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Original Article

Isolation and characterization of *Klebsiella oxytoca* from the rhizosphere of *Lotus corniculatus* and its biostimulating features

Isolamento e caracterização de *Klebsiella oxytoca* da rizosfera de *Lotus corniculatus* e suas características bioestimulantes

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Abstract

Significant food resource shortages are occurring worldwide. Plant growth-promoting rhizobacteria (PGPR) represent an ecofriendly and efficient approach for increasing soil fertility and plant productivity. The current study explored biostimulating traits of PGPR from the rhizosphere of *Lotus corniculatus* growing in the Al-Ahsa region. A bacterial isolate (LCK121) was obtained, characterized for phenotypic, and identified by 16S rRNA gene sequencing. In addition, its growth-stimulating effects on barley were investigated. The strain identity was confirmed via comparative analysis of the 16S rDNA sequences with *Klebsiella oxytoca* (99.3% similarity level). LCK121 exhibited multiple plant growth-promoting features, including indole-3-acetic acid (IAA) production (16.34 µg mL^{-1}), 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity (1.35±0.02 µmol α-ketobutyrate mg^{-1} h^{-1}), phosphate solubilization, and nitrogen fixation. Furthermore, in vitro inoculation of barley with LCK121 significantly increased the root and shoot dry weights. The results highlight the potential of LCK121 for developing green fertilizers for sustainable agriculture.

Keywords: plant biostimulation, rhizosphere *Lotus corniculatus*, *Hordeum vulgare*, *Klebsiella*.

Resumo

A escassez significativa de recursos alimentares está ocorrendo em todo o mundo. As rizobactérias promotoras de crescimento de plantas (PGPR) representam uma abordagem ecologicamente correta e eficiente para aumentar a fertilidade do solo e a produtividade das plantas. O presente estudo explorou características bioestimulantes de PGPR da rizosfera de *Lotus corniculatus* crescendo na região de Al-Ahsa. Um isolado bacteriano (LCK121) foi obtido, caracterizado quanto ao fenotípico, e identificado por sequenciamento do gene 16S rRNA. Além disso, seus efeitos estimulantes do crescimento na cevada foram investigados. A identidade da cepa foi confirmada por meio de análise comparativa das sequências de 16S rDNA com *Klebsiella oxytoca* (nível de similaridade de 99,3%). LCK121 exibiu várias características de promoção do crescimento de plantas, incluindo produção de ácido indol-3-acético (IAA) (16,34 µg mL-1), atividade de desaminase de ácido 1-aminociclopropano-1-carboxílico (ACC) (1,35±0,02 µmol α-cetobutirato mg −1 h−1), solubilização de fosfato e fixação de nitrogênio. Além disso, a inoculação in vitro da cevada com LCK121 aumentou significativamente os pesos secos da raiz e da parte aérea. Os resultados destacam o potencial do LCK121 para o desenvolvimento de fertilizantes verdes para a agricultura sustentável.

Palavras-chave: bioestimulação vegetal, Rhizosphere *Lotus corniculatus*, *Hordeum vulgare*, *Klebsiella*.

1. Introduction

Soil is considered a storehouse of extremely diverse microorganisms that establish various relationships within their own communities and with other associated living organisms. Plant roots are in direct contact with the soil and microorganisms, and the rhizosphere soil zone is directly influenced by roots and affects microbial activity (Kundan et al., 2015). Roots synthesize and release a variety of soluble organic substances, such as amino acids, sugars, growth factors, and enzymes, which are collectively known as "root exudates." Such nutrients have a direct impact on the microbial abundance and composition around plant roots. The quantity of microorganisms in rhizospheric soil

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is substantially higher than that in bulk soil (Gouda et al., 2018). Plant growth-promoting rhizobacteria (PGPR) play a paramount role in soil fertility by enhancing nutrient and hormone availability to plants and protecting against plant pathogens and abiotic stressors. Diverse bacterial genera have been documented as PGPR, including *Nocardia alni* from *Alnus glutinosa* (Nouioui et al., 2022), *Devosia rhizoryzae* and *Devosia oryziradicis* from the rhizosphere of rice (Chhetri et al., 2022), *Bacillus megaterium* from the root nodules of *Medicago sativa* (Khalifa and Almalki, 2015), *Enterobacter cloacae* from the roots of nonnodulating *Medicago sativa* (Khalifa et al., 2016), and *Pseudomonas monteilii* (Aldayel and Khalifa, 2021) *Streptomyces laurentii*, *Sinorhizobium* sp., *Bacillus safensis*, *Pseudomonas alcaliphila*, and *Pseudomonas hunanensis* from the rhizosphere of *Ocimum basilicum* (AlAli et al., 2021).

