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ARTICLE

Effects of Solution Applied and Different Encapsulation Method of Plant Growth Promoting Bacteria (PGPB) on the Physiological Response of Sunflower to Drought Stress

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ABSTRACT: Sunflower (*Helianthus annuus* L.) is an important food, oil and bioenergy crop frequently affected by drought stress. Plant growth-promoting bacteria (PGPB) can enhance plant growth and resilience to water deficit. However, their efficiency often diminishes over time under prolonged stress due to reduced bacterial survival. This study investigated whether encapsulated forms of *Bacillus subtilis*, with and without humic acid supplementation, improves bacterial viability and photosynthetic performance of a drought-sensitive sunflower variety under water deficiency. Seedlings were grown in a greenhouse, with water deficit imposed at 30% field capacity for 29 days. Treatments included solution (B) or encapsulated forms (alginate beads alone (EB) or supplemented with humic acid (HEB)) under well-watered (W) and drought (D) conditions. Encapsulation, particularly with humic acid, increased bacterial viability, mitigating damage to photosynthetic reaction centers as reflected by subsequent morphophysiological measurements. Under drought, all PGPB treatments enhanced plant height (BD: 15.2%, HEBD: 14.6%, EBD: 6.1% vs. D), above-ground biomass (HEBD and BD significantly higher), Gsw (EBD: 3-fold, HEBD: 10-fold vs. D), ΦPSII (HEBD: 10%, BD: 4% vs. D), ETR (HEBD: 31% vs. D), and *ChlF* indices like PIABS (BD: 95%, HEBD: 81%, EBD: 53% vs. D). Stable encapsulation without premature release, especially with humic acid, sustained bacterial viability and prolonged benefits by protecting the bacteria against stress, improving root interactions and photosynthetic resilience. These findings demonstrate that encapsulation, particularly with humic acid, optimizes PGPB delivery and sustains physiological benefits under drought, highlighting its strong potential as a strategy for sustainable agriculture in semi-arid regions.

KEYWORDS: *Bacillus subtilis*; bacterial encapsulation; drought stress; humic acid; photosynthetic efficiency

1 Introduction

Drought stress is a major abiotic factor limiting agricultural productivity worldwide and is expected to intensify as global temperatures rise and precipitation patterns become more erratic due to climate

change [1–4], posing critical threats to food security and agricultural sustainability [5,6]. Drought imposes physiological stress on plants that disrupts vital processes such as photosynthesis, transpiration, and nutrient uptake, ultimately leading to reduced growth, yield, and overall plant health [7–9]. Plant growth-promoting bacteria (PGPB) have been proposed to enhance drought tolerance in some crops [10–12], although other studies have shown that the function of PGPB declines as soil dries [13,14]. One potential explanation for this discrepancy may be the method of inoculation. Solution applications are widely used; however, encapsulation treatments may enable more sustained release of PGPB into the soil and provide nutrients and/or medium to prolong the viability of the PGPB. Enhanced viability and survival of PGPB would be of particular importance in terms of ensuring their beneficial effects on plant growth under water deficit conditions.

Under drought conditions, plants often reduce stomatal conductance (G_{sw}) to limit water loss through transpiration [15–17]. This physiological adjustment restricts the uptake of carbon dioxide, leading to decreased photosynthesis [15,18,19]. Additionally, increased leaf temperatures resulting from stomatal closure and transpirative water-loss [20] elevate the risk of heat stress [21,22]. During drought stress, energy used for photochemistry decreases [23,24], and if not dissipated via protective mechanisms such as non-photochemical quenching [25], excess energy can cause oxidative stress by generating reactive oxygen species (ROS), this can lead to oxidative stress if the production of ROS exceeds the protective capacity of antioxidant systems [26,27]. The thylakoid membranes are especially sensitive to oxidative stress, showing reduced photosystem II (PSII) electron transport, as evidenced by chlorophyll fluorescence (*ChlF*) measurements. Drought stress lowers the numbers of reaction centers and electron transport rate due to damage to PSII. Chlorophyll fluorescence (*ChlF*) measurement is an effective tool for evaluating PSII activity and electron transport, offering insights into plant physiological responses to stress [28–30].

Plant growth-promoting bacteria (PGPB) have emerged as a promising strategy to improve plant drought tolerance [31,32]. These beneficial microorganisms colonize the rhizosphere and root surfaces, promoting plant growth through various mechanisms, including the enhancement of nutrient acquisition, production of phytohormones, and modulation of plant stress responses [32,33]. Plant growth-promoting bacteria have been shown to improve root morphology, leading to increased root length and surface area [34–36], which are critical for water and nutrient absorption during periods of drought [36,37]. Moreover, PGPB can induce systemic tolerance mechanisms in plants, enabling plants to more effectively cope with the impacts of drought [31,38]. Plant growth-promoting bacteria play crucial role in synthesizing phytohormones (e.g., abscisic acid (ABA), ethylene (ET), cytokinins (CKs), salicylic acid (SA), gibberellic acid (GAs), Indole acetic acid (IAA), 1-aminocyclopane-1-carboxylate deaminase (ACCD) [11,39] that regulate plant growth under stress conditions. Both *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a emit two common volatile compounds (acetoin and 2,3 butanediol) that are linked to their strong plant growth-promoting effects, unlike other PGPB strains that do not enhance growth through volatile emissions [40]. Phytohormones activate signaling pathways that enhance the production of secondary metabolites such as proline, polyamines, soluble proteins, photosynthetic pigments, ascorbic acid, malondialdehyde (MDA), and antioxidant enzyme activities [41,42]. This includes the activation of antioxidant defense systems, reducing oxidative stress and maintaining chlorophyll integrity, which is vital for sustaining photosynthetic activity during periods of drought [21,27,43]. Studies of the crop-improving effects of various species within the *Bacillus* genus, including *Bacillus subtilis*, highlight their beneficial effects on plant growth, such as chlorophyll enhancement, secondary metabolites, antioxidant enzymes, photosynthesis, water use efficiency, root growth in species like poplar, radish, sugarcane, and rice [10,44–46]. *Bacillus* species are known for their diverse mechanisms of action, including the production of phytohormones, solubilization of

nutrients, and the enhancement of soil microbial health [38]. Liu et al. [47] showed that PGPB inoculation, particularly with *Bacillus cereus* L90, enhances drought resistance in walnut (*Juglans regia*) seedlings by boosting antioxidant enzymes and improving key photosynthetic traits such as photosynthesis and stomatal conductance under different stages of drought. This improved physiological performance, including PSII activity, not only enhances drought tolerance but also contributes to overall plant vigor and productivity. *Bacillus subtilis* is one of the most widely studied and commercially used plant growth-promoting bacteria due to its robust spore-forming ability, production of phytohormones and volatile compounds, and well-documented stress-alleviating mechanisms [10,44], while sunflower (*Helianthus annuus* L.) serves as an excellent model crop because it is a globally important oilseed, food, and bioenergy species that is highly sensitive to drought stress, particularly in semi-arid regions increasingly affected by climate change [48].

