



Unlocking the Drought-Relief Partnership: Exploring the Role of Sulfate-Reducing Bacteria and Salep Gum in Alleviating Drought Stress in Lettuce (*Lactuca sativa*)

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ABSTRACT

Drought stress is a critical factor limiting plant growth and productivity. In the present study, effects of three drought stress levels (40, 60, and 80%) and application of salep gum (SG) and sulfate reductase bacteria (SRB) on various physiological parameters of lettuce plants were investigated. Drought stress significantly reduced germination percentage, whereas SG and SRB positively influenced germination, particularly under severe drought conditions. Root growth increased under 60% drought stress, while SG and SRB treatments exhibited positive effects under 80% drought stress. Leaf area decreased under 80% drought stress but increased with SG and SRB treatments under milder drought levels. Relative water content (RWC) decreased under mild and moderate drought stress, but increased under severe drought stress with all treatments, particularly SG. H_2O_2 levels increased under 80% drought stress with SG and SRB treatments, accompanied by a decrease in catalase (CAT) activity under 80% stress conditions. Superoxide dismutase (SOD) activity increased under 80% drought stress with SG treatment. F_v/F_m decreased under 80% stress but recovered with SG and SRB treatments. Pi_{ABS} decreased with drought stress, while SG treatment increased Pi levels. ABS/RC and TR_0/RC decreased with drought stress but increased with treatments under severe drought conditions. Overall, SG and SRB treatments positively affected germination, root growth, leaf area, RWC, H_2O_2 levels, Antioxidant enzyme activity and photosynthetic functionality under different drought stress conditions. These findings contribute to understanding plant responses to drought stress and suggest the potential of SG and SRB treatments for mitigating its negative effects.

Introduction

Drought stress poses a significant challenge to plant agriculture, severely affecting crop growth, development, and overall productivity. (Das, 2005; Aliniaiefard et al., 2023). The scarcity of water availability during prolonged dry spells

poses a significant challenge, impeding vital physiological processes essential for plant vitality (Kambona et al., 2023; Zaib et al., 2023). This includes compromised photosynthesis, limited nutrient uptake, and hindered transpiration

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rates. As a consequence, plants exhibit stunted growth, reduced leaf area, and diminished root systems, resulting in decreased yields (Iqbal et al., 2020; Chada et al., 2023). Furthermore, drought stress disrupts metabolic pathways, leading to imbalances in nutrient assimilation and utilization (Kapoor et al., 2020). The susceptibility of drought-stressed plants to pest infestations and diseases has been frequently addressed, further provoke crop losses (Ray et al., 2020). The diverse impacts of drought stress highlight the pressing need for innovative mitigation strategies and the development of resilient crop varieties to maintain sustainable agricultural systems in an increasingly changing climate (Lastochkina et al., 2022; Seifikalhor et al., 2022). Lettuce (*Lactuca sativa*), a widely cultivated leafy vegetable, is particularly susceptible to water scarcity, resulting in reduced yield and compromised quality. Therefore, developing effective strategies to enhance plant resilience and alleviate drought stress is crucial for sustainable agriculture (Damerum, 2017; Shi et al., 2022).

In recent years, the exploration of beneficial microorganisms and natural compounds has emerged as a promising approach to enhancing plant tolerance to drought stress. Among these, sulfate-reducing bacteria (SRB), a diverse group of microorganisms, have demonstrated potential in improving plant resilience to various abiotic stresses (Abdelaal et al., 2021; Poudel et al., 2021). SRB can reduce sulfate to sulfide, which acts as a signaling molecule and triggers various protective mechanisms in plants, ultimately promoting drought tolerance (Kaya et al., 2018; Zhang et al., 2022).

Additionally, salep gam (SG), a natural polysaccharide derived from orchid tubers, has gained recognition for its unique properties. It exhibits water-holding capacity and acts as a natural hydrogel, promoting water retention in the soil. This enhances the availability of water to plants during periods of drought stress, mitigating the adverse effects of water scarcity (Kurt, 2021).

This study aims to examine the combined effects of SRB and SG in mitigating drought stress in lettuce plants. By investigating their synergistic interactions, the research seeks to enhance lettuce's adaptive responses to water scarcity and improve its overall tolerance to drought conditions. Due to SRB reducing sulfate to sulfide, which triggers protective mechanisms, while SG acts as a natural hydrogel to retain water in the soil, thereby improving plant water use efficiency, osmotic regulation, and stress tolerance mechanisms we hypothesize that the combined

application of SRB and SG enhances drought tolerance in lettuce plants more effectively than either treatment alone. To test this hypothesis, the research will address several key questions: Does the combined application of SRB and SG improve drought tolerance in lettuce plants compared to individual treatments and untreated controls? Additionally, how does this combined treatment affect plant growth, photosynthetic efficiency, water status, and water retention during drought stress? What impact do SRB and SG have on antioxidant defense mechanisms and osmolyte accumulation? Finally, how do SRB and SG work synergistically to enhance overall drought tolerance in lettuce plants?

The research protocol will involve subjecting lettuce plants to controlled drought conditions, both with and without the application of SRB and SG. A variety of parameters will be measured to evaluate the effectiveness of this combined treatment, including plant growth, photosynthetic efficiency, water status, antioxidant defense mechanisms, osmolyte accumulation, and stress-responsive gene expression. This study aims to uncover the mechanisms through which SRB and SG alleviate drought stress in lettuce. Understanding the interactions between these beneficial microorganisms and natural compounds will offer valuable insights into their synergistic effects on enhancing plant water use efficiency, osmotic regulation, and overall stress tolerance. The findings of this research have the potential to contribute to the development of sustainable agricultural practices. By harnessing the power of beneficial microorganisms and natural compounds, we can enhance crop resilience and productivity under drought conditions. Implementing these strategies in lettuce cultivation can not only mitigate the negative impacts of water scarcity but also promote sustainable food production in water-limited regions.