Lotus corniculatus (bird's-foot trefoil) is an important perennial herbaceous leguminous plant that can be used for pasture, silage, and hay. *L. corniculatus* exhibits remarkable adaptability to adverse climatic conditions, including salinity, drought, and heavy metals, and establishes symbiotic relationships with certain root-nodule rhizobial species, such as *Bradyrhizobium* sp., *Ensifer* sp., *Mesorhizobium loti*, and *Rhizobium* sp. (Lorite et al., 2018). This type of association has a significant role in satisfying host nitrogen needs and improving soil fertility and plant growth. In addition to rhizobia, non-rhizobial species have been reported in the root nodules of *L. corniculatus* such as *Paenibacillus kribbensis*, *Pseudomonas grimontii*, and *Bacillus subtilis* (El-Batanony et al., 2020). Additionally, a wide variety of microbial groups exists in the rhizospheric zone of *L. corniculatus*, including *Arthrobacter, Bacillus*, and *Pseudomonas* (Borozan et al., 2019). Beneficial microorganisms could be exploited as an inevitable and ecofriendly route for sustainable agriculture, agroforestry, and land reclamation to cope with the huge shortage of food resources around the globe.

Barley (*Hordeum vulgare* L.) is an important flowering herbaceous plant cultivated as a grain crop in many areas worldwide, including Saudi Arabia. Barley grains are used in human food, livestock feed, and malt beverages, and it is cultivated for soil improvement; however, it has few industrial and medicinal applications (El-Hashash and El-Absy, 2019). Taxonomically, it belongs to the Poaceae (Gramineae) family. The Saudi grain organization reports that 5-7 million tons of barley are imported each year, indicating its economic importance. Therefore, it is of premium importance to enhance the growth of barely locally using ecofriendly approaches. PGPR could be a promising strategy for maintaining barley health and yield.

Despite their economic and ecological importance, relatively few studies (El-Batanony et al., 2020; Kneževi ć et al., 2021; Das and Barik, 2022) have investigated the natural population of PGPR associated with the roots of *L. corniculatus* particularly in the Al-Ahsa region, which presents harsh conditions, such as drought, salinity, and elevated temperature. PGPR associated with the roots of *L. corniculatus* could reveal bacterial species that are previously unreported. Furthermore, they could have potential features that do not exist in other PGPR. Therefore, the current study aimed to explore the biostimulating features of PGPR obtained from the rhizosphere of *L. corniculatus* growing in the Al-Ahsa region. A bacterial isolate designated as LCK121 was obtained, characterized for phenotypic and identified by 16S rRNA gene sequencing. In addition, the growth-stimulating effects of this strain on barley were investigated.

2. Materials and Methods

2.1. Isolation of LCK121 from the rhizosphere of L. corniculatus

Rhizosphere soil samples from *L. corniculatus* plants were collected from Al-Ahsa, Saudi Arabia (25°37′35.60″″ N, 49°54′59.19″ E), on March 28, 2019. One gram of the rhizospheric soil was added to 9 mL physiological saline solution (0.85% NaCl) and serially diluted. Aliquots (0.1 mL) were streaked onto nutrient agar plates, which were then incubated at 30 °C for 48–72 h. Purification was performed by restreaking the obtained isolate onto fresh agar plates to obtain single colonies. Pure colonies were preserved in 15% glycerol at -80 °C. The isolates were maintained in agar slant test tubes at 4 °C for routine use and subcultured on fresh slants every 4 weeks. LCK121 was grown on nutrient agar plates and incubated at 30 °C for 48 h.

