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Bacillus subtilis and phosphorus improve wheat drought tolerance through water status and pigment stability

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Abstract

Background Drought stress is a major constraint to wheat (*Triticum aestivum* L.) productivity, creating a need for integrated and sustainable strategies to improve crop resilience. Although plant growth-promoting rhizobacteria (PGPR) and mineral nutrients are known to influence drought responses, limited evidence is available on whether *Bacillus subtilis* performs more effectively with phosphorus (P) or potassium (K) across contrasting growth stages and drought systems. This study compared the effects of *B. subtilis*, P, and K, alone and in combination, on drought tolerance in three wheat cultivars under seedling-stage hydroponic osmotic stress and grain-filling pot drought.

Results The combined *B. subtilis* + P treatment under drought produced the most consistent improvement compared with drought-only treatment and was generally more effective than *B. subtilis* + K. In hydroponics, *B. subtilis* + P improved relative water content, pigment stability, root–shoot growth, and biomass under PEG-induced drought. Faisalabad 2008 showed the strongest seedling-stage water-status response, while Dharab showed greater osmolyte accumulation through soluble sugars and proline. In the pot experiment, *B. subtilis* + P improved yield-contributing traits, particularly in Dharab and Faisalabad 2008, although cultivar-specific differences were evident.

Conclusion *B. subtilis* combined with phosphorus was the most effective drought-mitigation treatment across the measured physiological, biochemical, growth, and yield-related traits. These findings suggest that PGPR–phosphorus integration may be a useful strategy for improving wheat performance under drought, especially where water limitation occurs during early growth or grain filling. Field validation and direct measurements of bacterial colonization, nutrient uptake, and oxidative stress markers are needed to confirm the mechanisms underlying these responses.

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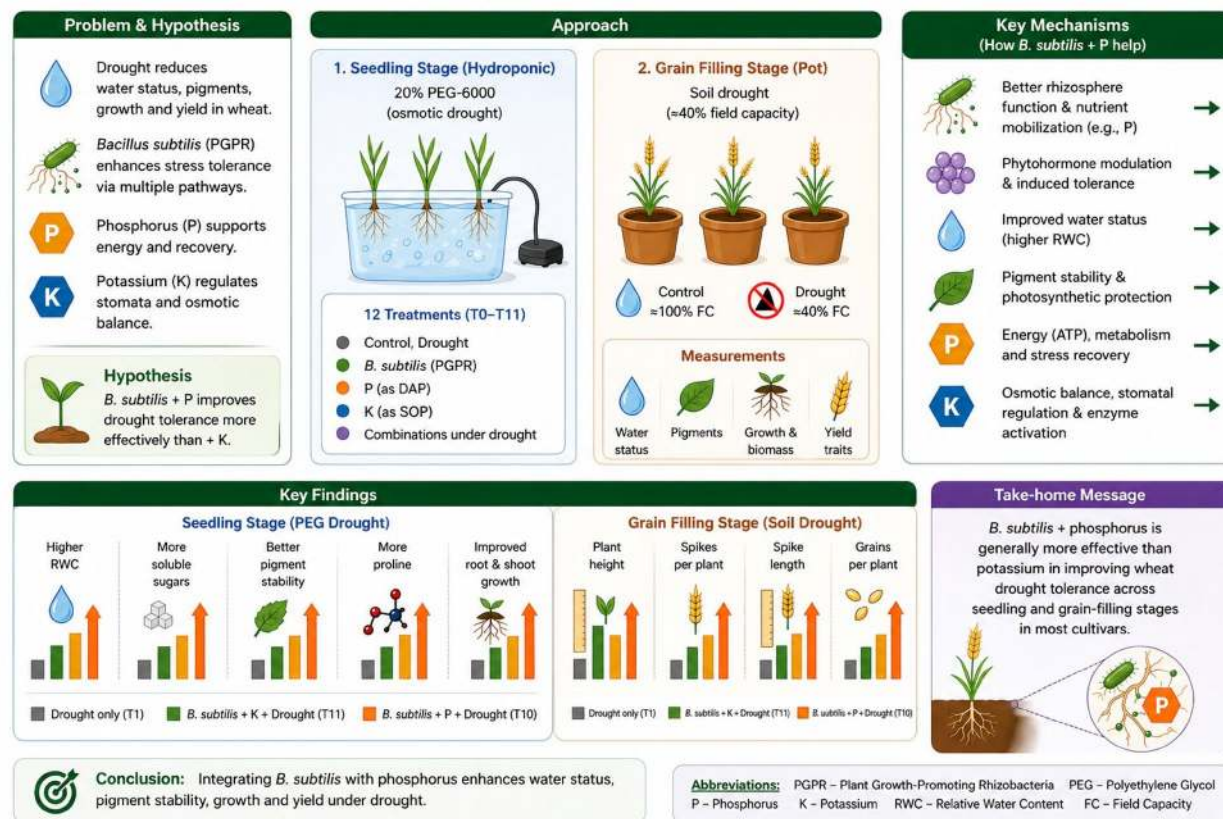
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Keywords Physiological stress, Microbial inoculation, Soil fertility, Water use efficiency, Plant stress adaptation

