# Abstract

Water scarcity is a major challenge for agriculture, particularly in water-scarce regions such as Saudi Arabia. This study evaluated the effects of chlorinated treated wastewater (TWW), alone and in combination with plant growth-promoting bacteria (PGPB), on the growth of red lettuce (*Lactuca sativa*) under soil-based and closed hydroponic systems. Two locally isolated bacterial strains, *Bacillus pumilus* (RT50) and *Pseudomonas reinekei* (RH30), were used as biological amendments. Lettuce plants were subjected to five treatments: tap water, TWW alone, TWW + RT50, TWW + RH30, and TWW + RT50 + RH30. Root, shoot, and total growth were measured, and treatment effects were evaluated using one-way ANOVA. In the soil system, TWW treatments generally improved plant growth compared with the tap-water control, with TWW alone producing the highest mean shoot (43.82) and total growth (49.89), and significant differences were observed among treatments. In the hydroponic system, RT50 produced the highest mean shoot growth (14.73) and total biomass (16.22); however, differences in shoot growth among treatments were not statistically significant ($F = 1.083$, $p = 0.386$). Overall, the findings suggest that treated wastewater has potential as an alternative irrigation source for lettuce and may reduce dependence on freshwater resources, while the additional effects of PGPB require further investigation using larger sample sizes and additional experiments. The use of TWW as an irrigation source and the potential benefits of PGPB are supported by previous studies on wastewater irrigation and plant growth-promoting bacteria [6,7,12]


# Introduction

Water is a currency of life. Plenty of human activities, such as agriculture, industrial development, and healthy ecosystems, are all highly dependent on the quality and quantity of usable water [1]. Furthermore, water scarcity is demand is higher than the available clean water, water scarcity occurs [3,4]. Water scarcity is a major ongoing catastrophe because it threatens all human activities, including human life itself [1,2]. Moreover, the agricultural sector is described as a highly water-demanding sector. Globally, agricultural water use accounts for approximately 70% of total freshwater use [5]. Furthermore, under certain circumstances, Saudi Arabia and the other countries of the Gulf Cooperation Council (GCC) are considered water-scarce nations according to the United Nations [3]. Consequently, the water crisis in these countries will obviously affect food security due to insufficient water supplies to support the agricultural sector. As a result, water-scarce nations must look for sustainable and applicable solutions to mitigate the effects of water shortages on their agricultural sectors.

Therefore, municipal treated wastewater is a renewable water source. It is characterized by a high nutrient content, which can eventually lead to increased harvesting yields, therefore reducing dependence on chemical fertilizers. Moreover, using treated wastewater (TWW) will mitigate the demand for potable water [6]. All these benefits, unfortunately, come with different consequences, such as soil salinity and chemical and microbial contamination of both the soil and crops [6]. Therefore, appropriate management is needed to alleviate the negative outcomes of using treated wastewater as an irrigation water source [6].

Plant Growth-Promoting Bacteria (PGPB) are a group of bacteria that have various beneficial effects on plant growth through different mechanisms, such as the production of Indole-3-acetic acid (IAA), which is a phytohormone that plants naturally secrete to promote root growth [7,8,11,12]. They also provide some insolubilized nutrients, such as nitrogen, phosphorus, and iron, to the plant [7,9,11,12]. PGPB can also play a role in reducing the adverse effects of recycled water by increasing antioxidant activity in the plant or by mitigating heavy-metal toxicity through various mechanisms, such as decreasing heavy-metal availability [10–12].

In this study, red lettuce (*Lactuca sativa*) belonging to the Asteraceae family was used as the experimental plant because of its high economic crop value [13,14], ease of cultivation, and suitability for use in both soil and hydroponic systems. Mainly, lettuce takes 65 to 135 days to harvest before flowering [14], which is considered a short period of time [14]. Moreover, two agricultural systems were used in this experiment to cultivate red lettuce: a regular soil system and a closed hydroponic system with air pumps. Both systems were maintained inside a greenhouse at 24°C and 50% humidity.