2.2. Colony characteristics and Gram staining for LCK121

LCK121 was grown on nutrient agar plates and incubated at 30 °C for 48 h. Subsequently, the color, margin, elevation, diameter, and shape of the developed colonies were determined. The reaction to Gram staining was also checked for LCK121 cells, as previously described (Claus, 1992).

2.3. Determination of biochemical traits using API-20 kits

To determine the biochemical traits of LCK121, the efficient micro-test strip API-20E, (bioMérieux, Marcy l'Etoile, France) was used. LCK12 was tested for its ability to consume various metabolites containing API-20E based on the manufacturer's recommendations. Results were reported after 24–48 h of incubation at 30 °C.

2.4. Plant growth-promoting activities

LCK12 was grown in YM medium supplemented with tryptophan (2.4×10^{-3} M) for 48 h at 30 °C and 120 rpm. IAA production was assessed as described by Glickmann and Dessaux (1995) with slight modifications. Briefly, LCK12 was grown in nutrient broth amended with tryptophan (0.2 mg mL^{-1}) and incubated in a shaking incubator for 72 h at 30 °C. The cells were collected by centrifugation at 10,000 × *g* for 10 min. Next, the supernatant (1 mL) was added to Salkowski's reagent (2 mL) with the following composition: concentrated sulfuric acid (150 mL), distilled water (250 mL), and 0.5 M $FeCl_3 \cdot 6H_2O$ (7.5 mL). The tubes were kept in the dark at 30 °C for 25 min, and the IAA content was determined using a spectrophotometer (OD_{530} nm) (Provider).

The 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity of LCK121 was determined following a previously described method (Penrose and Glick, 2003).

Briefly, LCK121 was grown in tryptic soy broth medium at 30 °C for 16 h, and then the cells were collected by centrifugation at 10,000 × *g* at 4 °C for 20 min. The cell pellets were washed with 5 mL of Dworkin–Foster salts minimal medium. Next, the cells were added to 7.5 mL of the same medium with 45 µL of 0.5 M ACC (Sigma, USA). The tubes were placed in a shaking incubator (150 rpm min^{-1}) at 30 °C for 24 h, and then the cells were further washed in 5 mL of 0.1 M Tris HCl (pH 7.6) and resuspended in 600 µL of 0.1 M Tris HCl (pH 8.5) in a 1.5-mL microcentrifuge tube. Thirty microliters of toluene (v/v) was added to the bacterial suspension and mixed vigorously. Then, 5 µL of ACC was added to 50 µL of toluenized cells and incubated at 30 °C for 30 min. Next, 500 µL of 0.56 M HCl was added to the mixture, mixed well, and centrifuged for 10 min at 10,000 × *g* at 25 °C to remove cell debris. Briefly, 500 µL of 0.56 M HCl and 150 µL of DNF solution (0.1 g of 2,4-dinitrophenylhydrazine in 100 mL of 2 M HCl) were added to 500 µL of supernatant in a glass test tube. The tubes were then incubated at 30 °C for 30 min. Then, 1 mL of 2 M NaOH was added to the sample and the absorbance at 540 nm was determined. The AAC activity of LC121 was determined from a standard curve using α-ketobutyrate.

LCK121 was tested for phosphate solubilization on Pikovskaya agar medium, which included tricalcium phosphate ($Ca_3(PO4)_2$) as the P source (Pikovskaya, 1948). Incubation of the inoculated plates was performed at 30 °C for 5 days. The appearance of clear halo zones around LCK121 colonies was considered a positive result.

2.5. 16S rRNA gene sequencing and phylogenetic analyses

LCK121 was identified using 16S rDNA gene sequencing analysis. Genomic DNA extraction, primers, PCR conditions, and sequencing were performed as previously described (Khalifa and Bekhet, 2018). The phylogenetic relation was inferred using the neighbor-joining method (Saitou and Nei, 1987). The optimal tree was done with a sum of branch length = 0.38845289. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al., 2004) and are expressed in units of the number of base substitutions per site. This analysis involved eight nucleotide sequences, and the included codon positions were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted using MEGA7 (Kumar et al., 2016). The 16S rRNA gene sequence of LCK121 has been deposited in the NCBI GenBank under accession number MN301350 (Table 1).