Graphical Abstract



Introduction

Wheat (*Triticum aestivum* L.) is among the most important cereal crops for global food security, but its productivity is increasingly threatened by drought stress under changing climatic conditions [1, 2]. Drought reduces water availability, stomatal conductance, CO₂ assimilation, photosynthetic efficiency, biomass production, and ultimately grain yield [3, 4]. At the physiological and biochemical levels, drought commonly causes reductions in chlorophyll content and relative water content, disrupts membrane stability, promotes osmotic adjustment through compatible solute accumulation, and increases reactive oxygen species formation and lipid peroxidation [5]. Therefore, drought tolerance in wheat should be evaluated through an integrated set of measurable traits, including photosynthetic pigments, water status, osmotic regulation, oxidative stress indicators, antioxidant capacity, root traits, growth, and yield components.

Plant growth-promoting rhizobacteria (PGPR) have gained attention as sustainable tools for improving crop performance under abiotic stress. Among them, *Bacillus subtilis* is particularly promising because it can enhance

plant growth and stress tolerance through multiple pathways, including phytohormone modulation, root system stimulation, induced systemic tolerance, improved rhizosphere functioning, and enhanced physiological stability under osmotic stress [6–11]. These mechanisms may help plants maintain better water status, pigment stability, membrane integrity, and osmotic balance during drought. However, many PGPR-based drought studies focus mainly on microbial inoculation alone, while less attention has been given to how PGPR effects interact with specific mineral nutrients across different wheat growth stages.

Balanced phosphorus (P) and potassium (K) nutrition is also central to drought resilience. Phosphorus supports ATP production, nucleic acid synthesis, early establishment, and metabolic recovery under stress, whereas potassium regulates enzyme activation, stomatal movement, osmotic adjustment, and photosynthetic efficiency [12–14]. Because P and K contribute to drought tolerance through different physiological roles, their interaction with *B. subtilis* may produce distinct synergistic responses. This comparison is important because the

relative benefit of *B. subtilis* + P and *B. subtilis* + K may vary between early seedling development and later reproductive stages, yet such stage-wise comparisons remain insufficiently explored.

Previous studies have separately examined wheat drought physiology, PGPR-mediated stress mitigation, and mineral nutrient management. However, fewer studies have integrated *B. subtilis* inoculation with targeted P and K supplementation under defined drought conditions while comparing responses across contrasting experimental systems and growth stages. In particular, limited information is available on whether *B. subtilis* is more effective in combination with P or K, and whether this synergistic effect is consistent across seedling-stage hydroponic drought stress and pot-grown plants at grain filling. This gap limits a clear understanding of how to combine microbial inoculation and nutrient management to improve drought resilience in wheat.

Therefore, the present study was designed to address the following research question: Does *Bacillus subtilis* improve drought tolerance in wheat more effectively when combined with phosphorus or potassium, and do these synergistic effects differ between early seedling and grain-filling stages? We hypothesized that: (i) drought stress would reduce water status, pigment stability, osmotic balance, growth, and yield-related traits; (ii) *B. subtilis* inoculation would mitigate drought injury by improving physiological and biochemical stress responses; (iii) combined *B. subtilis* + P or *B. subtilis* + K treatments would perform better than individual applications; and (iv) the relative superiority of P- or K-based synergy would differ across growth stages because P and K contribute to drought tolerance through different physiological functions. Thus, the objectives were to quantify drought-induced physiological and biochemical changes in wheat, evaluate the mitigating role of *B. subtilis*, compare the synergistic effects of *B. subtilis* with P and K, and assess whether these effects remain consistent between hydroponic seedling screening and soil pot evaluation at grain filling.

Materials and methods

Plant material, PGPR source, and experimental framework

Ten wheat (*Triticum aestivum* L.) varieties were obtained from the Plant Genetic Resource Institute (PGRI), Islamabad, Pakistan. All varieties were initially screened for germination percentage and seedling uniformity to identify suitable cultivars for detailed drought-response evaluation. Based on this preliminary screening, three cultivars—Dharab, Ujala, and Faisalabad 2008—were selected for subsequent experiments because they showed comparatively high germination percentage (>90%), uniform seedling emergence, and stable early growth performance. Their selection also enabled

comparison of drought responses among cultivars with reliable establishment under both controlled hydroponic and soil pot conditions.