Material and methods

SRB cultivation and application

SRB from *Desulfovibrio desulfuricans* species was performed in Postgate medium B culture in the laboratory of Industrial Microbiology at Shahid Beheshti University (Postgate, 1984; Reis et al., 1992). For the isolation of SRB bacteria, approximately 1 g of sediment sample from a marsh was added to 100 mL glass bottles containing 50 mL of specialized bacterial culture medium. The bottle lids were sealed to create anaerobic conditions. The samples were incubated in an incubator at a temperature of 30

°C for one week. In this culture medium, SRB cells grew and turned the culture medium black. Then, the optical density of the grown bacterial cells was measured, and the culture medium was adjusted to a desired value of 1 by adding more culture medium or allowing further growth. The culture medium containing bacteria was centrifuged at 5000 rpm for 10 min, and the bacterial sediment formed was gently dissolved in 10 mL of sterile distilled water. Finally, the optical density was adjusted to 0.8 by adding water. It should be noted that this bacterial density (optical density of 0.8) had been evaluated as the optimal bacterial quantity for pH modification in soil in a preliminary experiment. After 10-14 d, the maximum growth of the bacteria was observed at an optical density of 1.2-0.8 OD, and accordingly, a quantity of 3-4 L of this bacterium was inoculated for drought stress in large-scale experiments. One d before seed sowing, SRB (with an optical density equivalent to 0.8 in sterile water) were mixed with the soil in the pots.

Preparation of culture medium for plants

The soil culture medium was prepared, containing 50% cocopeat and 50% perlite (Kalhor et al., 2018). Additionally, this type of soil medium was recommended by reviewers during the evaluation of the proposed project. Seed trays and pots were prepared according to the overall estimation of all experiments in the project (seedlings were kept in seed trays until the three-leaf stage for faster and better growth, then transferred to pots). A scale was prepared to measure the weight of the soil.

Seed coating and treatment

The seeds were initially washed with running water, followed by treatment with 70% ethanol for 1-2 min and 1% sodium hypochlorite for 3 min. After each step, the seeds were rinsed with distilled water. For seed coating with SG, 1 g of SG powder was dissolved in 100 mL of distilled water along with 0.015 g of calcium gluconate and 0.09 g of sodium lauryl sulfate, resulting in a gel-like solution (Zahmati, 2016). For control coating, the seeds were treated with distilled water and then immersed in the desired solutions to form a coating layer around the seeds. Surfactants help the coating to adhere to the seed surface.

Based on previous research (Seifikalhor et al., 2020), in this experiment, a 3% SG concentration was initially considered, but due to poor seed germination, the SG concentration was reduced to 1%. Therefore, 1% SG was used for seed coating in this design.

The soil for each treatment was placed in the germination tray cells, and then the seeds were sown in the soils. The germination trays were kept in a growth chamber with an average temperature of 23 ± 2 °C and a light/dark cycle of 16 h of light and 8 h of darkness. Irrigation was done every other day with Hoagland solution. Irrigation for drought stress treatments was based on the severity of drought stress.

Transferring seedlings to pots

Three weeks after seed sowing, leaf samples were taken from three-leaf-stage seedlings, and the seedlings were transferred to pots with the same corresponding treatments.

Application of drought stress

A soil moisture meter was prepared to measure the soil moisture level under drought stress. Drought stress was applied at 60, 40, and 80% levels. The soil moisture meter indicated the soil moisture levels of 40% (soil with 60% moisture), 60% (soil with 40% moisture), and 80% (soil with 20% moisture) under drought stress. The control or optimal treatment for drought stress is 40% moisture level (60% soil moisture).

Sampling

Sampling was performed at the early growth stages (3-4 leaf stage) before transferring to pots and in mature plants (4th stage) in pots. For drought stress, sampling was carried out from 4 treatments with 40% drought stress, 4 treatments with 60% drought stress, and 4 treatments with 80% drought stress, with a minimum of 4 pots per treatment, totaling 48 pots.

Germination and growth measurement

The percentage of germination was measured by counting the number of germinated seeds after 7 d. Germination was recorded when the radicle emerged from the seed coat. The percentage of seed germination was calculated using the following equation:

$$\text{Germination percentage} = 100 \times \left[\left(\frac{\text{total number of seeds}}{\text{number of germinated seeds}} \right) \right]$$

To measure the root length and leaf area, samples were photographed, and the images were analyzed using ImageJ 1.44p software (National Institutes of Health; Bethesda, MD).

Relative water content (RWC)

To measure the RWC of the leaves, samples from

all experimental treatments were taken, and their fresh weight was immediately measured. All samples were placed in distilled water in darkness for 24 h. The saturated weight of the leaves was measured, and the leaves were dried in an oven at 70 °C for 24 h. The dry weight of the leaves was then measured. The RWC in leaf samples was calculated using the following formula:

$$RWC = \left(\frac{Fw - Dw}{Sw - Dw} \right) \times 100$$

Fw: Fresh weight of the leaves immediately after sampling

Dw: Dry weight of the leaves after drying

Sw: Saturated weight of the leaves after being placed in distilled water

Measurement of H₂O₂ concentration

For the measurement of H₂O₂ concentration, the method described by (Velikova et al., 2000) was utilized. Fresh plant tissue weighing 500 mg was homogenized with 5 mL of 1% (w/v) trichloroacetic acid (TCA) on ice. The homogenized tissue was centrifuged at 12,000 g for 15 min at 4 °C. 500 µL of 10 mM potassium phosphate buffer at pH 7 was added to 500 µL of the supernatant, followed by the addition of 1 mL of 1 M potassium iodide solution. The absorbance of the solution at a wavelength of 390 nm was measured using a spectrophotometer.