2.6. Cross-inoculation experiment

To assess the ability of LCK121 to promote plant growth, in vitro cross-inoculation of barley grains was carried out as described previously by Khalifa and Almalki (2015). Briefly, surface sterilization of healthy barley grains was carried out by immersing them in ethanol (70%) for 10 s, followed by H_2O_2 (3%) for 3 min. The grains were then repeatedly washed with sterilized distilled water to ensure that no traces of H_2O_2 were present. Under aseptic conditions, the grains were inoculated with an actively growing 5 × 10^6 CFU mL^{-1} LCK121 suspension for 24 h. Individually germinated grains were carefully transferred to sterilized petri dishes and incubated at 25 °C in a growth chamber with a 14 h light/10 h dark cycle. After seven days, the growth parameters (root and shoot dry weights and lengths) were recorded. Appropriate control grains were used, in which sterilized distilled water (5 mL) was added instead of the bacterial suspension. The experiment was repeated three times.

Table 1. Characterization of LCK121.

Feature	LCK121
Colony shape	Circular
Color	White
Colony diameter	2–3 mm
Colony elevation	Raised
Colony margin	Entire
Gram reaction	Gram-negative rods
ONPG *(beta-galactosidase)	+
Arginine (arginine dihydrolase)	+
Lysine (lysine decarboxylase)	+
Ornithine (ornithine decarboxylase)	+
Citrate (citrate utilization)	-
Na thiosulfate (H_2S production)	+
Urea (urea hydrolysis)	-
Tryptophan (deaminase)	+
Indole (indole production)	+
Na pyruvate (acetoin production)	+
Charcoal gelatin (gelatinase)	+
Glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin, or arabinose (fermentation/oxidation)	+
Nitrogen fixation	+
Solubilization of phosphate	+
IAA production (µg ml^{-1})	16.34 ± 0.44 µg mL^{-1}
ACCD activity (nmol of α-KB mg^{-1} Pr h^{-1})	396.20 ± 21.18
Closest strain	*Klebsiella oxytoca*
Identity (%)	99.3
Accession number	MW301350

*Ortho-nitrophenyl-β-galactoside.
(+) indicates positive results, and (-) indicates negative results.

2.7. Statistical analysis

Data are represented by the mean of three replicates ± standard deviation (SD). The results were subjected to one-way analysis of variance (ANOVA), and mean comparisons were performed by post hoc test using Microsoft Excel. Differences between means were considered statistically significant at $p < 0.05$.

3. Results

Out of the 12 morphologically distinct isolates, one bacterial isolate, LCK121, was selected for further phenotypic and genotypic characterization. The reason was because LCK121 displayed potential enhancement of barely germination during the initial screening. The phenotypic characteristics of LCK121 are presented in Table 1. LCK121 formed circular smooth colonies with entire margins (Figure 1). The cells were gram-negative rods without endospores (Table 1). These findings are identical to those reported for *Klebsiella oxytoca* (Miguel et al., 2020; Passet and Brisse, 2018).

LCK121 utilized 90% (18 out of 20) of the testers found in the API-20E strips (Table 1). LCK121 effectively metabolized all carbohydrates and amino acids tested, and growth was not observed with urea and citrate. The biochemical profile of LCK121 indicated its metabolic versatility.

LCK121 produced the plant growth-promoting hormone IAA (16.34 µg mL^{-1}) when the growth medium was supplemented with the amino acid tryptophan (1 mg mL^{-1}) (Table 1). LCK121 was qualitatively tested to determine its ability to solubilize inorganic phosphate based on a previously described procedure (Pikovskaya, 1948), and the results are shown in Table 1. A halo zone developed around the colonies of LCK121 grown on Pikovskaya agar medium, which was amended with tricalcium phosphate . This observation clearly indicates that LCK121 has a remarkable phosphate solubility capability.