A plant growth-promoting rhizobacteria strain identified as *Bacillus subtilis* was obtained from the Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore. The strain was provided as *B. subtilis* by the supplying laboratory; however, no specific strain accession number or molecular identification code was assigned. Therefore, the strain is reported here as *Bacillus subtilis* without a designated strain number. Functional traits such as phosphate solubilization, potassium solubilization, indole acetic acid production, siderophore production, ACC deaminase activity, or exopolysaccharide production were not independently quantified in the present study. Accordingly, interpretation of bacterial effects is based on plant-response traits rather than direct confirmation of specific microbial functional mechanisms.

The study consisted of two independent experiments: (i) a hydroponic seedling-stage experiment using PEG-6000 to impose controlled osmotic drought stress, and (ii) a pot experiment under a rainout shelter in which drought was imposed at grain filling by maintaining soil moisture near 40% field capacity compared with well-watered controls maintained near 100% field capacity. These two systems were used to compare early-stage physiological responses under controlled osmotic stress with later-stage drought responses under soil-based conditions.

Seed sterilization and germination screening

Seeds were surface-sterilized using 70% ethanol for 2 min followed by 1.5% sodium hypochlorite for 15 min and then rinsed three times with sterile distilled water. Sterilized seeds were placed on sterile Petri plates, with five seeds per variety per plate, and incubated for 7 days. Germination percentage was recorded daily and calculated after one week.

The germination screening was conducted to select cultivars with reliable early establishment for factorial evaluation under drought and nutrient treatments. Germination percentage ranged from 78 to 96% among the ten cultivars. Dharab, Ujala, and Faisalabad 2008 were selected because they showed comparatively high germination, uniform seedling emergence, and sufficient vigor for subsequent hydroponic and pot experiments.

PGPR culture, inoculum preparation, and seed inoculation

The *Bacillus subtilis* culture was grown on a medium containing agar (4.12 g L⁻¹), NaCl (2.5 g L⁻¹), tryptone (2.5 g L⁻¹), and yeast extract (1.25 g L⁻¹) prepared in 1 L distilled water. The pH was adjusted to 7.0–7.5 before autoclaving. The medium was poured into Petri plates

under laminar airflow, plates were streaked with the bacterial culture, sealed with parafilm, and incubated at 37 °C overnight.

A fresh bacterial suspension was prepared from overnight growth and standardized to approximately 1×10^8 CFU mL⁻¹ using serial dilution plating and colony counting. This inoculum concentration was used consistently in both experiments to improve reproducibility. For seed inoculation, surface-sterilized seeds were immersed in the standardized bacterial suspension for 30 min with gentle agitation. Seeds were then drained and air-dried under sterile conditions overnight. Non-inoculated control seeds were treated in the same manner using sterile suspension without bacteria.

Experiment 1: hydroponic seedling-stage PEG-induced drought experiment

Experimental design, treatments, and controls

The hydroponic experiment was conducted at the Department of Botany, University of Gujrat, using three cultivars: Dharab, Ujala, and Faisalabad 2008. The experiment followed a completely randomized design with 12 treatments and three biological replicates per treatment per cultivar.

The control treatment consisted of Hoagland nutrient solution without PEG, PGPR, phosphorus, or potassium supplementation. The drought-only treatment consisted of 20% PEG-6000 in Hoagland solution without PGPR or added P/K inputs. To improve readability and reduce excessive treatment-code complexity, treatments are presented with simplified labels while retaining the full factorial structure of PEG-induced drought, PGPR inoculation, and P/K supplementation (Table 1).

Hydroponic system setup and growing conditions

Seeds were germinated on Petri plates and transferred to static aerated hydroponic reservoirs. Each reservoir was

covered with a foam or thermocol sheet lid to minimize evaporation and contamination. Holes were made in the lid to support seedlings in an upright position using net cups and inert support material, allowing roots to remain submerged in nutrient solution while shoots remained above the lid. Aeration was maintained continuously using aquarium air pumps and air stones.

Hoagland nutrient solution was used as the basal solution throughout the hydroponic experiment [15]. Solution pH was maintained at 5.8 ± 0.2 , and electrical conductivity was maintained at 1.8–2.0 dS m⁻¹ using calibrated pH and EC meters. pH and EC were recorded every 48 h. Nutrient solution volume was replenished daily with distilled water to compensate for evapotranspiration losses, and the complete solution was replaced at 7-day intervals to minimize nutrient depletion and osmotic drift.

Plants were grown under controlled conditions at 25 ± 2 °C, 60–70% relative humidity, a 16 h light/8 h dark photoperiod, and approximately 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density.