The effectiveness of PGPB largely depends on their survival and persistence in soil [49]. Several studies have demonstrated inconsistent or limited positive effects of PGPB on plant growth, with either no measurable or only short-term benefits, exclusively under well-watered conditions [50,51]. When inoculated directly to the soil, PGPB populations decrease significantly due to harsh and fluctuating environmental conditions [52]. Factors such as desiccation, temperature changes, and competition with indigenous microorganisms can rapidly reduce bacterial viability, ultimately diminishing their plant growth–promoting effects. In this context, carriers are essential for shielding PGPB from adverse soil conditions, without such protection, stresses such as drought can severely impair bacterial survival and thereby reduce the effectiveness of the inoculant [14].

Encapsulation technologies offer a means to protect bacterial cells from these stresses while allowing their gradual release and sustained activity in the rhizosphere [53]. One of the most suitable methods for whole cell immobilization is entrapment within calcium alginate, because this technique is straightforward, non-toxic, and relatively cheap [14,54–56]. Sodium alginate is a readily available and non-toxic biological polymer; therefore, it is accepted as a suitable polymer for the encapsulation of bacteria [54]. Encapsulation, inspired by natural biofilms, embeds plant growth-promoting bacteria (PGPB) in polysaccharides such as alginate, chitosan, and cellulose derivatives [14]. This biomimetic approach protects inoculants under adverse conditions while maintaining their functionality, offering a promising solution for sustainable agriculture. Mendoza-Labrador et al. [52] demonstrated that encapsulating *B. subtilis* extended its activity to 70–89 days, ensuring steady release and prolonged plant-bacteria interactions. This method increased plant biomass by 22%, along with higher proline and crude protein content, compared to traditional solution applications under drought. Encapsulation performance can be improved by adding organic additives such as humic acid, skimmed milk, and whey protein, sugar [51,57,58]. Young et al. [57] showed that humic acid nourishes bacteria, supports their multiplication, and enhances plant growth by interacting with the root system in alginate beads.

Although encapsulation has been widely investigated for improving plant growth, secondary metabolites, and antioxidant enzyme activities under stress conditions [52,59–61], its impact on detailed photosynthetic mechanisms remains largely unexplored, a crucial omission if the real impact of PGPB on plant function is to be understood. A previous study explored the use of encapsulated *Pseudomonas libanensis* to improve plant growth and photosynthetic efficiency under drought stress in cowpea [62]. However, the results were inconclusive, suggesting that encapsulation efficacy may be influenced by several factors, such as plant, bacterial species and application method, underscoring the need for further research in this area. The literature reports numerous studies for encapsulation of different PGPB on plant growth, plant secondary metabolites and antioxidant enzyme activities of plants under different stress conditions [50,52,63] but crucially not photosynthetic performance.

To the best of our knowledge, very limited studies have compared solution-applied and encapsulated formulations of plant growth-promoting bacteria under drought stress, and none have specifically evaluated humic-acid-containing encapsulated *B. subtilis* in relation to OJIP-derived chlorophyll fluorescence parameters. Determining whether improved plant responses arise from the intrinsic bacterial activity or from enhanced persistence and controlled delivery provided by encapsulation remains a critical unsolved question which represents the novelty of the present work. In addition, although sunflowers have previously been treated with different plant growth-promoting bacteria delivered through various carrier systems, the use of a humic-acid-containing encapsulated *Bacillus subtilis* formulation under drought stress constitutes a novel study design [50,52,63].

Such distinction is essential for understanding the mechanistic basis of PGPB-mediated drought tolerance and for optimizing formulation strategies. Encapsulation not only protects microorganisms from environmental stresses but also enable their localization near the root zone and improve their establishment in the rhizosphere [64]. Furthermore, the inclusion of humic acid is hypothesized to serve a dual purpose: reinforcing the structural integrity of the bead to prevent non-specific leakage, while potentially acting as a bio-stimulant for the bacteria once established. Through sustained release, encapsulation technologies may enable the persistence of PGPB within the rhizosphere during changes in soil water status throughout the growing season. Although several studies have examined the encapsulation of various PGPB strains, direct comparisons between conventional solution application and different encapsulation strategies of the same bacterial strain under drought stress remain scarce, no previous work has simultaneously evaluated solution-applied versus alginate-encapsulated *Bacillus subtilis* (with and without humic acid) in a drought-sensitive sunflower variety, with a particular focus on detailed photosynthetic performance and OJIP-derived chlorophyll fluorescence parameters. The present study therefore addresses this critical knowledge gap by systematically comparing these delivery methods, providing new mechanistic insights into how encapsulation, particularly when supplemented with humic acid, enhances bacterial persistence and prolongs physiological benefits under prolonged water deficit.

The optimization and encapsulation of PGPB application have potential to advance sustainable agriculture and food security in semi-arid regions by protecting these microorganisms from adverse environmental conditions, ensuring their viability and sustained activity in the soil. This mechanism allows for prolonged interaction with plant roots, thereby maximizing their beneficial effects on plant physiology: (i) creating a more favorable microenvironment for microbial strains, thereby reducing the rapid decline in cell viability during storage; (ii) enhancing the ability of the introduced strains to compete against the native soil microfauna following their introduction; and (iii) minimizing losses due to predation by soil microfauna following soil introduction. These strategies collectively aim to provide a consistent and reliable source of viable cells that can engage with plants and the soil microbiome over an extended period.

This manuscript aims to investigate the effects of three different (*in free solution form (B), alginate beads-without humic acid (EB), alginate beads-supplemented with humic acid (HEB)*) delivery methods of a specific PGPB strain (*B. subtilis*) applications on plant growth parameters and physiological responses such as photosynthetic efficiency, chlorophyll fluorescence, stomatal conductance in a drought sensitive sunflower variety, *H. annuus* var. Deray subjected to drought stress. This study investigates; (i) if *B. subtilis* improves the resilience of drought-sensitive sunflower under drought conditions; (ii) whether encapsulation of *B. subtilis* maintains a superior protective microenvironment that sustains beneficial effects on plants over an extend period under drought conditions, and; (iii) whether the synergistic combination of *B. subtilis* with humic acid increases any potential capacity of *B. subtilis* to sustain a beneficial interaction with plants to alleviate the negative effects of drought stress over a prolonged period.

2 Materials and Methods

2.1 Plant Material and Growth Conditions

Sunflower (*Helianthus annuus* L.) seeds of the "Deray" cultivar, identified as drought-sensitive through field trial research, were obtained from the Trakya Agricultural Research Institute-Edirne, Türkiye. The experiment took place during autumn 2023 at Konya Food and Agriculture University in Konya, Türkiye, (GPS coordinates: 37.8762° N, 32.4742° E), under greenhouse conditions, with temperature and humidity logged every 30 min using a data logger (Cem DT-172). The average recorded temperature was 20°C, and the average relative humidity was 47%. Detailed temperature and relative humidity data during the experiment are given in Supplementary Material Fig. S1. Seeds were initially sown in trays filled with compost (Klasmann-Deilmann GmbH, Geeste, Germany). After two weeks, the seedlings were transplanted into individual 3-L pots containing a mixture of 10% compost and 90% sand.