Additionally, LCK121 exhibited an obvious ability to grow on Jensen's medium (Table 1), which lacks any added nitrogen and is used to detect bacteria with nitrogen-fixing capabilities (Sulistiyani and Meliah, 2017). PGPR is able to fix nitrogen and thus can adequately fulfill the plant requirements of nitrogen, thereby improving the fertility of soil and yield of plants.

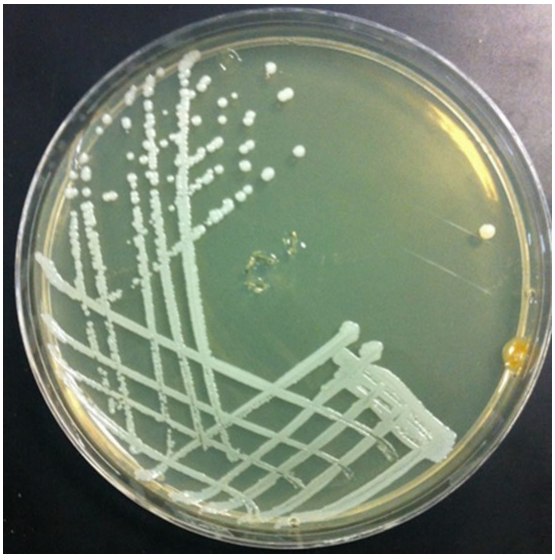

Figure 1. Pure colonies of the LCK121 strain from the rhizosphere of *L. corniculatus*.

The ACC deaminase activity of LCK121 is shown in Table 1. LCK121 exhibited relatively high ACC deaminase activity (1.35±0.02 µmol α-ketobutyrate mg^{-1} h^{-1}).

An analysis of the 16S rRNA gene sequencing results identified LCK121 (MW301350) as *Klebsiella oxytoca*, with 99.3% sequence homology (Table 1). Furthermore, LCK121 was clearly clustered with *K. oxytoca (*accession number AB004754) (Figure 2) based on the phylogenetic tree that was inferred after applying the neighbor-joining method to the 16S rRNA gene sequences of LCK121 and similar related sequences of reference strains (Figure 2). LCK121 was assembled with members of *Enterobacteriaceae*, thus confirming its taxonomic affiliation.

In vitro inoculation of barley plants with LCK121 significantly improved the growth parameters. Compared with the untreated plants, significant increases were observed in the root and stem lengths of the treated barley, which increased from 2.6 cm to 7.2 cm and from 2.2 cm to 6.3 cm, respectively, (Figures 3-4). LCK121 significantly improved the dry weights of the roots and shoots, which increased from 21.7 mg to 48.6 mg and from 20.6 mg to 57.3 mg, respectively (Figure 5).

4. Discussion

The aim of study was to explore the biostimulating activities of PGPR obtained from the rhizosphere of *L. corniculatus*. Owing to its positive biostimulating activities, LCK121 was chosen for subsequent in-depth characterization. The morphological characteristics of LCK121 presented in Table 1 are identical to those of *K. oxytoca* (Miguel et al., 2020; Passet and Brisse, 2018).

API-20E is a fast, cost-effective, and efficient tool for biochemical screening of various bacterial species. API-20E contained 20 different dried chemical substrates in microtubes, designed to target certain biochemical traits/enzymes. The biochemical profile of LCK121 was in general agreement with that reported for *Klebsiella* spp. (Brisse et al., 2006; Messaoudi et al., 2009; Budiarso et al., 2021). LCK121 can produce several important molecules with beneficial biological functions, such as ammonia, acetoin, and H_2S. Ammonia can serve as a nitrogen source

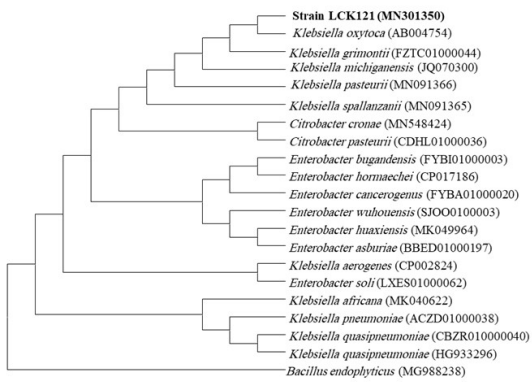

Figure 2. Evolutionary relationships of LCK121 and other related bacterial species as inferred using the neighbor-joining method.