PEG-induced drought stress imposition and duration

Drought stress in the hydroponic system was imposed by adding 20% PEG-6000 to Hoagland nutrient solution. This concentration was selected because PEG-6000 is widely used to create controlled osmotic stress in hydroponic and seedling-stage drought studies, and 20% PEG provides a sufficiently strong but non-lethal stress level that allows measurable physiological and biochemical responses to develop.

Seedlings were maintained under the assigned treatment regimes for 25 days. PEG-containing solutions were freshly prepared at each weekly nutrient solution replacement to maintain consistent stress intensity. Although PEG-induced osmotic stress is useful for controlled drought screening, it does not fully reproduce

Table 1 Treatment structure for the hydroponic seedling-stage experiment under PEG-induced osmotic drought stress

Code	Simplified treatment label	Hoagland solution	PEG-6000 20%	<i>B. subtilis</i>	<i>P</i>	<i>K</i>
T0	Control	+	–	–	–	–
T1	Drought	+	+	–	–	–
T2	PGPR	+	–	+	–	–
T3	P	+	–	–	+	–
T4	K	+	–	–	–	+
T5	PGPR+P	+	–	+	+	–
T6	PGPR+K	+	–	+	–	+
T7	PGPR+Drought	+	+	+	–	–
T8	P+Drought	+	+	–	+	–
T9	K+Drought	+	+	–	–	+
T10	PGPR+P+Drought	+	+	+	+	–
T11	PGPR+K+Drought	+	+	+	–	+

PGPR, plant growth-promoting rhizobacteria; PEG-6000, polyethylene glycol 6000; P, phosphorus; K, potassium. In the hydroponic experiment, 20% PEG-6000 was used to impose controlled osmotic stress. PEG-induced stress was interpreted as a controlled drought-like osmotic treatment and not as a complete substitute for soil drought

soil drought because it primarily lowers solution water potential and does not capture soil-related processes such as hydraulic resistance, soil–root interactions, nutrient diffusion constraints, or microbial activity in the rhizosphere. Therefore, PEG results were interpreted as controlled osmotic-stress responses rather than direct equivalents of field soil drought.

Measurements in the hydroponic experiment

After 25 days of treatment exposure, plants were sampled to quantify growth, biomass allocation, water status, and biochemical drought-response indicators. Sampling was performed at the same time of day to reduce diurnal variation in leaf water status and metabolite levels.

Seedlings were carefully removed from the support ports, roots were rinsed briefly with distilled water to remove residual nutrient solution and PEG, and tissues were gently blotted before weighing. Root length was measured from the crown to the tip of the longest root, and shoot length was measured from the crown to the tip of the longest fully expanded leaf. Root and shoot fresh weights were recorded immediately, while dry weights were obtained after oven-drying tissues to constant weight.

Leaf relative water content was determined using fresh weight, turgid weight, and dry weight according to the formula [16, 17]:

$$\text{RWC (\%)} = [(\text{FW} - \text{DW}) / (\text{TW} - \text{DW})] \times 100$$

Soluble sugar content was quantified using the phenol–sulfuric acid method [18]. Photosynthetic pigments, including chlorophyll a, chlorophyll b, and carotenoids, were determined spectrophotometrically using ethanol extraction [19]. Proline content was measured using the

ninhydrin method [20] and expressed on a fresh weight basis.

Experiment 2: Pot experiment at grain filling under soil-moisture drought

Experimental design, treatments, and drought definition

The pot experiment was conducted at the Botanical Garden, University of Gujrat, under a rainout shelter to prevent rainfall interference. The same three cultivars and 12-treatment structure used in the hydroponic experiment were evaluated, with three biological replicates per treatment per cultivar in a completely randomized design.

The control treatment consisted of fully irrigated pots maintained near 100% field capacity throughout the experiment. Drought treatments were imposed during grain filling by withholding irrigation until soil moisture reached approximately 40% field capacity, which was then maintained during the drought period. The same treatment logic was used in the pot experiment to enable comparison between seedling-stage hydroponic responses and grain-filling soil-drought responses, with treatment labels simplified for clarity (Table 2).

Soil preparation, fertilization, and crop establishment

Soil was collected from the Botanical Garden, air-dried, sieved through a 2 mm mesh, and filled into pots at 6.5 kg soil per pot. Baseline soil analysis indicated sandy loam texture, near-neutral pH, low electrical conductivity, moderate organic matter, and low-to-moderate available phosphorus and potassium, confirming suitability for evaluating nutrient supplementation effects under drought.