Standard solutions in the range of 0-100 µM were prepared, and the corresponding standard curve was plotted based on the absorbance of the solutions at a wavelength of 390 nm. The H₂O₂ concentration of each sample was then calculated using the formula obtained from the standard curve. Finally, by dividing the calculated concentration by the weight of the plant tissue, the H₂O₂ content was expressed in mg per g of tissue weight.

Determination of antioxidant enzyme activity

Catalase (CAT)

The CAT was extracted according to the method described by Maehly and Chance (1954). 0.25 g of plant tissue was finely powdered in liquid nitrogen. The powdered tissue was homogenized in 1.5 mL of cold 100 mM phosphate buffer (pH 7) containing 1% polyvinylpyrrolidone (PVP) and 1 mM EDTA. The homogenate was centrifuged at 10,000 g for 15 min at 4 °C. The supernatant was used for enzyme measurement.

The CAT activity was measured using the method described by (Aebi, 1974) 100 µL of the diluted

enzyme extract and 1 mL of 50 mM potassium phosphate buffer (pH 7) were mixed, and the reaction was initiated by adding 100 µL of 100 mM H₂O₂. The changes in absorbance at 240 nm were measured for 2 min at 15-second intervals using a spectrophotometer.

The CAT activity (U mL⁻¹) was calculated using the following formula:

$$\text{Enzyme activity (U mL}^{-1}\text{)} = \frac{[\text{Change in absorbance (}\Delta\text{)} \times \text{Reaction mixture volume (1.2 mL)} \times \text{H}_2\text{O}_2 \text{ coefficient (2)}]}{[\text{Extinction coefficient (39.4)} \times \text{Path length (1 cm)} \times \text{Extract volume (0.1 mL)} \times \text{t}\Delta \text{ (2)}]}$$

The specific activity of the CAT enzyme (U mg⁻¹ protein) was calculated by dividing the enzyme activity by the total protein content.

Superoxide dismutase (SOD)

The activity of SOD enzyme was determined using the method described by (Giannopolitis and Ries, 1977). Fresh or frozen plant tissue weighing 0.5 g was homogenized with 3 mL of 50 mM sodium phosphate buffer at pH 7.8, containing 2% polyvinylpyrrolidone (PVP) and 1 mM EDTA. The homogenate was centrifuged at 13,000 g for 10 min at 4 °C. The reaction mixture buffer consisted of 50 mM potassium phosphate buffer at pH 7.8, 0.1 mM EDTA, 75 µM NBT (nitro blue tetrazolium), 13 mM methionine, and 4 µM riboflavin.

To initiate the reaction, 100 µL of the sample solution was added to 3 mL of the reaction mixture buffer, and the combination was exposed to a 40-watt fluorescent light for 8 min. After 8 min, the absorbance of the reaction mixtures at a wavelength of 560 nanometers was measured using a spectrophotometer. The control sample consisted of 3 mL of the reaction mixture without the extract, which was kept in the light.

The activity of the SOD enzyme was calculated using the following formula:

$$\text{Superoxide Dismutase Activity (U mg}^{-1}\text{ protein)} = 2 \times \frac{(\text{Absorbance Control} - \text{Absorbance Sample})}{\frac{\text{Absorbance Control}}{\text{Total Protein}}}$$

Slow and fast induction of chlorophyll fluorescence

The F_v/F_m parameter, representing the maximum quantum efficiency of photosystem II, was measured using intact leaves and an advanced chlorophyll fluorescence imaging system (Handy Fluor-Cam FC 1000-H; Photon System Instruments, Brno, Czech Republic). The measurements were conducted eight weeks after sowing, following a 20 min period of dark adaptation. Two sets of fluorescence data were

collected: one during short flashes in darkness to determine minimum fluorescence (F_0), and the other during a saturating light flash (F_m) with a light intensity of $3900 \mu\text{mol m}^{-2} \text{s}^{-1}$. Variable fluorescence (F_v) was calculated as the difference between F_m and F_0 . The average data and standard deviation for F_v/F_m were recorded using version 7 of the Fluor-Cam software (Kalhor et al., 2018).

To analyze the polyphasic chlorophyll fluorescence (OJIP) transient, a convenient fluorometer system (FluorPen FP 100-MAX, Photon Systems Instruments, Drasov, Czech Republic) was used. Similar to the gradual induction of chlorophyll fluorescence, the OJIP transient measurements were performed on plants that underwent a 20 min period of dark adaptation. A saturating light with an intensity of $3000 \mu\text{mol m}^{-2} \text{s}^{-1}$ was used to induce the transient. The fluorescence data were collected at various time points, including fluorescence intensities at $50 \mu\text{s}$ (F_0), 2 ms (F_I or J-step), 60 ms (F_I or I-step), and the maximum fluorescence (F_m) (Esmaeili et al., 2022).

To assess the performance index on an absorption basis (Pi_{ABS}), the following equation was employed:

$$Pi_{ABS} = \left[\frac{1}{\left(\frac{ABS}{RC}\right)} \right] \times \left[\frac{\frac{F_v}{F_m}}{\left(1 - \frac{F_v}{F_m}\right)} \right] \times \left[\frac{\psi 0}{(1 - \psi 0)} \right]$$

ABS/RC represents the energy fluxes per reaction center (RC) for energy absorption, and $\psi 0$ denotes the probability (at $t = 0$) of electron transport beyond Quinone A-. Additionally, other calculated data such as specific energy fluxes per reaction center (RC) for energy absorption (ABS/RC), trapped energy flux (TR_0/RC), electron transport flux (ET_0/RC), and dissipated

energy flux (DI_0/RC) were derived based on the parameters obtained from the OJIP fluorescence transient (Kalhor et al., 2018).