The experiment consisted of 64 pots arranged in a factorial design with two irrigation treatments (well-watered and drought) and four bacterial treatments (control, solution-applied, encapsulated, humic acid-encapsulated), and eight replicates in each group. The experiment performed once and each plant considered an independent biological replicate, without sample pooling. After plants were assigned to one treatment group, bacterial solution (10^8 CFU/mL, 1 mL), the alginate beads (Alg.) and humic acid beads (H-Alg.) were applied to the root zone of each designated plant during seedling transplantation. Each pot received 10^8 CFU/mL of bacteria for both suspension and encapsulation treatments. All groups were irrigated daily with distilled water to 70% of field capacity (FC) for two weeks. Before transplantation of the seedlings, pot field capacity was determined by saturating the soil mixture and allowing it to drain for 48 h, with the remaining water content considered as 100% FC and used as the baseline for daily irrigation levels. During the initial two-week period after seedling transplantation, seedlings received NPK fertilizer (10.30.10+ ME, Gübretaş, Türkiye) once a week. After this period, seedlings were 4 weeks-old and drought stress was applied by irrigating designated seedlings daily to 30% FC for 29 days, while well-watered groups continued to receive irrigation at 70% FC until the end of the experiment [65].

2.2 Bacterial Strain and Culture Conditions

Soil isolated *Bacillus subtilis* was obtained from the Bacterial Strain Collection of Microbiology and Molecular Biology Laboratory at Konya Food and Agriculture University. Bacterial culture was stored at −80°C in Luria-Bertani (LB) broth supplemented with 50% glycerol. It was streaked onto LB agar medium containing 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, and 12 g/L bacteriological agar; incubated at 37°C overnight.

For encapsulation, the bacterial culture was prepared by suspending a single colony in LB medium and incubating it at 37°C with constant shaking at 180 rpm overnight. It was then sub-cultured and incubated to reach the exponential growth phase. Bacteria were harvested with centrifugation at 4000× *g* for 15 min, and the pellet was resuspended and adjusted to OD_{600} = 0.5 (app. 10^8 CFU/mL) with physiological saline.

2.3 Encapsulation

2.3.1 Preparation and Characterization of Alginate Beads

Alginate beads were prepared following the slightly modified version of the method of Souza-Alonso, Rocha [62]. Two different calcium alginate bead formulations were prepared. For the first formulation (Alg.), 500 µL of bacterial suspension was mixed with 2.5 mL 1% (w/v) alginate solution (Sigma, Germany). For the second formulation (H-Alg.), alginate solution and humic acid (Biomol, Genta Tarim, Türkiye) were

mixed 1:1 ratio (v/v), and the same amount of bacterial suspension was added. Humic acid was incorporated into the alginate beads not only as a structural additive but also because it functions as a biostimulant that enhances bacterial viability by serving as a carbon and energy source, promotes biofilm formation and bacterial proliferation within the beads, improves root colonization through its chelating and hormone-like properties, and synergistically augments plant stress tolerance by stimulating antioxidant systems and nutrient uptake [66–68].

For the formation of beads, the alginate-bacteria mixture was dropped into the 2% calcium chloride solution with a Pasteur pipette. The resulting Alginate (Alg.) beads were collected, washed with sterile 0.85% saline, and used for further evaluation. The same procedure was applied to form the Humic Acid-Alginate (H-Alg.) beads. The beads (Alg. and H-Alg.) were then transferred to the root zone of each plant at the time of transplantation. The bead morphology was examined using a stereomicroscope and a bright-field microscope. The diameter of the beads was determined by processing the images in ImageJ software.

2.3.2 Determination of Viable Cell Recovery from Alginate Beads

To determine viable cell counts after encapsulation, 10 Alg. or H-Alg. beads were added into 10 mL of 200 mM sodium citrate buffer (pH 5.5) and stirred gently for 30 min. Serial dilutions were made with physiological saline and enumerated with the spread plate technique onto LB agar plate.

2.4 Time-Course Evaluation of Bacterial Release

The release of bacteria from the beads was monitored over 10 days by incubation in physiological saline. 10 beads were transferred to 5 mL of 0.85% saline and incubated at 37°C for 10 days with gentle shaking. Samples of the saline solution were taken on days 1, 2, 3, 5, 7, 10 and spread onto an LB agar plate.

2.5 Plant Morphological and Physiological Measurements

After transplantation of the seedlings into the pots when they were two weeks old, seedling height was recorded every two days to monitor plant growth. Cumulative water loss as evapotranspiration was calculated by weighing the pots daily from the onset of drought stress, summing the daily water losses. At harvest, the number of fully developed leaves and above-ground and below-ground (root) biomass were measured.

Dark-adapted chlorophyll fluorescence was measured using a Pocket Plant Efficiency Analyzer (PEA, Hansatech Instruments Ltd., King's Lynn, UK) after a 20-min dark adaptation period. Dark-adapted Chlorophyll Fluorescence measurements were collected following the protocol used for PEA measurements at the end of the experiment, when seedlings were two-month-old [69]. Plants were then exposed to a saturating light pulse (intensity > 3000 μmol m^{-2} s^{-1}, with excitation at 650 nm). Fluorescence readings were taken from the uppermost fully expanded third leaf of each plant between 10:00 and 12:00 AM, at the conclusion of the experiment. The data were analyzed using the OJIP curve, which depicts a polyphasic fluorescence transient of chlorophyll a (*Chl* a) on a logarithmic timescale, providing insights into the activity of photosystem II (PSII). The fluorescence response is marked by distinct phases: O (origin) represents the initial fluorescence intensity (F*o*) at 30 μs, J and I are the fluorescence intensities (F_J and F_I) at 2 and 30 ms, respectively, and P (peak) represents the maximum fluorescence intensity (F_M) at 1 s [70]. Definitions and formulas for the PEA parameters used in this study are given in Supplementary Material Table S1.

Stomatal conductance of water vapor (G_{sw}) and light-adapted chlorophyll fluorescence parameters were measured using a Li-600 porometer and fluorimeter using a light flash intensity of 7000 μmols m^{-2} s^{-1} and a flow rate of 150 μmols s^{-1} (Li-Cor Inc., Lincoln, NE, USA). The third fully developed leaf on each plant

was chosen for these measurements. Stomatal conductance to water vapor (G_{sw}, mmol/m^2·s) was assessed to evaluate plant water regulation and photosynthetic physiology. This was determined by measuring the rate of water vapor released from a leaf within a chamber, based on the difference in water vapor concentration between incoming and outgoing air. The quantum sensor on the Li-600 was used to measure ambient photosynthetic photon flux density (PPFD) at the time of the measurement—these values ranged from 200 to 850 μmol m^{-2} s^{-1}, with a mean PPFD of 520 μmol m^{-2} s^{-1}. Maximum fluorescence (F_M) represents the peak fluorescence yield in a light-adapted state, reflecting the efficiency of PSII under light conditions [71]. Quantum efficiency of PSII (ΦPSII) indicates the fraction of absorbed light energy used by PSII for electron transport, calculated from maximum and steady-state fluorescence yields in a light-adapted leaf [72]. The electron transport rate (ETR, μmol m^{-2} s^{-1}) quantifies the rate of electron flow through the photosynthetic electron transport chain, providing an indicator of photosynthetic activity and overall plant performance [73].