Phosphorus and potassium were applied as diammonium phosphate and sulphate of potash, respectively. Full phosphorus was applied at 3.33 g DAP pot^{−1}, and full

Table 2 Treatment structure for the pot experiment under grain-filling soil-moisture drought

Code	Simplified treatment label	Irrigated control ≈ 100% FC	Drought ≈ 40% FC	<i>B. subtilis</i>	P as DAP	K as SOP
T0	Control	+	−	−	−	−
T1	Drought	−	+	−	−	−
T2	PGPR	+	−	+	−	−
T3	P	+	−	−	+	−
T4	K	+	−	−	−	+
T5	PGPR + P	+	−	+	+	−
T6	PGPR + K	+	−	+	−	+
T7	PGPR + Drought	−	+	+	−	−
T8	P + Drought	−	+	−	+	−
T9	K + Drought	−	+	−	−	+
T10	PGPR + P + Drought	−	+	+	+	−
T11	PGPR + K + Drought	−	+	+	−	+

FC, field capacity; PGPR, plant growth-promoting rhizobacteria; DAP, diammonium phosphate; SOP, sulphate of potash. Phosphorus was applied as 3.33 g DAP pot^{−1} and potassium as 0.075 g SOP pot^{−1}. Because DAP supplies both phosphorus and nitrogen, nitrogen was not fully balanced across all treatments. Therefore, responses in DAP-based phosphorus treatments should be interpreted mainly in relation to phosphorus supplementation, but with possible contribution from additional nitrogen supplied through DAP

potassium was applied at 0.075 g SOP pot⁻¹. Fertilizers were incorporated uniformly into the soil before sowing.

Because DAP supplies both phosphorus and nitrogen, its use may introduce nitrogen as a potential confounding factor in phosphorus treatments. Nitrogen levels were not fully balanced across all treatments with an equivalent non-phosphorus nitrogen source. Therefore, responses observed under DAP-based phosphorus treatments should be interpreted as primarily associated with phosphorus supplementation but with possible contribution from the additional nitrogen supplied through DAP. This limitation was considered when interpreting nutrient effects.

Seeds were surface-sterilized and inoculated with standardized *B. subtilis* suspension before sowing, following the same procedure described above. Seeds were sown at 2 cm depth on December 4, 2018. Pots were maintained under the rainout shelter and received uniform agronomic care across treatments, including weed removal and pest monitoring.

Grain-filling drought imposition and moisture monitoring

Drought stress was imposed during grain filling by withholding irrigation in drought-designated treatments. Field capacity was determined gravimetrically before drought initiation. Irrigated control pots were maintained near 100% field capacity, whereas drought-stressed pots were reduced to and maintained near 40% field capacity.

Soil moisture was monitored daily by pot weighing. Irrigation volumes were calculated to restore control pots to the target field capacity while maintaining drought pots near the reduced field-capacity threshold. Drought stress was applied for 14 days beginning at early grain filling [21, 22]. If cultivars differed phenologically, drought was applied cultivar-wise at the same developmental stage to ensure stage-equivalent comparison.

Measurements in the pot experiment

Measurements in the pot experiment were conducted to quantify vegetative performance, physiological drought status, biochemical adjustment, and yield formation under grain-filling drought. Plants were carefully removed from pots at designated sampling points, roots were washed gently to remove soil particles, and tissues were blotted before weighing.

Vegetative traits were measured using the same procedures as in the hydroponic experiment, including root length, shoot length, root fresh weight, shoot fresh weight, root dry weight, and shoot dry weight. Leaf relative water content, soluble sugars, proline, and photosynthetic pigments were also quantified using the same methods described for Experiment 1 to maintain cross-system consistency.

Yield and yield-contributing traits were recorded at maturity. Plant height was measured from the soil surface to the tip of the tallest spike. The number of spikes per plant was counted manually. Spike length was measured excluding awns. The number of grains per plant was determined after harvesting, manual threshing, and counting total grains per plant.

Statistical analysis

Data from the hydroponic and pot experiments were analyzed separately because the two experiments differed in growth system, drought-imposition method, and developmental stage. Each experiment followed a completely randomized design with three biological replicates per treatment per cultivar.

For each experiment, factorial analysis of variance was performed to test the main and interaction effects of cultivar, drought condition, PGPR inoculation, and nutrient supplementation. The general ANOVA model included cultivar, drought, PGPR, nutrient treatment, and their two-way, three-way, and higher-order interaction terms as fixed effects. In the hydroponic experiment, drought was represented by PEG-6000 treatment, whereas in the pot experiment drought was represented by soil moisture level. Nutrient treatment represented phosphorus or potassium supplementation depending on the treatment combination.

Interaction effects were examined to determine whether the response to *B. subtilis* depended on drought condition, nutrient supplementation, cultivar, or their combinations. Particular attention was given to PGPR × nutrient, drought × PGPR, drought × nutrient, cultivar × drought, and drought × PGPR × nutrient interactions because these directly addressed the study objective of comparing the synergistic effects of PGPR–P and PGPR–K under drought.