Statistical Analysis of Data

The data obtained from the experiment were analyzed using analysis of variance (ANOVA) based on a completely randomized design (CRD) with a minimum of three replicates per treatment. The effects of the treatments on the measured traits were evaluated using statistical software such as SPSS and PRISM. The means of the treatments were compared using the Tukey method, and the grouping of treatments was performed using the 2 way ANOVA and the Duncan's multiple range test at a significance level of $P \leq 0.05$. This level was considered as the threshold for determining the significance of differences.

Results

In general, the findings demonstrate a significant reduction in germination percentage due to drought stress (Fig. 1A). However, the utilization of SG and SRB exhibited a remarkably positive influence, particularly when SRB was applied alone or in combination with SG, resulting in a substantial increase in germination percentage, particularly under severe drought conditions (Fig. 1A).

Under 60% drought stress without any interventions, root growth displayed an augmentation, implying a potential plant physiological response towards water acquisition. While the combined application of SG and SRB demonstrated a positive effect under 40 and 80% drought stress, other treatments did not significantly affect root growth under various stress conditions (Fig. 1B).

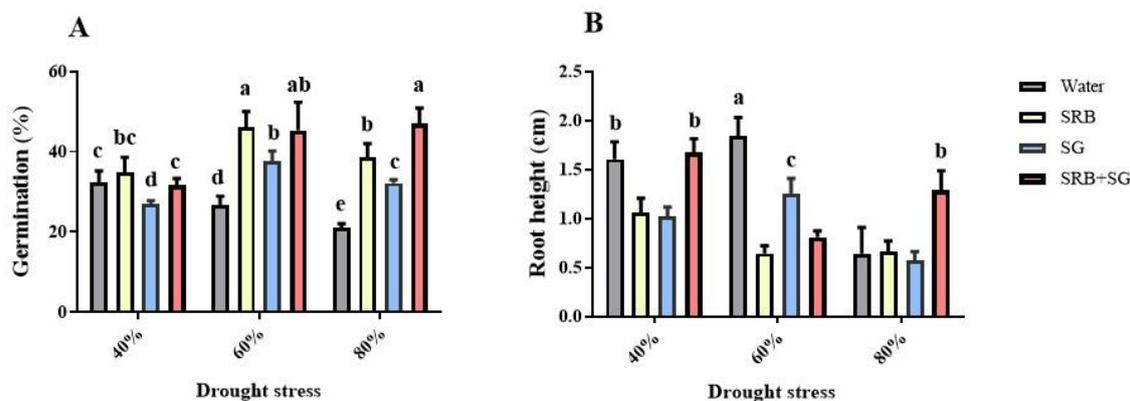


Fig. 1. Effects of exogenous sulfate reducing bacteria (SRB) and salep gum (SG) applications on seed germination (A) and root height growth (B) of the lettuce plants under three different drought stress levels. Values are the means of three replicates and bars indicate means \pm standard error. Different letters indicate a significant difference between the means at the probability level of $P < 0.05$.

A considerable decline in leaf area was observed under 80% drought stress. Conversely, the simultaneous application of SG and SRB under 40% drought stress led to an increase in leaf area (Fig. 2A). Specifically, SRB alone elicited an increase in leaf area under 60% drought stress. Intriguingly, under 80% drought stress, SRB alone or in combination with SG significantly augmented leaf area, surpassing that of plants grown under 40% drought stress (Fig. 2A). RWC experienced a decrease following the application

of drought stress, despite the treatments exhibiting no positive effects and resulting in its reduction under mild and moderate drought stress (Fig. 2B). Nonetheless, under severe drought stress, all treatments, particularly SG, enhanced the RWC in treated plants compared to untreated ones. Notably, SG demonstrated the ability to elevate the RWC in treated plants under drought stress to a level akin to that of plants grown under mild and moderate drought stress conditions (Fig. 2B).

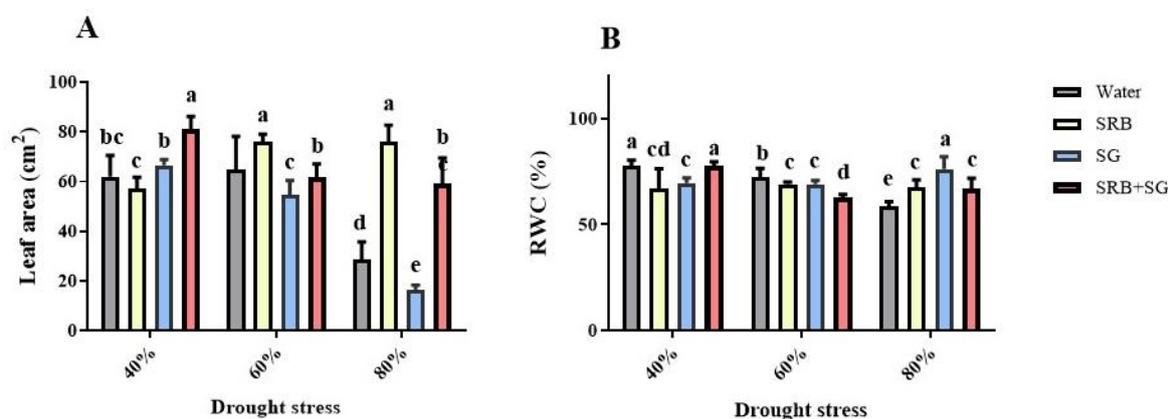


Fig. 2. Effects of exogenous sulfate reducing bacteria (SRB) and salep gum (SG) applications on leaf area (A) and RWC (B) of the lettuce plants under three different drought stress levels. Values are the means of three replicates and bars indicate means \pm standard error. Different letters indicate a significant difference between the means at the probability level of $P < 0.05$.