Furthermore, chlorinated treated wastewater from the KAUST Aerobic Membrane Plant was used as the water source in both soil and hydroponic systems, combined with PGPB. The PGPB used were *Bacillus pumilus* (RT50), which is a rod-shaped, Gram-positive, spore-forming bacterium [17] that was isolated from the **rhizosphere soil** of olive trees cultivated under reduced-water irrigation in the Al Jouf desert farm. *B. pumilus* has the potential to promote plant growth and can also be used as a biocontrol agent against fungi [16,19]. The other bacterium is *Pseudomonas reinekei* (RH30), which is a rod-shaped, Gram-negative bacterium that was isolated from the internal root tissue of the same mentioned olive tree, making it an endophytic bacterium [18]

Therefore, this study aimed to evaluate the effects of treated wastewater irrigation, alone and in combination with locally isolated PGPB, on the growth and performance of red lettuce cultivated under soil-based and closed hydroponic systems.

# Materials & Methods

### Lettuce Preparation

Seeds were sown in a potting at and perlite mixture in black seedling trays. Th mixture was irrigated with tap water before and after seed sowing to ensure adequate soil moisture for germination. The seeded trays were then placed in a growth chamber maintained at **24°C**, **50% relative humidity**, and a **8 h light/16 h dark photoperiod**. The seeds were covered for the first three days after sowing. One week after germination, healthy and uniform seedlings were transplanted into the prepared pots and Hydroponic system in KAUST **greenhouse** for further growth.

### Treatments

Five treatments were evaluated in both soil (eight replicates per treatment) and hydroponic (six replicates per treatment) systems. The treatments included: tap water control, aerobic membrane-treated wastewater (TWW) alone, TWW + RH50, TWW + RT30, and TWW + RH50 + RT30.

### Soil System Preparation and Irrigation

**Eight** black plastic pots (5 L) with side drainage holes were prepared for each respective treatment, by placing a layer of crepe bandage-like cloth at the bottom of each pot to prevent soil loss while allowing excess water to drain freely. The pots were then filled with potting soil, gently shaken to distribute the soil evenly, and lightly compressed

to eliminate large air pockets, which can increase drainage and reduce water retention. After seedling transplanting, the pots were placed in a KAUST greenhouse growth chamber maintained at **22–24°C** and **50% relative humidity**. Furthermore. After seedling plantation into the prepared pots, initial irrigation aims to reach the point of soil capacity which was about **250ml to 350ml** per pot. The further irrigation was nearly twice a week with 100ml of receptive treatment. Moreover after 21 days the plant get very stressed due to nitrogen deficiency, so urea fertilizer which is highly concentrated nitrogen fertilizer [20], was added to the soil by mix one gram of it with one liter of tap water and gave to each plant a 15 ml of the mentioned fertilizer solution.

**Hydroponic System Preparation and Feeding**

A plastic container was used as a hydroponic growth system. A white cork board was covered with aluminum foil; to prevent mold formation, and six holes were perforated to accommodate the net cups. The prepared cork board was then placed on top of the container. Lettuce seedlings were removed from the potting soil, and their roots were wrapped with rockwool substrate before being inserted into the net cups. Each container was filled with 4 L of the respective water treatment solution supplemented with Murashige and Skoog (MS) nutrient medium at a concentration of **3.5 g $L^{-1}$**. Finally, an air pump connected to a transparent tube to continuously aerate the nutrient solution, ensuring an adequate oxygen supply to the root system. Moreover after 2 weeks the old water of each respective water treatment were changed, MS medium was added as mentioned above.

**Bacterial preparation**

Two bacterial strains*, B. pumilus and P. reinekei***,** were first retrieved from an **−82°C glycerol stock**. Using a sterile inoculating loop, a small amount of each bacterial culture was streaked onto **Tryptic Soy Agar (TSA)** plates prepared by dissolving **30 g $L^{-1}$ Tryptic Soy Broth (TSB)** and **15 g $L^{-1}$ agar** in distilled water. The inoculated plates were incubated at **37°C for 24 h** to obtain isolated colonies. Following incubation, a single well-isolated colony was transferred into **Tryptic Soy Broth (TSB)** using a sterile inoculating loop. The broth cultures were then incubated in a **shaking incubator at 37°C for 24 h** to obtain actively growing bacterial cultures for subsequent experiments.