Before ANOVA, data were checked for normality and homogeneity of variance using residual diagnostics. Normality was assessed through normal probability plots and/or Shapiro–Wilk tests, while homogeneity of variance was evaluated using residual plots and Levene's test where appropriate. When ANOVA indicated significant treatment effects at $\alpha=0.05$, treatment means were separated using Tukey's honest significant difference test. Results were expressed as mean ± standard error from three biological replicates. Statistical analyses were performed using Minitab, and graphs were prepared using Microsoft Excel 2010.

Results

Water relations and osmotic adjustment under PEG-induced drought in hydroponic seedlings

PEG-induced drought reduced relative water content (RWC) in all cultivars compared with the control

(Fig. 1a). Under drought-only treatment (T1), RWC decreased to 32.22% in Faisalabad 2008, 14.44% in Ujala, and 28.00% in Dharab. The combined *Bacillus subtilis* + phosphorus + drought treatment (T10) increased RWC to 76.00% in Faisalabad 2008, 41.00% in Ujala, and 50.07% in Dharab. Compared with *B. subtilis* + potassium + drought (T11), T10 produced higher RWC in Faisalabad 2008 and Dharab, whereas Ujala showed higher RWC under T11. Tukey's lettering indicated significant treatment differences within cultivars.

Soluble sugar content differed among treatments and cultivars (Fig. 1b). In Faisalabad 2008, T10 and T11 recorded similar sugar contents of 0.0957 and 0.0970 mg g⁻¹ FW, respectively. In Ujala, T10 produced the highest sugar content among the drought-combination treatments, reaching 0.1433 mg g⁻¹ FW compared with 0.0860 mg g⁻¹ FW under T11. In Dharab, soluble sugar content was slightly higher under T11 (0.1580 mg g⁻¹ FW) than T10 (0.1520 mg g⁻¹ FW).

Photosynthetic pigments and proline under PEG-induced drought

Chlorophyll a content varied among treatments and cultivars (Fig. 2a). In Faisalabad 2008, chlorophyll a decreased from 0.0301 mg g⁻¹ FW under control conditions to 0.0268 mg g⁻¹ FW under drought-only treatment. Under drought-combination treatments, T11 recorded 0.0291 mg g⁻¹ FW, while T10 recorded 0.0266 mg g⁻¹ FW. In Ujala, chlorophyll a increased from 0.0199 mg g⁻¹ FW under T1 to 0.0254 mg g⁻¹ FW under T10 and 0.0234 mg g⁻¹ FW under T11. In Dharab, T10 and T11 recorded 0.0209 and 0.0195 mg g⁻¹ FW, respectively.

Chlorophyll b showed treatment-dependent variation (Fig. 2b). In Faisalabad 2008, T10 recorded 0.0537 mg g⁻¹ FW compared with 0.0482 mg g⁻¹ FW under T11. In Ujala, T10 recorded 0.0464 mg g⁻¹ FW, while T11 recorded 0.0389 mg g⁻¹ FW. In Dharab, T10 and T11 recorded similar values of 0.0275 and 0.0276 mg g⁻¹ FW, respectively.

Carotenoid content differed among cultivars and treatments (Fig. 2c). In Faisalabad 2008, carotenoids were higher under T10 (0.4954 mg g⁻¹ FW) than T11 (0.4387 mg g⁻¹ FW). In Ujala, T10 recorded 0.4632 mg