The application of 80% drought stress resulted in a reduction in H_2O_2 levels (Fig. 3A). The treatments exhibited no significant positive effect on H_2O_2 content under mild and moderate drought conditions, except for the 80% drought stress condition, where a remarkable increase in H_2O_2 content was observed with the simultaneous application of SG and SRB treatments, leading to the highest recorded H_2O_2 levels (Fig. 3A). SOD activity demonstrated a decrease under 60% drought stress without any treatments, but exhibited an increase under 80% drought stress (Fig. 3B). In mild and moderate stress conditions, both SRB alone and in combination with SG significantly enhanced SOD activity. However, under 80% drought stress, only SG was able to increase SOD activity, while the other treatments had a negative impact (Fig. 3B). CAT activity showed a notable decrease under 80% stress conditions (Fig. 3C). Specifically, only the simultaneous application of SG and SRB at this stress level further reduced CAT activity. In contrast, the other treatments resulted in a decrease in CAT activity compared to

control conditions across different stress conditions (Fig. 3C).

The variable F_v/F_m exhibited a significant reduction under 80% stress conditions (Fig. 4A). However, the concurrent application of SG and SRB treatments resulted in its recovery, reaching levels comparable to those observed in plants grown under non-stressful conditions. This restorative effect was also evident across other levels of drought stress. Sole SG treatment, on the other hand, caused a decrease in F_v/F_m under 60 and 40% drought stress, but it increased this parameter under severe drought conditions when compared to untreated plants (Fig. 4A).

The levels of Pi_{ABS} demonstrated a remarkable decrease upon the application of drought stress (Fig. 4B). Nonetheless, SG treatment increased Pi levels under all drought conditions, effectively alleviating the detrimental effects of drought stress. Notably, in 40 and 60% drought stress conditions, SG treatment surpassed the Pi levels observed under normal drought conditions, indicating a further enhancement of Pi accumulation (Fig. 4B).

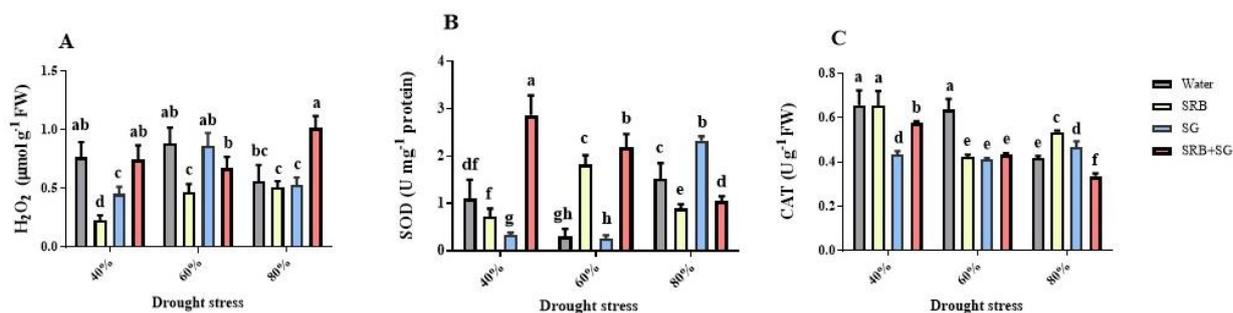


Fig. 3. Effects of exogenous sulfate reducing bacteria (SRB) and salep gum (SG) applications on H_2O_2 content (A), SOD activity (B) and CAT activity (C) of the lettuce plants under three different drought stress levels. Values are the means of three replicates and bars indicate means \pm standard error. Different letters indicate a significant difference between the means at the probability level of $P < 0.05$.

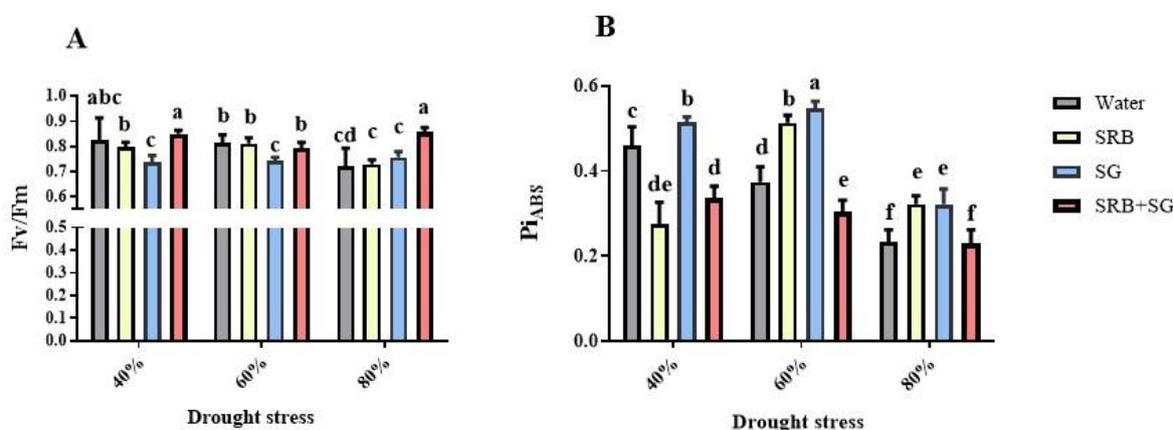


Fig. 4. Effects of exogenous sulfate reducing bacteria (SRB) and salep gum (SG) applications on F_v/F_m (A) and Pi_{ABS} (B) of the lettuce plants under three different drought stress levels. Values are the means of three replicates and bars indicate means \pm standard error. Different letters indicate a significant difference between the means at the probability level of $P < 0.05$.