2.6 Statistical Analysis

Data were first tested for normality using the Shapiro-Wilk test (see Supplementary Material Table S2A,B). ANOVA Summary Table for df and F-value, see Supplementary Material Table S3A,B. The effects of solution application bacterial treatment, encapsulation and drought stress on various parameters were analyzed using one-way ANOVA, with post-hoc comparisons via Duncan's multiple range test and LSD test. Analyses were conducted using SPSS 27.0 (IBM Corp., Armonk, NY, USA) or GraphPad Prism 10.4. (GraphPad Software, San Diego, California USA). The differences in the size of the beads were determined by Student's *t*-test. The statistical threshold was $p < 0.05$.

The experiment assessed the effects of solution-applied and encapsulated bacterial inoculation under drought stress on sunflower seedlings, using eight replicates for each of the eight treatment groups:

i. Water Regime: Drought (D) or Well-watered (W).
ii. Bacterial Treatment: None (W and D), Solution-Applied (BW and BD), Encapsulated (EBW and EBD), Humic Acid-Encapsulated (HEBW and HEBD).

3 Results

Under both well-watered and drought conditions, the application of *Bacillus subtilis* in solution and encapsulated forms (H-Alg. and Alg.) led to significantly improved morphological parameters compared to their control conditions (Well-watered (W) and Drought (D)).

3.1 Plant Growth and Water Use Efficiency

The application of *Bacillus subtilis* in solution (BW) and the encapsulated form with and without humic acid (HEBW and EBW) under well-watered conditions resulted in a gradual increase in plant height throughout the experiment. This increase was statistically significant compared to their control (well-watered-W) counterparts (Fig. 1). At the conclusion of the experiment, the BW and EBW treatments exhibited the highest plant height under well-watered conditions. Meanwhile, the solution-applied *Bacillus subtilis* (BD) treatment demonstrated a significantly greater and sustained increase in plant height compared to the well-watered control group (Fig. 1a). Although above-ground growth slowed towards the end of the experiment, BD maintained the highest plant height that was 15.2% greater in comparison to their control group (D) under drought conditions. Under drought conditions, the encapsulated *Bacillus subtilis* treatments (HEBD and EBD) showed the highest plant height by 14.6 and 6.1% respectively, compared to the control group (D) (Fig. 1a and Supplementary Material Table S4).

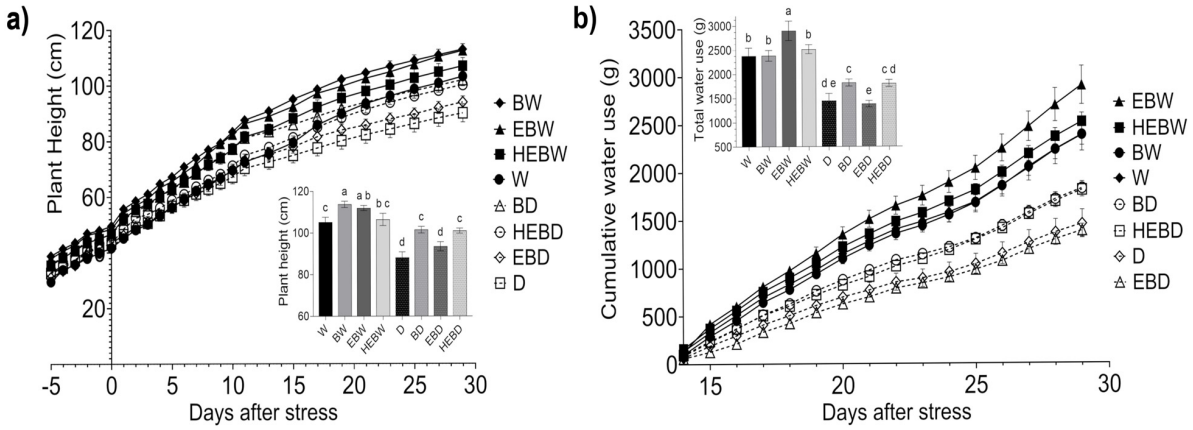

Figure 1: (**a**) Time course of plant height during the experiment and final plant height at the end of the experiment (minor graph lower inset), (**b**) Cumulative water loss as evapotranspiration of each treatment during the experiment and final cumulative water use at the end of the experiment (minor graph upper inset). *Bacillus* + Well-watered (BW, ●), Encapsulated-*Bacillus* + Well-watered (EBW, ▲), Humic Acid Encapsulated-*Bacillus* + Well-watered (HEBW, ■), Well-watered (W, ♦), *Bacillus* + Drought (BD, ○), Humic Acid Encapsulated-*Bacillus* + Drought (HEBD, □), Encapsulated-*Bacillus* + Drought (EBD, △), Drought (D, ◇). Values represent means ± SE (n = 8 independent plants). Different and identical letters above histograms indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Duncan's test, n = 8.

Similarly, the EBW treatment exhibited the highest cumulative water use/loss throughout the experiment ($p < 0.05$); moreover, the result at the end of the experiment (when seedlings were two months-old) was significantly higher than other groups (HEBW, BW and W). The application of humic acid-encapsulated *Bacillus subtilis* (HEBW) resulted in relatively higher values compared to the control group (W), although the difference was not statistically significant. Under drought conditions, BD and HEBD treatments exhibited prolonged higher evapotranspirative water use compared to the D and EBD groups (Fig. 1b). Furthermore, water use, plant height, and G_{sw} values for EBW, HEBW, and BW were significantly higher than those observed in the control (W) treatment, which exhibited the lowest values for these parameters.

In terms of above-ground and below-ground biomass the BW and HEBW treatments showed relatively higher biomass accumulation under the well-watered (W) condition. The application of the BW treatment resulted in a significant increase in above-ground biomass ($p < 0.05$) comparison to control (W) application. However, no statistically significant differences in above-ground biomass were observed between other treatments (BW, EBW and HEBW) (Fig. 2). Under drought stress, HEBD exhibited significantly higher above-ground biomass than drought (D) and also EBD treatment ($p < 0.05$). Solution applied *Bacillus subtilis* under both well-watered and drought (BW and BD) conditions increased above-ground biomass significantly ($p < 0.05$) compared to their uninoculated control (W and D) treatments (Fig. 2a). Below-ground biomass was not significantly affected by any of the treatments under either condition. However, the BW treatment showed a relatively higher root mass under well-watered conditions, while the HEBD treatment exhibited a comparatively higher root mass under drought conditions (Fig. 2b). Under drought conditions, BD and HEBD showed the highest morphological values than other groups (D and EBD).

