 MPN/gm in Year 1 and 7.5 × 10^5

MPN/gm in Year 2. , The combination of *Enterobacter cloacae* and *Pantoea agglomerans* further increased *Enterobacter cloacae* counts ($14 \times 10^5 \times 10^6$ MPN/gm in Year 1 and 17×10^5 MPN/gm in Year 2), The combination of both microbes with 30N kg/fed^{-1} resulted in the highest counts overall (54×10^5 CFU/gm in Year 1 and 60×10^5 MPN/gm dry soil in Year 2) . At the highest nitrogen level (45N kg/fed^{-1}), *Enterobacter cloacae* counts were slightly lower compared to the 30N treatment. However, the combination of both microbes with 45N kg/fed^{-1} still yielded higher counts (45×10^5 CFU/gm in Year 1 and 49×10^5 MPN/gm in Year 2) compared to the control and lower nitrogen treatments.

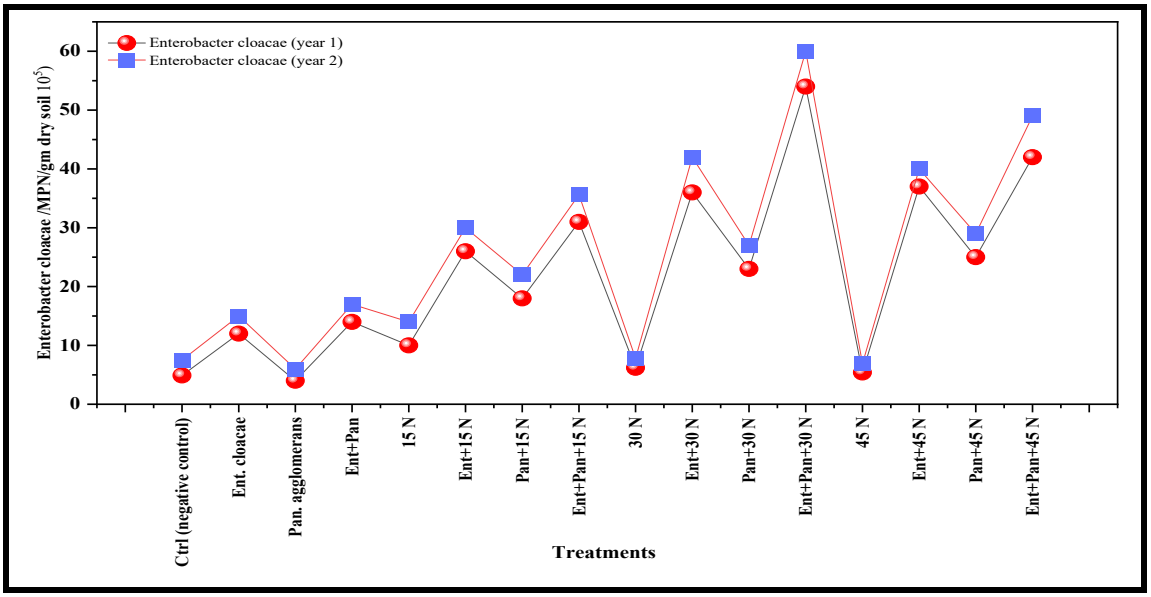


Fig. 2b: Biological activity in the rhizosphere in barley, b) *Enterobacter cloacae* counts (CFU/gm of dry soil).

4. Discussion

The current study aimed to characterize and identify two bacterial strains isolated from agricultural fields at the South El-Hossinia Research Farm Station in El-Sharkia Governorate, Egypt. Comprehensive morphological, genetic, and physiological analyses were conducted to achieve this goal, building on the work of. Mokrani et al. (2020) Additionally, the study investigated the effects of plant growth-promoting bacteria (PGPB) and varying nitrogen fertilizer rates on the agronomic traits of barley plants

The study involved partial sequencing of the 16S rRNA gene for the isolated strains, which was analyzed using NCBI's BLAST software. The strains were identified as belonging to two plant growth-promoting groups: *Pantoea agglomerans* and *Enterobacter cloacae*. The analysis, employing the neighbor-joining method, demonstrated a high similarity of 94% to 100% between the isolates and their respective reference strains.bOverall, the sequencing and phylogenetic analysis provided valuable insights into the taxonomic classification of the isolated strains, highlighting their close relationship to known reference strains. These findings are consistent with those previously reported. (Ortega-Ortega *et al.,* 2024).

The objective was to assess the plant growth-promoting rhizobacterial (PGPR) traits, with a particular focus on their ability to solubilize inorganic phosphate in the form of calcium phosphate ($Ca_3(PO_4)_2$, the phosphorus released by these strains in a liquid medium was quantified *Enterobacter cloacae* and *Pantoea agglomerans* also showed the highest released phosphorus value of 878 and 949 µg/mL respectively in NBRIP broth after 5 days of incubation. Furthermore, the results are consistent with those reported by (Prittesh Patel *et al.,* 2017). IAA stimulates the growth of lateral and adventitious roots, loosens the plant cell wall, and releases exudate that provides nutrition for rhizospheric microbes (Kumar *et al.,* 2025). The obtained results displayed the quantitative analysis of IAA production, which showed a concentration *of* 23.410 *and* 16.145 µg/mL respectively

for *Enterobacter cloacae* and *Pantoea agglomerans* This trend is consistent with findings by Atika et al. (2024).