**Bacterial Inoculation**

The bacterial cell concentration was determined using a hemocytometer (Thoma counting chamber) after preparing a 1:100 dilution. For RT50, the average cell count was 2.6, while RH30 had an average count of 1.5. Using the hemocytometer calculation, the estimated concentrations were $10.4 \times 10^8$ cells/mL for RT50 and $6 \times 10^8$ cells/mL for RH30. The target concentration was $10^9$ cells per plant, with each plant receiving 5 mL of bacterial suspension. Based on the calculated concentrations, 1 mL of RT50 suspension and approximately 1.66 mL of RH30 suspension were required per plant. To prepare the inoculum for 10 plants, 10 mL of RT50 and 16.6 mL of RH30 were needed. The bacterial suspensions were then centrifuged, and the resulting pellets were resuspended in 50 mL of physiological solution. For the combined RT50 + RH30 treatment, the two bacterial suspensions were mixed in a 1:1 ratio.

**Harvesting**

The Experiment was conducted for hydroponic system for 28 days from 20 July to 17 Aug. and for soil system the take 29 days form 19 July to 18 Aug. The plant for both the soil and hydroponic clean carefully to remove all the attached substance, then the root and shoot weighted separately.

**Water Analysis**

To evaluate the differences among the treatments, the following parameters were measured after one week: electrical conductivity, total dissolved solids, and salt concentration were measured using a conductivity meter, while pH was measured using a pH meter.

**Statistical design**

The experiment was conducted following a Completely Randomized Design (CRD) comprising five distinct irrigation treatments: tap water (control), treated wastewater (TWW), TWW inoculated with bacteria (RH50), TWW inoculated with bacteria (RH30), and TWW co-inoculated with a consortium of bacteria (RH50 + RH3). The soil treatment has a **8** replicate and the Hydroponic contains **6** replicates.

All collected data for the measured plant parameters were subjected to a one-way Analysis of Variance (ANOVA) using to evaluate the main effects of the water and bacterial treatments on the lettuce. Statistical analyses were performed using the Data Analysis Toolpak in Microsoft Excel. Differences among treatment means were considered statistically significant at a probability level of $p < 0.05$.

# Result

**Soil system Analysis**

The application of different irrigation treatments noticeably impacted the root, shoot, and total growth parameters of the lettuce (Table 1). Overall, plants irrigated with tap water (control) exhibited the lowest performance across all measured parameters, yielding average root, shoot, and total values of 3.92, 28.57, and 32.48, respectively. In contrast, the use of treated wastewater (TWW), both with and without bacterial inoculation, consistently enhanced plant growth.

The uninoculated TWW treatment **("Ae only")** produced the highest average shoot (43.82) and total (49.89) measurements. While bacterial inoculation altered the growth distribution—specifically, the TWW inoculated with

bacteria 2 (B2) produced the highest average root measurement (7.03)—all TWW and bacteria combinations outperformed the tap water control.

A one-way ANOVA was performed to compare shoot growth among the five treatment groups (Table 2) (Tap, TWW alone, TWW+RT50, TWW+RH30, and TWW+RH50+RT30). The results showed a statistically significant difference in shoot values among the groups, $F(4, 35) = 12.20$, $p = 2.59 \times 10^{-6}$, which is far below the 0.05 significance threshold, and the calculated F-value (12.20) greatly exceeds the critical F-value (2.64). This indicates that the type of irrigation/treatment had a significant effect on shoot growth. Looking at the group means, plants irrigated with Tap water alone had the lowest shoot average (28.57), while all TWW-based treatments produced markedly higher shoot growth, with TWW alone giving the highest mean (43.82), followed by TWW+RT50 (40.70), TWW+RH30 (39.38), and TWW+RH50+RT30 (37.56). This suggests that treated wastewater (TWW), whether applied alone or combined with the RT/RH amendments, enhanced shoot growth compared to tap water, though the differences among the TWW-based treatments themselves appear smaller.