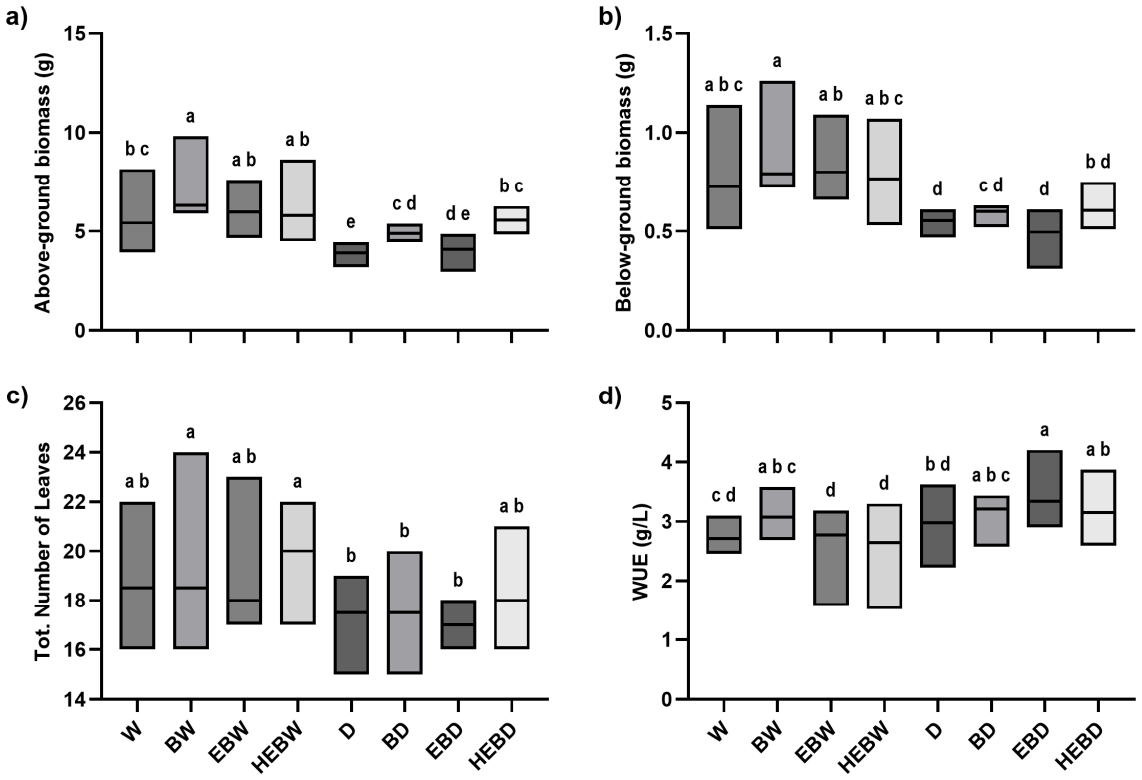

Figure 2: (**a**) Above-ground dry biomass (one-way ANOVA $F_{7,56} = 9.2$; $p = 1.7 \times 10^{-7}$) (**b**) Below-ground (root) dry biomass (one-way ANOVA $F_{7,56} = 8.8$; $p = 2.8 \times 10^{-7}$), (**c**) Total leaf number (one-way ANOVA $F_{7,56} = 2.6$; $p = 0.024$), and (**d**) Water use efficiency (WUE) (one-way ANOVA $F_{7,56} = 3.8$; $p = 0.002$) of each treatment at the end of the experiment. Well-watered (W), Bacillus + Well-watered (BW), Encapsulated-*Bacillus* + Well-watered (EBW), Humic Acid Encapsulated-*Bacillus* + Well-watered (HEBW), Drought (D), *Bacillus* + Drought (BD), Encapsulated-*Bacillus* + Drought (EBD), Humic Acid Encapsulated-*Bacillus* + Drought (HEBD). Values represent means ± SE (n = 8 independent plants). Different and identical letters above histograms indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Duncan's test.

3.2 Photochemical Efficiency

Stomatal conductance of water vapor (G_{sw}) increased significantly with both solution and encapsulated forms of *Bacillus subtilis* applications (BW, EBW, and HEBW) under well-watered conditions. Specifically, the BW treatment resulted in a 24% increase in G_{sw}, the EBW treatment led to a 45% increase, and the HEBW treatment caused a 38% increase, compared to the control group (W). Under drought conditions, G_{sw} decreased by 93% in comparison to the well-watered control (W) group. However, this reduction was mitigated by the application of alginate-encapsulated *Bacillus subtilis* (EBD) and humic-acid-encapsulated *Bacillus subtilis* (HEBD) (Supplementary Material Tables S5–S7). The EBD treatment increased G_{sw} threefold, while the HEBD treatment resulted in a nearly tenfold increase, compared to the control (D) treatment (Fig. 3a). Fig. 3b demonstrates that encapsulation of *Bacillus subtilis* with both with and without humic acid (EBW, HEBW and EBD, HEBD) reduce leaf temperature under both well-watered and drought conditions. This reduction is consistent with the observed increase in stomatal conductance of water vapor (G_{sw}) (Fig. 3a).