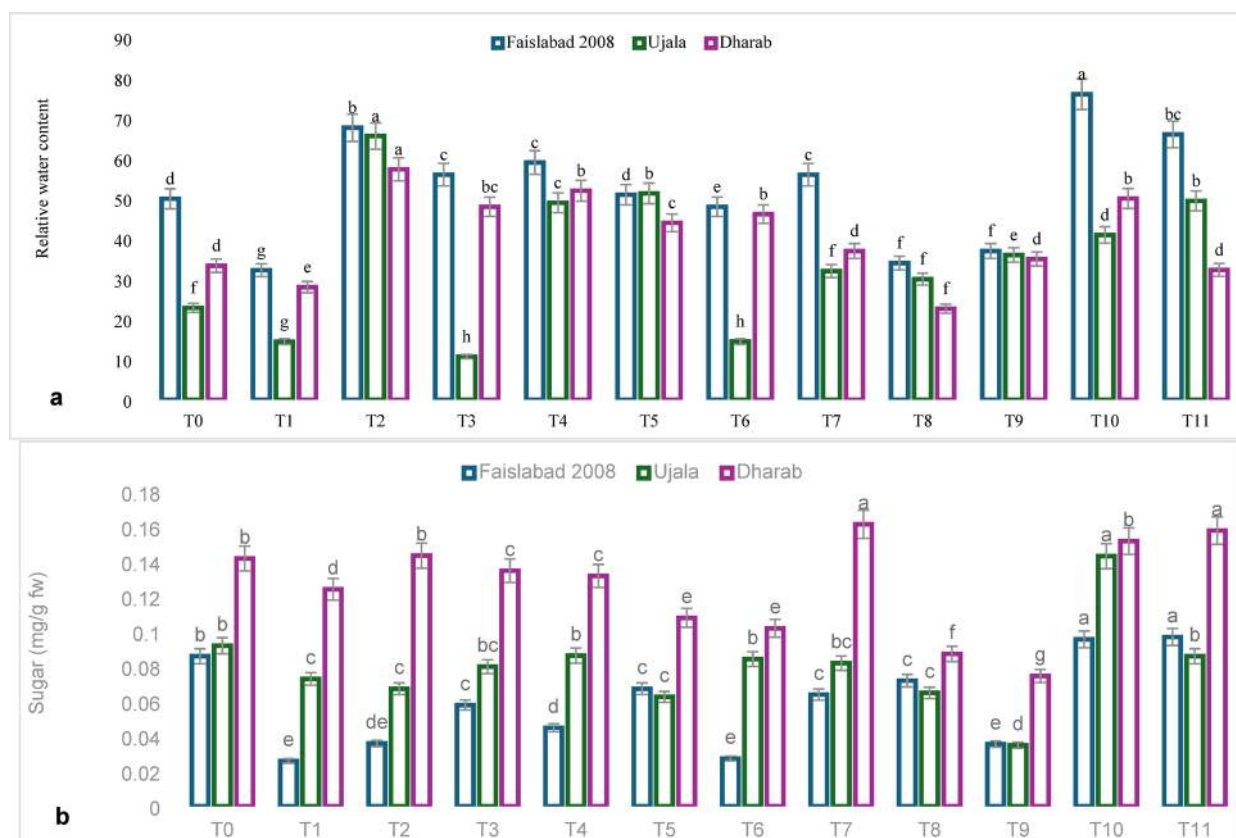


Fig. 1 Effects of PGPR and nutrient treatments on **a** relative water content (RWC, %) and **b** soluble sugar content (mg g⁻¹ FW) of wheat seedlings exposed to 20% PEG-6000-induced osmotic drought stress. Bars represent mean $\pm$ SE ($n=3$). Different letters above bars indicate significant differences among treatments within each cultivar at $p \leq 0.05$ according to Tukey's HSD test. Treatment labels: T0, Control; T1, Drought; T2, PGPR; T3, P; T4, K; T5, PGPR + P; T6, PGPR + K; T7, PGPR + Drought; T8, P + Drought; T9, K + Drought; T10, PGPR + P + Drought; T11, PGPR + K + Drought