A one-way analysis of variance (ANOVA) was conducted to examine the effect of treatment on the measured parameter across four groups. The analysis revealed a statistically significant difference among the treatment groups, $F(3, 28) = 225.93$, $p = 1.02 \times 10^{-19}$, which is far below the conventional significance threshold of 0.05. The between-groups sum of squares (SS = 6345.31) was substantially larger than the within-groups sum of squares (SS = 262.13), indicating that the variation among treatments accounted for the vast majority of the total variability (SS = 6607.43), while variation within replicates of the same treatment was comparatively minimal. Furthermore, the calculated F-value (225.93) greatly exceeded the critical F-value (2.95) at the 0.05 significance level, confirming that the observed differences among groups are highly unlikely to be due to random chance.

**Hydroponic System Analysis**

**Root growth** (Table 3 ) was lowest in the Tap water control (1.08), improved with TWW alone (1.45) and RH30 (1.36), and was highest in RT50+RH30 (1.31) and RT50 (1.49) — indicating that RT50, whether alone or combined with RH30, tended to promote slightly more root development than tap water, though the differences among the amended treatments are relatively small.

**Shoot growth** followed a similar pattern, with Tap water producing the lowest average shoot value (8.71), while RT50 (14.73) and RH30 (12.88) produced markedly higher shoot growth, and TWW alone (13.16) and RT50+RH30 (13.33) fell in an intermediate-to-high range. This suggests that all treated wastewater–based amendments enhanced shoot development relative to the tap water control.

**Total biomass** (Root + Shoot) mirrors the shoot trend, since shoot values dominate the total: Tap gave the lowest total (9.79), while RT50 (16.22), RT50+RH30 (14.64), RH30 (14.23), and TWW alone (14.61) all showed clear improvements over the control, with RT50 alone producing the highest overall biomass among the treatments.

Overall, these descriptive results suggest that treated wastewater and its amendments (RT50, RH30, and their combination) had a positive effect on both root and shoot growth compared to tap water irrigation, with RT50 alone appearing to be the most effective treatment for promoting total plant growth in this dataset. To confirm whether these differences are statistically significant, a one-way ANOVA (as performed for the previous datasets) should be run on this data, followed by a post-hoc test to compare specific treatment pairs.

The one-way ANOVA was conducted (Table 4) to determine whether there were significant differences in the mean values among the five groups: **Tap shoot, TWW Alone, RT50, RH30, and RT50 + RH30**.

The results showed that the mean values varied among the groups, ranging from **8.71 in the Tap shoot group** to **14.73 in the RT50 group**. However, the ANOVA indicated that these differences were **not statistically significant**.

The calculated **F-value was 1.083**, which was lower than the critical F-value of **2.759**. In addition, the **P-value was 0.386**, which is greater than the commonly used significance level of **0.05**. Therefore, the null hypothesis is **not rejected**.