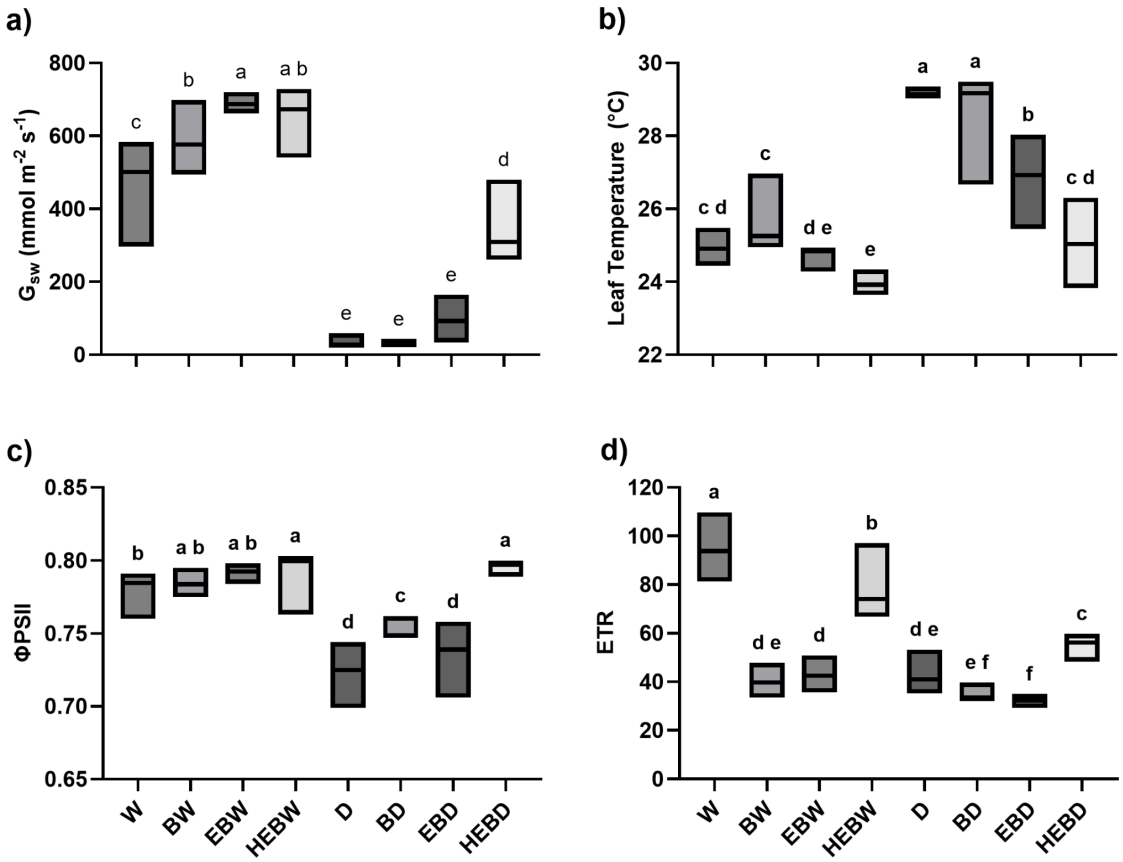

Figure 3: (**a**) Stomatal Conductance (G_{sw}) (one-way ANOVA $F_{7,40} = 62.6$; $p = 1.5 \times 10^{-19}$), (**b**) Leaf temperature-T_{leaf} (°C) (one-way ANOVA $F_{7,40} = 41.8$; $p = 2.0 \times 10^{-16}$), (**c**) Quantum efficiency of PSII (ΦPSII) (one-way ANOVA $F_{7,40} = 8.2$; $p = 3.0 \times 10^{-6}$), (**d**) Electron transport rate (ETR) of each treatment at the end of the experiment (one-way ANOVA $F_{7,40} = 61.7$; $p = 2.0 \times 10^{-19}$). Well-watered (W), *Bacillus* + Well-watered (BW), Encapsulated-*Bacillus* + Well-watered (EBW), Humic Acid Encapsulated-*Bacillus* + Well-watered (HEBW), Drought (D), *Bacillus* + Drought (BD), Encapsulated-*Bacillus* + Drought (EBD), Humic Acid Encapsulated-*Bacillus* + Drought (HEBD). Values represent means ± SE (n = 8 independent plants). Different and identical letters above histograms indicate significant ($p < 0.05$) and no significant differences ($p > 0.05$), respectively, between the means according to Duncan's test.

All *Bacillus subtilis* applications (BW, EBW, and HEBW) increased the quantum yield of photosystem II (ΦPSII) under well-watered conditions. Notably, the HEBW treatment exhibited a significantly higher ΦPSII compared to the control (W) group ($p < 0.05$). Under drought stress, the HEBD treatment significantly increased ΦPSII by 10%, while the BD treatment increased ΦPSII by 4% compared to the control (D) group ($p < 0.05$) (Fig. 3c). An analysis of the electron transport rate (ETR) under both well-watered and salt stress conditions, across various treatments (solution bacteria and encapsulated bacteria: BW, EBW, BD, and EBD), revealed a negative impact on ETR. However, under well-watered conditions, the humic-acid encapsulated bacteria treatment (HEBW) exhibited the most significant effect among all treatments (Fig. 3d). Under drought conditions, the solution and encapsulated-bacteria (BD and EBD) treatments exhibited reductions in ETR similar to those observed under well-watered conditions. However, the HEBD treatment increased ETR by 31% compared to the control group ($p < 0.05$) (Supplementary Material Tables S5–S7).

The analysis of chlorophyll fluorescence induction transient (OJIP) curves revealed that the drought treatment significantly affected the OJIP curves of sunflower leaves. Encapsulated *B. subtilis* without

humic acid (EBD) exhibited the lowest maximum fluorescence at the end of the experiment. In contrast, solution-applied *B. subtilis* (BD) and humic acid-encapsulated *B. subtilis* (HEBD) showed the highest maximum fluorescence under drought conditions, suggesting lower damage to photosystem II (PSII). Under well-watered conditions, both solution-applied and encapsulated *B. subtilis* (BW and EBW) did not show significant differences compared to the control group (W). Humic acid-encapsulated *B. subtilis* (HEBW and HEBD) displayed similar results under both conditions, with a slight reduction in fluorescence under drought stress (D) (Fig. 4a).

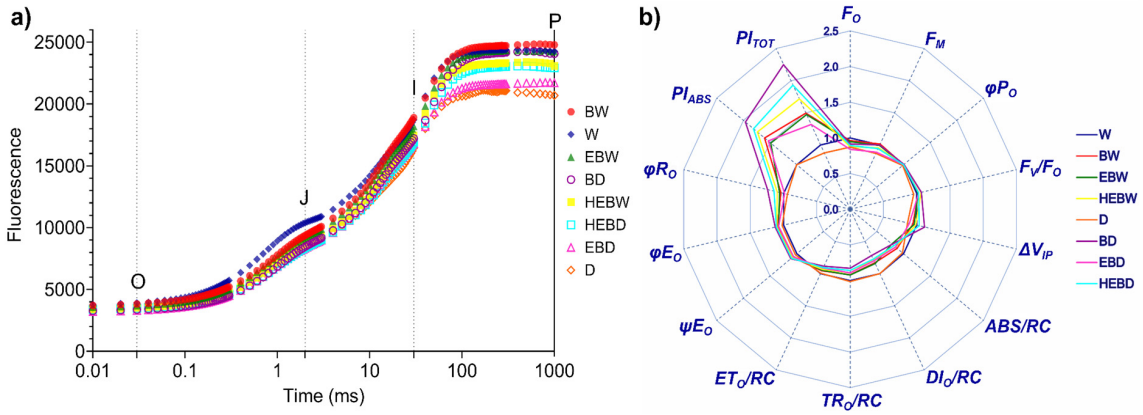

Figure 4: (**a**) Average OJIP induction curves of sunflower subjected to different treatments, (**b**) Spider plot of Chlorophyll fluorescence (*ChlF*) Parameters measured at the end of the experiment (see Supplementary Material for definitions and descriptions) extrapolated from the OJIP transient curve. Well-watered (W, ♦), *Bacillus* + Well-watered (BW, •), Encapsulated-*Bacillus* + Well-watered (EBW, ▲), Humic Acid Encapsulated-*Bacillus* + Well-watered (HEBW, ■), Drought (D, ◇), *Bacillus* + Drought (BD, ○), Encapsulated-*Bacillus* + Drought (EBD, △), Humic Acid Encapsulated-*Bacillus* + Drought (HEBD, □) n = 8.

Under drought conditions, solution-applied *B. subtilis* (BD), encapsulated *B. subtilis* with humic acid (HEBD), and encapsulated *B. subtilis* without humic acid (EBD), induced significant increases in the performance index based on the photochemical and non-photochemical energy absorption of chlorophyll antennae (PI_{ABS}) by 95%, 81%, and 53%, respectively, compared to drought treatment (D). Under well-watered conditions, solution-applied *B. subtilis* (BW), encapsulated *B. subtilis* with humic acid (HEBW), and encapsulated *B. subtilis* without humic acid (EBW) showed increases in PI_{ABS} by 60%, 74%, and 47%, respectively, compared to the well-watered control group (W). Drought (D) treatment did not result in any significant changes in PI_{ABS} compared to the well-watered treatment (W). The performance index incorporating the concentration of reaction centers (PI_{TOT}) was highest in solution-applied *B. subtilis* (BD), encapsulated *B. subtilis* with humic acid (HEBD), and encapsulated *B. subtilis* without humic acid (EBD), with increases of 157%, 121%, and 51%, respectively, compared to drought treatment (D). These rises were notably higher than the increases observed in PI_{TOT} under well-watered conditions, where *B. subtilis* applications (BW, HEBW, and EBW) led to increases of 50%, 72%, and 47%, respectively (Fig. 4b and Supplementary Material Tables S5–S7).