The parameter ABS/RC experienced a substantial reduction in response to drought stress (Fig. 5A). Specifically, under 40% drought stress, the application of SG treatment resulted in an increase in ABS/RC, while the inclusion of SRB treatment, either alone or in combination with SG, led to a decrease in this parameter. Conversely, under 60% drought stress, SG treatment contributed to an elevation in ABS/RC (Fig. 5A). However, in the presence of severe drought conditions, all treatments exhibited a noteworthy enhancement in light absorption by reaction centers. Likewise, TR_0/RC displayed a decline upon the imposition of drought stress. Although the treatments did not generate positive effects during mild drought conditions, they were capable of increasing TR_0/RC under severe drought conditions (Fig. 5B). Notably, the SRB treatment did not achieve the highest value

compared to the other treatments. Additionally, the application of drought stress did not significantly affect the ET_0/RC (Fig. 5C). SG treatment, on the other hand, effectively elevated this parameter under both low and moderate drought levels. Conversely, the remaining treatments failed to induce any noticeable alterations in TR_0/RC , with the exception of the combined SG and SRB treatment under 80% drought stress, which led to a decrease in this parameter. Furthermore, DI_0/RC exhibited a decrease as drought intensity increased (Fig. 5D). In mild and moderate drought conditions, SRB treatment, whether administered alone or in conjunction with SG, resulted in an increase in this parameter. Under severe drought stress, all treatments, particularly SRB, induced an augmentation in DI_0/RC (Fig. 5D).

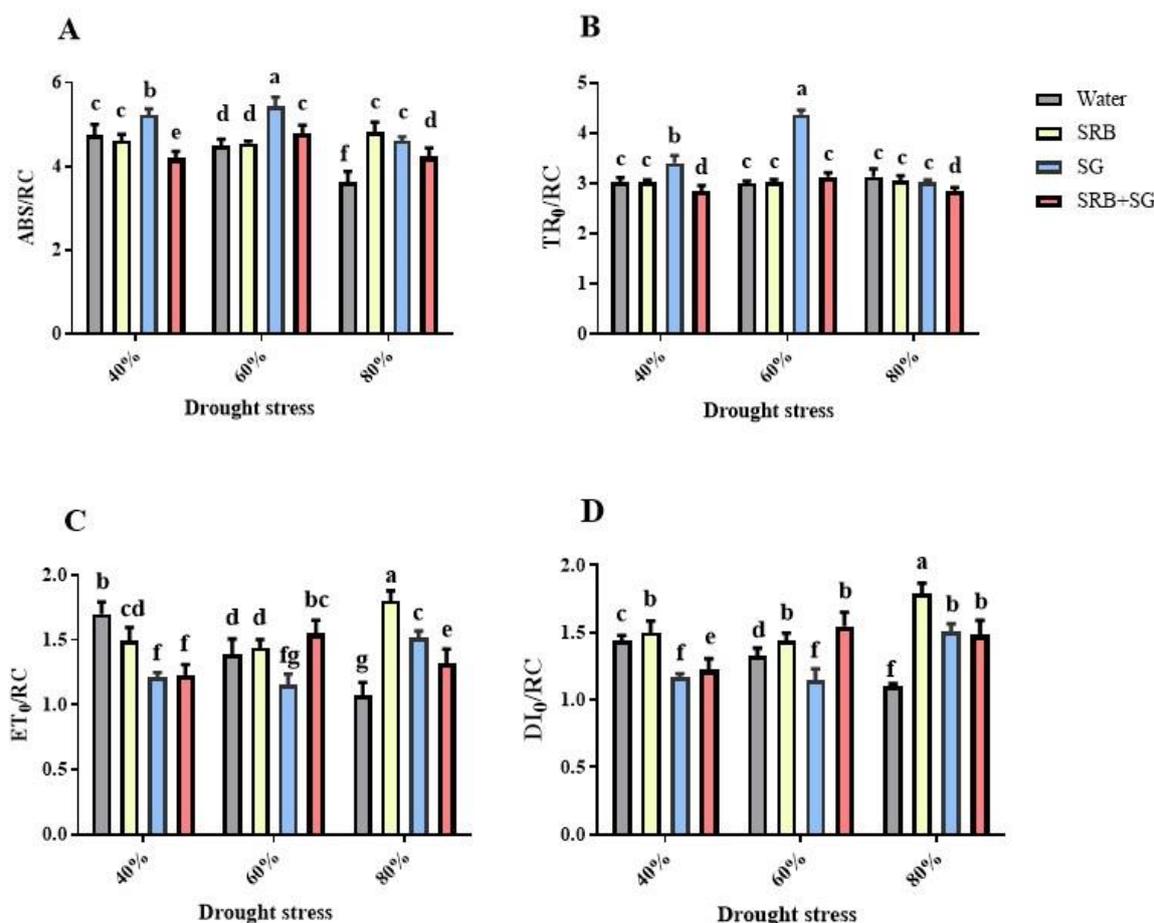


Fig. 5. Effects of exogenous sulfate reducing bacteria (SRB) and salep gum (SG) applications on ABS/RC (A), TR₀/RC (b), ET₀/RC (C) and DI₀/RC (D) values of the lettuce plants under three different drought stress levels. Values are the means of three replicates and bars indicate means \pm standard error. Different letters indicate a significant difference between the means at the probability level of $P < 0.05$.

Discussion

The findings of this study enhance our understanding of plant responses to drought stress and highlight the potential of SG and SRB treatments in mitigating its negative effects. The results demonstrate that SG and SRB treatments positively influenced germination, root growth, leaf area, RWC, hydrogen peroxide levels, CAT and SOD activity and photosynthesis functionality. The observed increase in germination percentage under severe drought conditions with the application of SG and SRB treatments can be attributed to various physiological and biochemical processes. SG treatment, which is likely a drought-tolerant agent, may have influenced germination by enhancing water uptake and retention in seeds (Ashraf et al., 2011; Hotta et al., 2016; Hoseini et al., 2022). SG treatment increases the water-holding capacity of seeds, improving their ability