| Tap | Root | Shoot | Totoal | TWW alone | Root | Shoot | Totoal | TWW+RT50 | Root | Shoot | Total | TWW+RH30 | Root | Shoot | Total | TWW+RH50+RT30 | Root | Shoot | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 4 | 27.5 | 31.5 | 1 | 5.11 | 41.19 | 46.3 | 1 | 6.24 | 40.12 | 46.36 | 1 | 7.74 | 43.83 | 51.57 | 1 | 5.58 | 43.05 | 48.63 |
| 2 | 5.55 | 21.46 | 27.01 | 2 | 6.33 | 50.52 | 56.85 | 2 | 6.07 | 41.46 | 47.53 | 2 | 7.16 | 39.38 | 46.54 | 2 | 5.57 | 38.44 | 44.01 |
| 3 | 7.65 | 34.59 | 42.24 | 3 | 7.12 | 36.12 | 43.24 | 3 | 5.36 | 42.36 | 47.72 | 3 | 6.16 | 40.51 | 46.67 | 3 | 7.71 | 43.85 | 51.56 |
| 4 | 2.74 | 21.18 | 23.92 | 4 | 6.28 | 50.72 | 57 | 4 | 5.61 | 39.08 | 44.69 | 4 | 6.66 | 36.47 | 43.13 | 4 | 9.06 | 39.11 | 48.17 |
| 5 | 4.55 | 32.25 | 36.8 | 5 | 4.75 | 40.32 | 45.07 | 5 | 4.33 | 39.78 | 44.11 | 5 | 8.5 | 42.52 | 51.02 | 5 | 6.3 | 29.51 | 35.81 |
| 6 | 1.49 | 24.8 | 26.29 | 6 | 8.1 | 44.2 | 52.3 | 6 | 4.53 | 44.34 | 48.87 | 6 | 8.92 | 35.82 | 44.74 | 6 | 8.71 | 41.02 | 49.73 |
| 7 | 2.45 | 27.36 | 29.81 | 7 | 5.78 | 44.57 | 50.35 | 7 | 6.75 | 39.07 | 45.82 | 7 | 4.86 | 38.95 | 43.81 | 7 | 3.48 | 29.5 | 32.98 |
| 8 | 2.9 | 39.39 | 42.29 | 8 | 5.08 | 42.89 | 47.97 | 8 | 3.45 | 39.36 | 42.81 | 8 | 6.26 | 37.59 | 43.85 | 8 | 6.77 | 36.03 | 42.8 |
| Avarge | 3.91625 | 28.56625 | 32.4825 | Avarage | 6.06875 | 43.81625 | 49.885 | Avarage | 5.2925 | 40.69625 | 45.98875 | Avarage | 7.0325 | 39.38375 | 46.41625 | Avarage | 6.6475 | 37.56375 | 44.21125 |

Table1. **Table 1. Raw measurements and calculated averages of lettuce growth parameters across five irrigation treatments.** Data represents root, shoot, and total growth metrics recorded for eight replicates per treatment. The experimental groups evaluate the efficacy of different water sources and biological amendments under the Microbes Promoting Plant Project, including tap water control, treated wastewater alone (Ae only), and treated wastewater inoculated with individual bacterial strains (RT50, RH30) or a bacterial consortium (RT50+RH30).

| *Source of Variation* | *SS* | *df* | *MS* | *F* | *P-value* | *F crit* |
|---|---|---|---|---|---|---|
| Between Groups | 1057.593 | 4 | 264.3982 | 12.19804 | 2.59E-06 | 2.641465 |
| Within Groups | 758.6413 | 35 | 21.67547 | | | |
| | | | | | | |
| Total | 1816.234 | 39 | | | | |

*Table 2: This table presents a one-way ANOVA comparing shoot growth across five treatment groups (Tap, TWW alone, TWW+RT50, TWW+RH30, and TWW+RH50+RT30), each with 8 replicates. The Summary section reports the count, sum, mean, and variance of shoot values for each group, while the ANOVA section partitions the total variation (SS = 1816.23, df = 39) into Between Groups (SS = 1057.59, df = 4) and Within Groups (SS = 758.64, df = 35) components. The resulting F-value (12.20) far exceeds the critical F-value (2.64), and the P-value ($2.59 \times 10^{-6}$) is well below 0.05, indicating a statistically significant difference in shoot growth among the treatment groups.*