Although the increases in other parameters were not as pronounced as in PI_{ABS} and PI_{TOT}, the efficiency of electron chain flux (ΔV_{IP}) was enhanced by 30% in solution-applied *B. subtilis* (BD) and 20% in encapsulated *B. subtilis* with humic acid (HEBD) under drought conditions. This parameter did not show significant changes under well-watered conditions. Additionally, the quantum yield of the final stage acceptor reduction of PSI (φRo) increased by 32% in BD and 22% in HEBD under drought stress.

The absorption of chlorophyll antennae per reaction center (ABS/RC), the flux of energy dissipated for each reaction center (DI_o/RC), and the flux of trapped energy per reaction center leading to the reduction of plastoquinone A (TR_o/RC) were reduced by 15–20% with solution-applied *B. subtilis* (BD) and encapsulated *B. subtilis* with (HEBD) and without humic acid (EBD), particularly under drought conditions. These reductions indicate less damage to the reaction centers under drought stress. In contrast, under well-watered conditions, these decreases were much less pronounced (Fig. 4b, Supplementary Material Tables S5 and S7).

3.3 Characterization of Alginate Beads

Quantitative analysis showed that Alg. beads had an average major axis of 4.55 + 0.56 mm and a minor axis of 3.94 + 0.12 mm (n = 40), while H-Alg. beads had an average major axis of 4.56 + 0.39 mm and a minor axis of 3.78 + 0.08 mm (n = 40) (Table 1). No statistically significant difference was observed in the major axis between Alg. Beads and H-Alg. beads (p = 0.093), whereas the minor axis differed significantly between the two groups (p < 0.0001). The aspect ratio, calculated as the ratio of the major axis to the minor axis, was used to assess the uniformity of bead morphology. Beads formulated with only alginate had an average ratio of 1.15 ± 0.11, while those from alginate w/humic acid showed a slightly higher average aspect ratio of 1.21 ± 0.08 and were statistically different from each other (p = 0.006). These results suggest that the beads were moderately elliptical, with H-Alg. beads exhibiting more elongation compared to Alg. beads. Morphological observations of the beads revealed rather smooth and uniform structure (Fig. 5).

Table 1: Size measurements and aspect ratios of Alg. and H-Alg. beads. Data represent mean ± SD (n = 40).

Bead Formulation (Type)	Major Axis (mm)	Minor Axis (mm)	Aspect Ratio
Alg. Beads	4.55 + 0.56	3.94 + 0.12	1.15 + 0.11
H-Alg. Beads	4.56 + 0.39	3.78 + 0.08	1.21 + 0.08

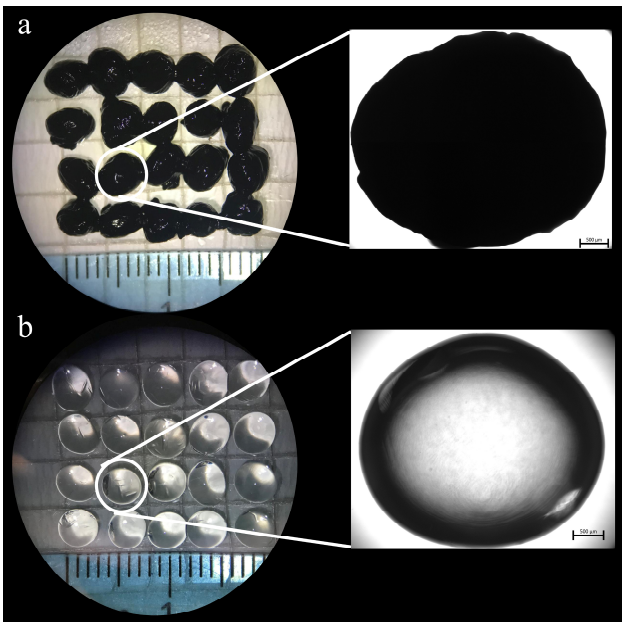

Figure 5: Stereomicroscopic (left) and brightfield microscopy (right) images of (**a**) H-Alg and (**b**) Alg beads. Left panels include a physical millimeter scale; right panel scale bar represent 500 µm.

In the Alg.bead formulation, the number of viable cells recovered from the beads was calculated as 1.08 ± 0.45 × 108. In the H-Alg. beads, the recovered bacterial load was estimated to be 6.72 ± 3.56 × 107 CFU (Table 2). Over the 10-day storage period tested, no measurable bacterial release was observed in the 0.85% NaCl as storage media. Although no bacterial release was detected from the alginate or humic acid-alginate beads when incubated in 0.85% NaCl for 10 days, this outcome confirms excellent storage stability and protection against premature leakage during handling and short-term storage. This stability is advantageous for practical formulation and application. However, bacterial release in soil is expected to follow different kinetics. In the soil environment, bead degradation and subsequent bacterial liberation are driven by multiple factors absent in simple saline solution, including ion exchange with soil cations (e.g., Na^+, K^+, Mg^{2+}), enzymatic breakdown of the alginate matrix by native soil microorganisms, and physical disruption by growing roots and soil aggregates. These mechanisms enable a more gradual and sustained release of *B. subtilis* into the rhizosphere compared to the highly stable conditions observed in the laboratory saline test. Future studies should quantify actual release rates and colonization dynamics directly in soil to further validate this process.

Table 2: Bacterial cell counts in Alg. and H-Alg. bead. Data represent SD ± mean.

Bead Formulation (Type)	Total CFU Added (Input)	Total CFU Recovered
Alg. Beads	$6.28 \pm 3.50 \times 10^8$	$1.08 \pm 0.45 \times 10^8$
H-Alg. Beads	$3.85 \pm 2.33 \times 10^8$	$6.72 \pm 3.56 \times 10^7$

4 Discussion

The application of *B. subtilis* as a plant growth-promoting bacterium (PGPB) in solution or encapsulated forms significantly enhanced the morphological and physiological performance of the drought-sensitive 'Deray' sunflower variety under water deficit. This suggests that the beneficial effects of the PGPB were evident under water deficit conditions in contrast to previous studies [49,74,75]. Under drought stress, solution-applied *B. subtilis* (BD) increased plant height by 15.2%, while encapsulated forms (HEBD and EBD) improved plant height by 14.6% and 6.1%, respectively, compared to the water deficit control (D) (Fig. 1a; Supplementary Material Table S4). These gains likely stem from PGPB-induced phytohormone production (e.g., IAA, ABA) and enhanced root morphology, facilitating better water and nutrient uptake [11,38]. Similarly, above-ground biomass increased significantly in BD and HEBD treatments (Fig. 2a), corroborating prior studies indicating the positive impact of *B. subtilis* on plant biomass gain in water deficit stressed crops such as sugarcane and rice by modulating antioxidant systems and nutrient solubilization [10,45].

Total water use and evapotranspiration were higher in PGPB-treated groups under both regimes (Fig. 1b), yet water use efficiency (WUE) improved under water deficit, particularly in HEBD (Fig. 2d), suggesting a degree of stomatal closure to prevent excessive water loss [17]. This is consistent with the putative role of PGPB in inducing systemic tolerance via EPS production and biofilm formation, which retain soil moisture [76,77]. Stomatal conductance was significantly higher under water deficit conditions with the encapsulated *B. subtilis* treatment (EBD: 3-fold, HEBD: 10-fold vs. D; Fig. 3a), this higher G_{sw} reduced leaf temperature (Fig. 3b) and potentially alleviated photooxidative stress associated with heat stress [15,21]. Plant transpiration functions as an evaporative cooling mechanism for leaves; therefore, higher leaf temperatures indicate stomatal closure, which is reduction in G_{sw}, in response to increased stress. These changes mitigated the deleterious impacts observed following the 93% decrease in G_{sw} observed in

the control plants, that likely impaired CO_2 uptake and photochemical energy usage leading to oxidative stress [26,27].