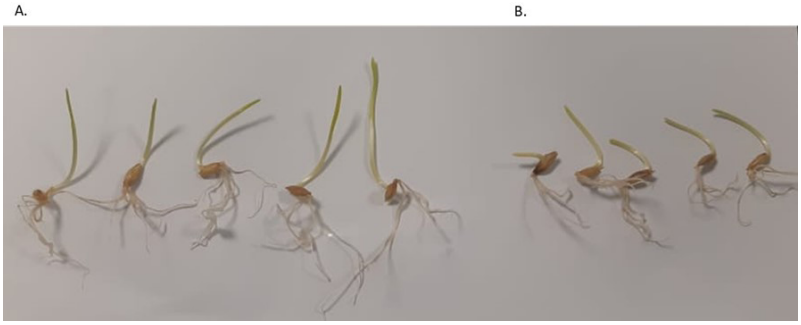

Figure 3. Enhancement of the root and shoot lengths of barley when inoculated with LCK121, A. LCK121-treated ones; B. control seedlings.

for plant growth, and ammonia production by LCK121 is more likely to be mediated by arginine dihydrolase. Acetoin production is mediated via acetolactate synthase and α-acetolactate decarboxylase and is considered an indirect mechanism to enhance plant growth. The physiological role of acetoin as an elicitor relies on its ability to induce plant resistance to phytopathogens (Meng et al., 2022). Interestingly, LCK121 produced H_2S from sodium thiosulfate (Table 1). H_2S is a multifunctional gas that regulates many vital processes within plant cells, including alleviating the harmful effects of biotic (plant microbial pathogens) and abiotic (e.g., drought, salinity, and heat) stressors and regulating seed germination and root development in plants. Detailed information has recently been provided on the roles of H_2S (Liu et al., 2021). The findings on LCK121 highlight its metabolic versatility and potential role in promoting plant growth via versatile features.

LCK121 produced a comparable amount of IAA (16.34 µg mL^{-1}) relative to that estimated by Sachdev et al. (2009), who showed that *Klebsiella pneumoniae* from the rhizosphere of wheat (*Triticum aestivum*) produced IAA (27 µg mL^{-1}), although the values were lower than that reported by Kang et al. (2020), who highlighted that *K. pneumoniae* YNA12 isolated from seeds of *Oenothera biennis* produced 120 µg mL^{-1} IAA. The variability in IAA quantities could be attributed to the bacterial strain tested, growth conditions, and measurement parameters. IAA is a common type of auxin that enhances cell elongation, cell division, root hair emergence and secondary root growth, thus allowing plants to obtain adequate water and nutrients from the soil. Additionally, IAA helps in loosening plant cell walls, thereby increasing the quantities of root exudates that favor PGPR colonization of roots (Tzipilevich et al., 2021). Furthermore, IAA increases ACC production mediated by ACC synthetase (Glick et al., 1998) and LCK121 enhances plant growth via IAA production

Phosphorus (P) is an essential element required for normal plant growth. Although the P content in soil may be relatively high, it may be in a form that cannot be accessed by plants. PGPR solubilize P compounds, which increases plant growth (Yarzábal et al., 2018). LCK121 exhibited P solubilization in our experiments, which is consistent with the findings reported for *Klebsiella* sp. (Gupta et al., 2018), *Klebsiella variicola* (Yang and Yang, 2020), and *K. oxytoca* (Miguel et al., 2020). Many other PGPR species have been reported to solubilize phosphate, including *Bacillus* spp. (Khalifa and Almalki, 2015; Khalifa et al., 2020) and *Enterobacter cloace* (Khalifa and Almalki, 2015). Phosphate solubilization by LCK121 may be mediated via organic acid production and phytase secretion (Sorty et al., 2018). This PGP trait facilitates the absorption of P by the plant roots to sustain growth and productivity.