| Tap | Root | Shoot | Totoal | TWW alone | Root | Shoot | Totoal | RT50 | Root | Shoot | Total | RH30 | Root | Shoot | Total | RT50+RH30 | Root | Shoot | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.89 | 7.85 | 8.74 | 1 | 1.93 | 17.05 | 18.98 | 1 | 1.6 | 15.95 | 17.55 | 1 | 2.99 | 27.54 | 30.53 | 1 | 2.4 | 21.52 | 23.92 |
| 2 | 1.01 | 8.84 | 9.85 | 2 | 0.67 | 7.19 | 7.86 | 2 | 1.12 | 13.2 | 14.32 | 2 | 1.03 | 8.81 | 9.84 | 2 | 2.25 | 19.39 | 21.64 |
| 3 | 0.5 | 5.86 | 6.36 | 3 | 1.6 | 13.68 | 15.28 | 3 | 1.36 | 16.73 | 18.09 | 3 | 2.08 | 17.27 | 19.35 | 3 | 0.39 | 7.81 | 8.2 |
| 4 | 1.75 | 10.83 | 12.58 | 4 | 2.19 | 16.71 | 18.9 | 4 | 3.25 | 22.55 | 25.8 | 4 | 0.91 | 10.33 | 11.24 | 4 | 0.62 | 8.52 | 9.14 |
| 5 | 1.4 | 11.26 | 12.66 | 5 | 1.25 | 12.71 | 13.96 | 5 | 0.82 | 10.74 | 11.56 | 5 | 0.56 | 6.93 | 7.49 | 5 | 1.39 | 13.76 | 15.15 |
| 6 | 0.95 | 7.6 | 8.55 | 6 | 1.07 | 11.62 | 12.69 | 6 | 0.78 | 9.23 | 10.01 | 6 | 0.57 | 6.37 | 6.94 | 6 | 0.78 | 8.99 | 9.77 |
| Avarage | 1.08333 | 8.70667 | 9.79 | Avarage | 1.45167 | 13.16 | 14.6117 | Avarage | 1.48833 | 14.7333 | 16.2217 | Avarage | 1.35667 | 12.875 | 14.2317 | Avarage | 1.305 | 13.3317 | 14.6367 |

*Table 3 : All treated wastewater (TWW) treatments and amendments positively impacted plant growth compared to the tap water control, which consistently yielded the lowest averages for root (1.08), shoot (8.71), and total biomass (9.79). In contrast, RT50 emerged as the most effective overall treatment, producing the maximum root (1.49), shoot (14.73), and total biomass (16.22) values. While TWW alone, RH30, and the RT50+RH30 combination all markedly improved growth metrics over the control.*

| Source of Variation | SS | df | MS | F | P-value | F crit |
|---|---|---|---|---|---|---|
| Between Groups | 123.7574 | 4 | 30.93936 | 1.083396 | 0.385944532 | 2.75871 |
| Within Groups | 713.9439 | 25 | 28.55776 | | | |
| | | | | | | |
| Total | 837.7013 | 29 | | | | |

*Table 4 : The one-way ANOVA showed that there was **no statistically significant difference among the five groups** ($F = 1.083$, $p = 0.386 > 0.05$). The mean values ranged from **8.71 in the Tap shoot group** to **14.73 in the RT50 group**. Although the RT50 group showed the highest mean value, followed by RT50 + RH30 (13.33), TWW Alone (13.16), RH30 (12.88), and Tap shoot (8.71), these differences were not significant. Therefore, the treatments **did not have a significant effect on the measured parameter** under the conditions of this experiment.*

# Discussion

The results of this study indicate that treated wastewater (TWW) can support the growth of red lettuce and may provide better growth than tap water under both soil and hydroponic conditions. In the soil system, TWW treatments generally produced higher root, shoot, and total growth than the tap-water control, with TWW alone showing the highest average shoot and total growth. The soil ANOVA also showed significant differences among treatments, indicating that the type of irrigation treatment had an important effect on plant growth. In the hydroponic system, the treated wastewater treatments also showed higher growth than the tap-water control. RT50 produced the highest mean shoot growth (14.73) and total biomass (16.22), suggesting that this bacterial treatment may have promoted plant development. However, the ANOVA for shoot growth showed no statistically significant difference among the five treatments ($F = 1.083$, $p = 0.386$). Therefore, although RT50 showed the highest average value, the observed differences cannot be confirmed as an effect of treatment and may be related to variation among the replicates. Overall, the results suggest that TWW has potential as an alternative irrigation source for lettuce, while the beneficial effects of bacterial inoculation may require further investigation with larger sample sizes and additional experiments.

# Conclusion

In conclusion, the study demonstrated that treated wastewater can be used to support red lettuce growth and generally resulted in better growth than tap water. In the soil system, significant differences among treatments were observed, while in the hydroponic system the differences in shoot growth were not statistically significant. RT50 produced the highest mean shoot and total biomass in the hydroponic system, although this difference was not statistically significant. These findings indicate that treated wastewater has potential as a sustainable water source for lettuce cultivation, particularly in water-scarce regions, while the contribution of PGPB should be further evaluated through additional experiments and larger numbers of replicates.

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