to endure drought stress. This effect is attributed to the presence of hydrocolloids in SG, which form a gel-like matrix upon hydration. This matrix helps retain water and creates a protective barrier against desiccation, supporting the seeds during periods of water scarcity. (Western, 2012). The hydrocolloids in SG may also contribute to osmotic adjustment, helping seeds maintain cellular hydration and integrity during drought stress. Furthermore, SG may stimulate the accumulation of compatible solutes, such as sugars and amino acids, in seeds (Wilson, 2015). These compatible solutes act as osmoprotectants, maintaining cellular osmotic balance and protecting cellular structures from damage caused by water deficit (Singh et al., 2015). Conversely, SRB treatment, recognized for its growth-promoting potential, may have indirectly influenced seed germination by improving overall plant health and vigor. SRB can enhance

nutrient availability, stimulate hormone production, and boost stress tolerance, contributing to better seedling establishment and growth. (Park et al., 2017). These factors can positively impact seed germination and early seedling growth under drought conditions. The combined treatment might have acted through a combination of improved water uptake, nutrient availability, hormone regulation, and stress tolerance mechanisms, thereby creating a favorable environment for seed germination despite severe drought stress (Lastochkina et al., 2019; Shomali and Aliniaiefard, 2020; Lastochkina et al., 2021a; Lastochkina et al., 2021b). In addition to its direct effects on seeds, SG treatment has been reported to promote root growth and development. Research indicates that SG-like structures can stimulate root elongation and branching, resulting in a larger root surface area and enhanced water absorption capacity. (Dickinson et al., 2019). This can be attributed to the presence of bioactive compounds in SG that promote root growth, such as plant growth regulators and signaling molecules (Benítez García et al., 2020).

The observed increase in root growth under 60% drought stress without interventions suggests an adaptive response by plants to optimize water acquisition. In moderate drought conditions, plants often undergo physiological and biochemical changes that promote enhanced root development. This allows them to explore a greater soil volume, improving their ability to access and uptake water more effectively in response to the stress. (Fang et al., 2017). This response is driven by various mechanisms, including altered hormone signaling, changes in root architecture, and increased production of root growth-promoting compounds (López-Bucio et al., 2003).

The decline in leaf area under 80% drought stress aligns with the well-established response of plants to severe water limitation (Wang et al., 2020). During drought stress, plants typically reduce leaf expansion to minimize water loss through transpiration (Chen et al., 2020; Seleiman et al., 2021). The reduction in leaf area indicates that the plants experienced significant water stress, leading to a decrease in leaf growth (Sousaraei et al., 2021). The simultaneous application of SG and SRB under 40% drought stress resulting in increased leaf area suggests a potential ameliorating effect of the combined treatment on low drought stress. SG, known for its drought-tolerant properties, likely contributed to enhanced water retention and uptake in the plants. This could be achieved through mechanisms such as improved osmotic

adjustment and increased accumulation of compatible solutes, enabling the plants to maintain optimal hydration levels and support leaf expansion (Aliniaiefard et al., 2016a; Aliniaiefard et al., 2016b). Additionally, SRB, as a growth-promoting bacterium, might have facilitated nutrient availability and hormonal regulation, further supporting leaf growth under moderate drought stress (Abdelaal et al., 2021; Fadji et al., 2022).

The increase in leaf area observed with SRB treatment alone under 60% and 80% drought stress suggests that SRB can positively influence leaf growth, even without the presence of SG. The growth-promoting effects of SRB likely contribute to nutrient mobilization, hormone production, and activation of stress tolerance mechanisms, allowing for leaf expansion despite restricted water availability. These findings underscore SRB's potential as a beneficial microorganism for promoting leaf growth under drought conditions. Drought stress typically limits water availability, causing dehydration in plant tissues, which is evidenced by the reduced relative water content (RWC). (Aliniaiefard and Van Meeteren, 2016). However, intriguingly, under severe drought stress, all treatments, particularly SG, enhanced the RWC in treated plants compared to untreated ones. This indicates that SG, specifically, had a positive impact on water retention and maintenance of cellular hydration under severe water stress conditions. Leaf area and RWC change by SG are the different sides of the same coin enabling the treated plants to maintain a relatively higher water content and leaf expansion compared to the untreated plants. The capacity of SG to effectively maintain plant hydration levels, even under severe water stress, resembles the conditions with milder stress levels. In current study, under 80% drought stress, there was a remarkable increase in H_2O_2 content in non-treated plants. This increase could be attributed to the disruption in the plant's antioxidant defense system, potentially due to the severe water scarcity conditions (Aalifar et al., 2020). Concurrently, CAT activity showed a notable decrease under 80% stress conditions, suggesting a compromised ability to break down H_2O_2 . These findings indicate a potential imbalance between H_2O_2 production and detoxification, resulting in the accumulation of H_2O_2 . H_2O_2 can act as a signaling molecule in plants. When plants are exposed to stress, such as drought, heat, or pathogens, they often produce more H_2O_2 (Carvalho and Silveira, 2020; Dikilitas et al., 2020). Increased H_2O_2 levels can trigger various signaling pathways that help the plant to adapt and defend against the stress (Sachdev et

al., 2021). It can activate genes involved in stress responses, antioxidant production, and repair mechanisms. This result was concurrent with reduced CAT activity suggesting that this disruption can lead to altered gene expression patterns, hormonal regulation, and impaired cellular signaling, further impacting the plant's ability to adapt to stress.