Additionally, the ability of LCK121 to grow on Jensen's medium, which is nitrogen-free, might indicate the

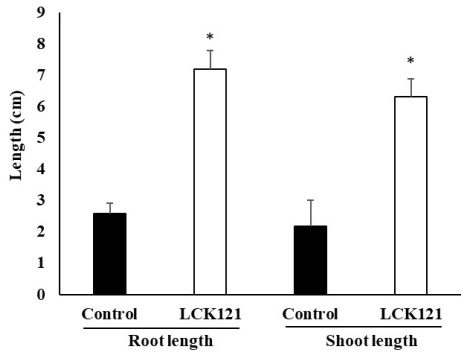

Figure 4. Enhancement of the root and shoot lengths of barley when inoculated with LCK121. *Indicates a significant effect (*P* value < 0.05).

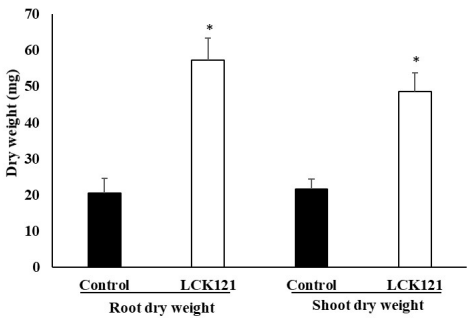

Figure 5. Enhancement of the root and shoot dry weight of barley when inoculated with LCK121. *Indicates a significant effect (*P* value < 0.05).

existence of nitrogen fixation features (Sulistiyani and Meliah, 2017). The nitrogen fixation process is mediated by a unique enzyme complex called nitrogenase. This result is in agreement with many previous studies that unequivocally confirmed the nitrogen-fixing capability of *Klebsiella* spp. (Gupta et al., 2021; Sharma et al., 2021; Sheoran et al., 2021). PGPR can fix nitrogen and thus can adequately fulfill the plant requirements of nitrogen, thereby improving the fertility of soils and yields of plants.

LCK121 displayed relatively higher ACC deaminase activity (1.35±0.02 µmol α-ketobutyrate mg^{-1} h^{-1}) than that reported for *Klebsiella* sp. from *Acacia farnesiana* (1.2 µmol α-ketobutyrate mg h^{-1}) (Carlos et al., 2016) but lower activity than that reported for *Klebsiella* sp. from *Oryza sativa* (Mukherjee et al., 2020) (0.1±0.02 µmol α-ketobutyrate mg h^{-1}); thus, wide differences in ACC deaminase activities exist among PGPR. However, PGP has been reported to be achieved with very low activity (20 nmol α-ketobutyrate mg^{-1} h^{-1}) by ACC-deaminase-producing bacteria (Penrose and Glick, 2003).

LCK121 was identified as *K. oxytoca* at 99.3% identity level based on 16S rRNA gene sequencing and confirmed by grouping with clustered *K. oxytoca* based on phylogenetic analyses. LCK121 was assembled with members of *Enterobacteriaceae*, thus confirming its taxonomic affiliation. These findings highlight that comparative analysis of the 16S rRNA gene is an efficient taxonomic approach not only for identifying bacterial strains but also for inferring phylogenetic relationships.

In vitro inoculation of barley plants with LCK121 significantly improved the growth parameters (barley root and stem lengths and dry weights of roots and shoots). These findings are in accordance with those of previous studies, which showed that *K. oxytoca* enhances growth parameters in mung bean seedlings (Buddhi et al., 2014) and wheat (Ebrahimi et al., 2022). The enhancing effects of LCK121 on barley may be explained by the multifunctional PGP activities of this bacterium. Such beneficial effects can be mediated by direct, indirect, or synergistic mechanisms. For example, LCK12 could provide growing seedlings with nitrogen via nitrogen fixation and P via phosphate solubilization; moreover, it protects seedlings against pathogens via the production of acetoin. However, further field trials are required to confirm the plant growth biostimulating activities of LCK121.

5. Conclusions

Collectively, the findings described herein highlight that the rhizosphere of the wild legume *L. corniculatus* harbors *Klebsiella oxytoca that* has multiple features that promote the growth of barley. Thus, this bacterium is a promising candidate for developing green fertilizers for sustainable agriculture in the future.

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