Ammonia (NH_3) is vital for plant nutrition because it can be directly absorbed by plants as ammonium ions (NH_4^+). This form of nitrogen is readily accessible and improves the efficiency of nitrogen absorption, resulting in enhanced plant growth and crop quality. Significantly, approximately 80% of the ammonia produced worldwide is used in fertilizers, highlighting its crucial role in agriculture (Zayed *et al.,* 2023). Both isolated strains exhibited the ability to produce ammonia, offering several benefits to plants, including enhanced nitrogen absorption and the inhibition of phytopathogens (Chen *et al.,* 2025).

The Effect of Bio-Fertilizers and Nitrogen Fertilizer Rates on the Agronomic Traits of Barley Plants, Plant growth-promoting bacteria (PGPB) can serve as a potent alternative or supplement to nitrogen fertilizers. Recent years have seen a growing body of research on these bacteria in field conditions. To date, several studies have documented the successful use of PGPB in barley cultivation.(Kumar *et al.,* 2024). The data reveal that the Ent+Pan+30 N treatment produced the highest grain yield (GY) in Year 1 (2285.57 kg/fed^{-1}), demonstrating that moderate nitrogen application (30 N) combined with bio-fertilizers is highly effective for maximizing grain yield. Similarly, the highest straw yield (SY) was achieved under Ent+Pan+45 N in both years (6683.64 kg/fed^{-1} in Year 1 and 7066.66 kg/fed^{-1} in Year 2), and the highest biological yield (BY) was also observed under Ent+Pan+45 N (8514.29 kg/fed^{-1} in Year 1 and 8780.95 kg/fed^{-1} in Year 2). However, higher nitrogen levels (45 N) did not always lead to proportional yield increases, likely due to nitrogen saturation or reduced bio-fertilizer efficiency at very high N levels. These findings align with research on nitrogen use efficiency, emphasizing the importance of optimizing nitrogen application to minimize environmental losses (Shahwar *et al.,* 2023) Overall, the improved yields with bio-fertilizers and moderate nitrogen levels highlight the potential of integrated nutrient management for sustainable crop productivity.

The maximum 1000-grain weight was achieved with the *Ent+Pan+30 N* treatment, yielding 70.00 g in Year 1 and 66.00 g in Year 2. Similarly, the highest number of spikes per square meter was observed in treatments combining bio-fertilizers and nitrogen fertilizers, particularly at the 30 N nitrogen level, with 499.20 spikes/m² in Year 1 and 501.60 spikes/m² in Year 2. The longest spikes were also recorded in treatments combining bio-fertilizers and nitrogen fertilizers, especially at the 30 N level, measuring 9.50 cm in Year 1 and 9.70 cm in Year 2. However, the highest plant height was observed in the combined treatment of *Enterobacter cloacae* and *Pantoea agglomerans* with 45 N and 30 N, reaching 125.00 cm in Year 1 and 121.67 cm in Year 2. This can be attributed to the fact that increasing nitrogen fertilization promotes vegetative growth, sometimes at the expense of grain weight. These findings align with the results reported by several researchers(Çağlar *et al.,* 2024).

The highest protein levels (11.50% and 11.63% in Year 1 and Year 2, respectively) were achieved when microbes were combined with a nitrogen fertilizer application (Yin *et al.,* 2022). Moreover, the Ent+Pan+30 N treatment yielded the most substantial increases in Chlorophyll b content, reaching levels of 29.240 in Year 1 and 29.223 in Year 2(Dolkhani *et al.,* 2022).

Biological activity in the rhizosphere of barley was significantly influenced by microbial integration and nitrogen input. The highest microbial counts for *Pantoea agglomerans* were achieved by combining microbial treatments with a nitrogen input of 30 kg N/ha, reaching peaks of 20.0×10^6 CFU/g in Year 1 and 22.0×10^6 CFU/g in Year 2. Similarly, for *Enterobacter cloacae*, the integration of microbes with 30 kg N/ha resulted in the highest overall counts, measured at 54×10^5 CFU/g in Year 1 and 55×10^5 MPN/g dry soil in Year 2 (Alotaibi,, *et al.,* 2024 a&b)

5. Conclusion

A field experiment was carried out at the Malawi Agricultural Research Station in the Minya Governorate during the agricultural seasons of 2022/2023 and 2023/2024, focusing on the Egyptian barley cultivar Giza 138. to assess the effectiveness of two bacterial strains, *Enterobacter cloacae* and *Pantoea agglomerans*, alongside three levels of nitrogen fertilizer (15, 30, and 45 kg N/fed^{-1}). The findings indicated that the combined application of *Enterobacter cloacae* and *Pantoea agglomerans* at a nitrogen level of 30 kg N/fed^{-1} significantly enhanced barley cultivar Giza 138 performance which had highest grain yield, 1000-grain weight, longer spike length, a greater number of grains per spike,

and improved chlorophyll a and b content, as well as protein levels. So we concluded that co-inoculation with these plant growth-promoting rhizobacteria (PGPR) strains, coupled with a reduced application of chemical nitrogen fertilizer, can significantly enhance both the yield and quality of barley.

Credit authorship contribution statement
Karima R. Ahmed, and Heba Abdel-motaal Mohamed: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources. Shaima Abozaed, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

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Data availability statement: Data availability in the National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). Other datasets generated are available from the corresponding authors upon request.

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