Under mild and moderate drought conditions, both SRB alone and in combination with SG significantly enhanced superoxide dismutase (SOD) activity, indicating that these treatments activated the plant's defense mechanisms to counteract the production of superoxide radicals and improve their scavenging. However, under more severe drought stress (80%), only SG was able to increase SOD activity. This suggests that the effects of SRB and SG on SOD activity may vary based on the intensity of drought stress. The regulation of the antioxidant defense system, such as through SOD activation, can have a profound impact on plant growth and development, primarily by supporting photosynthetic processes under stress conditions. (Pospíšil, 2016; Li et al., 2017). Investigation of photochemical processes, such as the OJIP test, has demonstrated significant insights into this area in current study. P_i_{ABS} refers to the parameter called "absorbance-based quantum yield of PSII electron transport" in plant photosynthesis research (Kumar et al., 2022). It is a measure used to assess the efficiency of photosystem II (PSII) in converting absorbed light energy into chemical energy, specifically the quantum yield of electron transport in PSII (Ashrostaghi et al., 2022). The application of SG and SRB treatments may have enhanced water uptake and improved water status in the plants. This improved water availability could have positively influenced the functioning of the PSII and the efficiency of energy trapping, leading to the recovery of P_i_{ABS} as it can be observed in both moderate and severe drought condition (Sousaraei et al., 2021). Moreover, drought stress often leads to stomatal closure as a protective mechanism to reduce water loss through transpiration (Arve et al., 2011). The SG and SRB treatments might have influenced stomatal regulation, allowing for a more balanced gas exchange, improved CO_2 availability, and enhanced photosynthetic efficiency, leading to the recovery of P_i_{ABS} . In a different scenario, drought stress can induce oxidative stress in plants, resulting in the accumulation of reactive oxygen species (ROS) that can damage cellular components, including the photosynthetic machinery. As discussed previously the SG and SRB treatments might have stimulated

antioxidant defense mechanisms, such as the production of enzymes like SOD, CAT, and peroxidase. The enhanced antioxidant capacity could have protected the PSII from oxidative damage, maintaining its efficiency and contributing to the recovery of P_i_{ABS} . Another crucial factor that warrants consideration is the F_v/F_m ratio, which holds significant relevance in plant photosynthesis research (Shomali et al., 2021). Drought stress can lead to photoinhibition, which is the reduction in the maximum quantum efficiency of photosystem II (PSII) indicated by a decrease in F_v/F_m . Photoinhibition occurs when the rate of damage to PSII exceeds the rate of repair (Tikkanen et al., 2014; Shomali et al., 2023). The SG and SRB treatments may have enhanced the repair mechanisms of PSII, such as the synthesis and replacement of damaged protein, thereby restoring the maximum quantum efficiency and resulting in the recovery of F_v/F_m . Stimulated the production of antioxidants by SG and SRB treatments might have similar effect on F_v/F_m similar to P_i_{ABS} . Drought stress can disrupt the electron transport chain, leading to inefficient energy conversion and decreased F_v/F_m . The SG and SRB treatments might have facilitated the maintenance of a functional electron transport chain by ensuring the availability of electron carriers, such as plastoquinone and ferredoxin. This would enhance the flow of electrons and the efficiency of energy conversion, resulting in the recovery of F_v/F_m . However, the mechanistic underpinnings of this process remain to be elucidated. Nevertheless, the functionality of photosynthesis can be regarded as an indicative measure for assessing the impact of both SG and SRB.

The impact of SG and SRB treatments on the absorbance-based quantum yield of PSII electron transport (ABS/RC) varied with the severity of drought stress. Under moderate drought stress, SG treatment increased ABS/RC , suggesting improved light absorption by the reaction centers. In contrast, under more severe drought conditions, all treatments led to a significant enhancement in ABS/RC . As previously noted, elevated H_2O_2 levels in SG and SRB treatments can activate specific signaling pathways, including mitogen-activated protein kinases (MAPKs) and calcium-dependent protein kinases (CDPKs), which transmit stress signals to downstream targets. (Lv et al., 2018). These pathways can trigger the activation of stress-responsive genes, leading to the synthesis of protective proteins, antioxidants, and osmoprotectants, as well as the adjustment of stomatal behavior and photosynthetic activity (Shomali et al., 2024). The presence of high levels of H_2O_2 in severe drought

stress conditions by applying SG and SRB may induce signaling pathways that promote the enhancement of ABS/RC. These pathways could involve the upregulation of genes associated with the photosynthetic apparatus, such as those involved in the synthesis of chlorophyll, light-harvesting complexes, and reaction center proteins. The activation of these genes would result in an increased capacity for light absorption by reaction centers, ultimately leading to an enhanced ABS/RC ratio.

The application of drought stress alone did not significantly affect the TR_0/RC parameter, suggesting of the plant's natural response to drought stress that may not directly involve changes in the dissipation of excess energy from reaction centers (Seif et al., 2021). However, increased TR_0/RC was observed by SG application. The combined effect of increased TR_0/RC and DI_0/RC by SRB application suggests an overall improvement in the efficiency of energy capture, electron transport, and utilization within the photosynthetic system. This can result in enhanced photosynthetic performance and potentially higher rates of carbon assimilation.

In conclusion, this study demonstrates that SG and SRB treatments effectively mitigate the negative effects of drought stress on plants, and in some cases, can help plants recover to near-normal conditions under high stress. Both SG and SRB enhance germination, root growth, and leaf area, while maintaining higher water content during drought stress. They also modulate reactive oxygen species and antioxidant enzyme activities, bolstering stress defense mechanisms. Additionally, SG and SRB treatments improve photosynthetic efficiency and the maximum quantum efficiency of photosystem II. These findings underscore the potential of SG and SRB as effective strategies for alleviating drought stress and boosting plant performance. However, further research is needed to confirm their practical applications. Investigating long-term effects of SG and SRB treatments on plant growth, development, and yield under prolonged drought conditions will provide insights into the sustainability and practical applicability of these treatments. Moreover, detailed mechanistic studies to elucidate the underlying biochemical and molecular mechanisms by which SG and SRB treatments improve plant tolerance to drought stress may explore their effects on gene expression, signaling pathways, and metabolic changes associated with drought response. Finally, field trials are essential to evaluate the effectiveness of SG and SRB treatments across

various crop species and under different environmental conditions. These trials will assess their impact on crop productivity, water-use efficiency, and overall crop performance.

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Conflict of Interest

The authors indicate no conflict of interest in this work.

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