Photosynthetic efficiency parameters further underscored the potential benefits of PGPB application of leaf level physiological performance. The quantum yield of PSII (ΦPSII) and electron transport rate (ETR) increased under drought (HEBD: 10% and 31% vs. D; Fig. 3c,d), indicating preserved thylakoid membrane integrity and reduced oxidative damage [27,78]. Chlorophyll fluorescence (*ChlF*) analysis via OJIP curves revealed higher maximum fluorescence in BD and HEBD (Fig. 4a), with elevated performance indices such as PI_{ABS} (BD: 95%, HEBD: 81%, EBD: 53% vs. D) and PI_{TOT} (BD: 157%, HEBD: 121%, EBD: 51% vs. D) (Fig. 4b; Supplementary Material Table S5). Reductions in ABS/RC, DIo/RC, and TRo/RC (15–20% in PGPB treatments) suggest fewer damaged reaction centers and efficient energy dissipation [28,30]. Enhanced ΔVIP and φRo in BD and HEBD (30% and 32%; 20% and 22%) highlight improved electron flux to PSI in contrast to the lower values observed under water deficit conditions [79].

The improved performance of plants grown with encapsulated *B. subtilis*, especially with humic acid, was likely associated with increased bacterial viability (Tables 1 and 2), protecting the bacteria against stressors such as desiccation, salts, and native microbes [14,52]. Humic acid likely enhanced bead stability and nutrient provision, reducing bead size slightly while boosting elongation (aspect ratio 1.21 vs. 1.15), consistent with enriched alginate formulations [57,63]. The carboxylic acid groups in humic acid have the ability to form hydrogen bonds with the alginate chains in the matrix. This would create a denser matrix, where the bacteria are entrapped within the polymer chains which prevents the premature release more efficiently [57]. The reduction in the average bead size with the inclusion of humic acid may be attributed to the lower final alginate concentration and reduced viscosity of the gel matrix (Table 1), which is consistent with previous studies reporting that decreasing alginate concentration or incorporating additional components can result in smaller bead formation due to a change in solution viscosity [80,81].

Humic acid may contribute to plant performance under drought through multiple mechanisms, including stimulation of antioxidant defense, improved nutrient bioavailability, and support of beneficial rhizosphere interactions [82–84]. Therefore, its role in the present system is likely associated not only with direct plant effects, but also with improved bacteria–plant–soil interactions under water-deficit conditions. Following these, mechanistically, humic acid serves as an electron shuttle [66,67,85] which maintains the physiological readiness of the bacteria. Moreover, the humic acid released from the matrix during degradation, may help the plant's own stress-defense along with its ability to induce chemotaxis for the bacteria in the rhizosphere [86].

These results indicate that although the encapsulation process may slightly reduce the viability of the initial inoculum, a remarkable number of live cells were successfully retained within the beads (Table 2). As previously reported, chemical and mechanical stress during the encapsulation process may hinder the viability of bacteria rather than ineffective cell entrapment. This interpretation is supported indirectly by the insignificant bacterial growth in $CaCl_2$ solution following bead preparation Importantly, the bacterial dose used in both free-cell and encapsulated treatments was standardized at the application stage, minimizing concern that the observed plant responses were confounded by differences in inoculum amount. Although colonization was not measured directly, the physiological responses observed in plants are consistent with the applied bacterial level is sufficient for soil colonization.

The absence of premature release is quite crucial, as it reduces the risk of bacterial loss before plant application. This release pattern is partially consistent with the previous reports [87,88]. It is confirmed that bacteria are retained in the beads during the storage process and released specifically into the soil environment. The mechanism of lackness of release in physiological saline is due to the "egg-box" model of

alginate-based beads. In this model, the divalent Ca^{+2} ions crosslink the alginate chains and the achievement of release is possible when these sites are destabilized by chelating agents such as citric acid. In a saline environment, however, Cl-ions lack this chelating ability, resulting in closed, dense confirmation of the beads kept intact during storage conditions. In addition, the nanometer-scale pore structure of calcium alginate beads is considerably smaller than the micrometer-scale size of bacterial cells, which further limits passive bacterial diffusion from the intact matrix [68].

The soil intrinsically contains ionic components such as citrate [89,90] and microbial populations, which are responsible for the dissociation of crosslinks between calcium and alginate. Once the beads applied to soil, these chelators remove the divalent ion from the structure which increases the pore size gelation and swelling of the bead polymer network [52,91]. This facilitates the localized high density delivery of the bacteria. The absence of premature release is quite crucial, as it reduces the risk of bacterial loss before plant application. This release pattern is partially consistent with the previous reports [87,88]. The viable cell recovery within the beads (1.08×10^8 CFU in Alg; 6.72×10^7 in H-Alg) and minimized premature loss over 10 days creates an advantage for the PGPB application and are consistent with biomimetic approaches mimicking biofilms [88,91]. This contrasts with solution applications, where efficacy wanes due to rapid decline [92], potentially accounting for the observed benefits of HEBD application under water deficit. While direct quantification of root colonization (e.g., via qPCR or CFU/g root tissue) was not performed in this study, the improvement in photosynthetic efficiency and drought resilience in treated plants indicates the functional bacterial establishment.

The results of the present study extend beyond growth metrics (e.g., [52,62] by detailing the effect of PGPB encapsulation on *ChlF* in sunflower. While *Pseudomonas* encapsulation yielded mixed results [62], the resilience of *B. subtilis* to water deficit was evident, potentially due to species-specific traits. The present study was conducted in pots under glass house conditions. Future studies should explore long-term field trials and microbiome shifts utilizing molecular assays and gene expression analysis to validate scalability for semi-arid agriculture (e.g., [93]). Overall, encapsulation optimizes PGPB delivery, which enables symbiotic plant-bacteria interactions amid rhizosphere changes [64], offering a sustainable tool against drought in sunflower cultivation.

5 Conclusions

This study demonstrates that *Bacillus subtilis* application, particularly in encapsulated forms with or without humic acid, significantly mitigates water deficit stress in sunflower by enhancing morphological growth (height, biomass) and physiological parameters (G_{sw}, ΦPSII, ETR, and dark adapted *ChlF* indices). Encapsulation prolonged bacterial viability, outperforming solution application methods under water deficit, with humic acid further amplifying these benefits through improved bead stability and nutrient support. These PGPB strategies enhanced photosynthetic resilience and water use efficiency, reducing oxidative damage to PSII. By promoting drought tolerance without chemical inputs, the encapsulation of PGPB may be a viable approach for sustainable agriculture, enhancing crop productivity in water-scarce semi-arid regions. Further field validation could integrate the findings of this study into broader sustainable agriculture practices that contribute to food and energy security within the context of climate change and population growth.

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