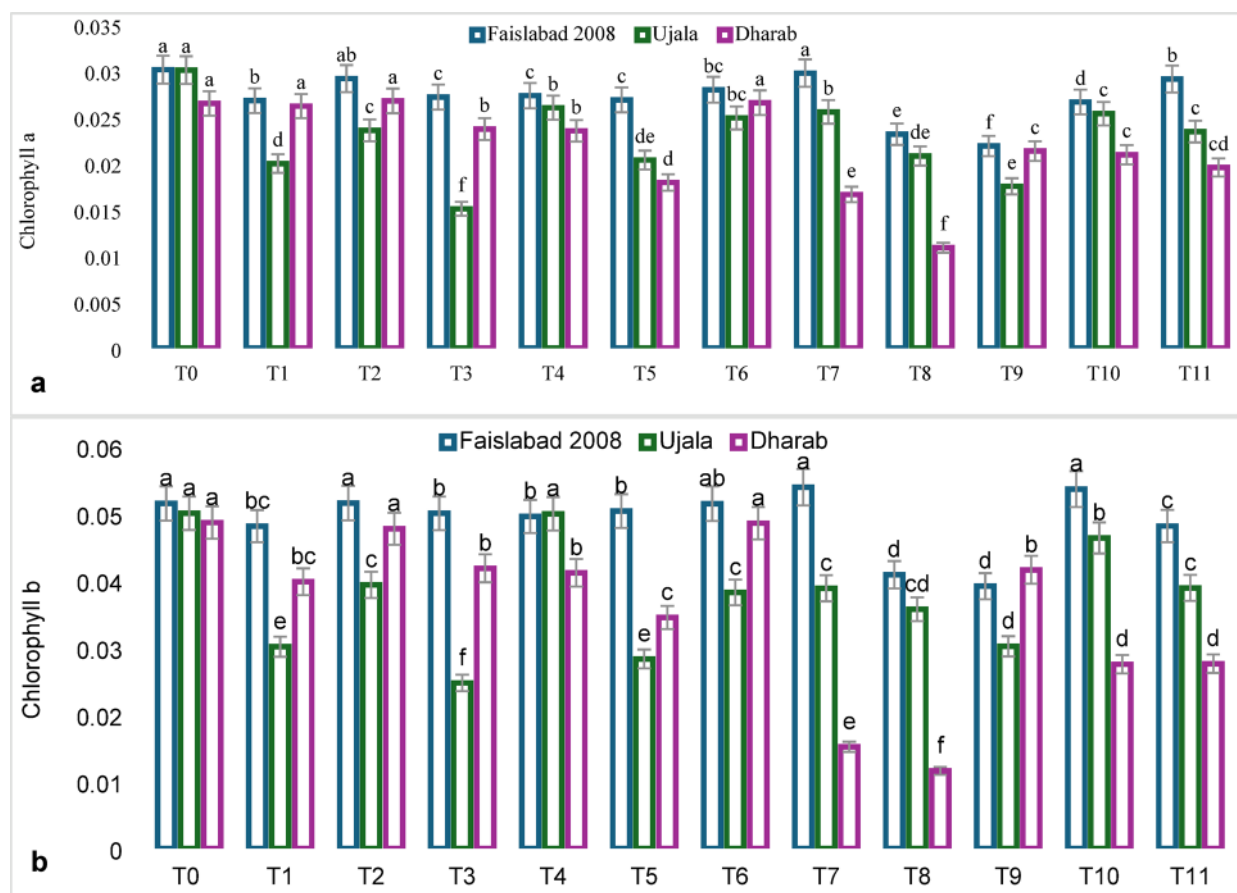


Fig. 2 Effects of PGPR and nutrient treatments on (a) chlorophyll a, (b) chlorophyll b, (c) carotenoids, and (d) proline content of wheat seedlings exposed to 20% PEG-6000-induced osmotic drought stress. Values are expressed as mg g^{-1} FW. Bars represent mean $\pm$ SE ($n=3$). Different letters above bars indicate significant differences among treatments within each cultivar at $p \leq 0.05$ according to Tukey's HSD test

g^{-1} FW compared with 0.4122 mg g^{-1} FW under T11. In Dharab, carotenoid content was 0.1683 mg g^{-1} FW under T10 and 0.1471 mg g^{-1} FW under T11.

Proline content showed cultivar-specific differences (Fig. 2d). In Faisalabad 2008, T11 recorded the highest proline value among the PGPR–nutrient drought combinations (0.4328 mg g^{-1} FW), followed by T10 (0.4115 mg g^{-1} FW). In Ujala, T11 recorded 0.3981 mg g^{-1} FW, while T10 recorded 0.2732 mg g^{-1} FW. In Dharab, T10 recorded 0.6885 mg g^{-1} FW compared with 0.6423 mg g^{-1} FW under T11.

Root–shoot growth and biomass under PEG-induced drought

Root length differed among treatments and cultivars (Fig. 3a). Under drought-only treatment, root length was 10.30 cm in Faisalabad 2008, 9.63 cm in Ujala, and 9.10 cm in Dharab. In Faisalabad 2008, T10 produced the highest root length (25.60 cm), compared with 20.20 cm under T11. In Ujala, T10 and T11 recorded 21.60 and 20.20 cm, respectively. In Dharab, T11 produced greater root length (18.10 cm) than T10 (4.40 cm). ANOVA

indicated a significant treatment $\times$ cultivar interaction for root length ($p < 0.05$).

Shoot length also varied among treatments and cultivars (Fig. 3b). In Faisalabad 2008, shoot length increased from 25.40 cm under T1 to 35.00 cm under T10 and 30.00 cm under T11. In Ujala, shoot length under T10 and T11 was 29.47 and 27.00 cm, respectively. In Dharab, T11 recorded 26.43 cm compared with 25.00 cm under T10. Treatment and cultivar effects were significant for shoot length ($p < 0.01$).

Root fresh weight showed cultivar-dependent responses (Fig. 3c). In Dharab, T10 recorded the highest root fresh weight (0.20 g), compared with 0.16 g under T11. In Faisalabad 2008, T10 and T11 recorded 0.06 and 0.04 g, respectively. In Ujala, T10 and T11 recorded 0.04 and 0.02 g, respectively.

Shoot fresh weight differed among cultivars and treatments (Fig. 3d). In Faisalabad 2008, T10 and T11 recorded 0.16 and 0.17 g, respectively. In Ujala, both T10 and T11 recorded 0.06 g. In Dharab, T11 recorded the highest shoot fresh weight (0.17 g), while T10 recorded 0.04 g.

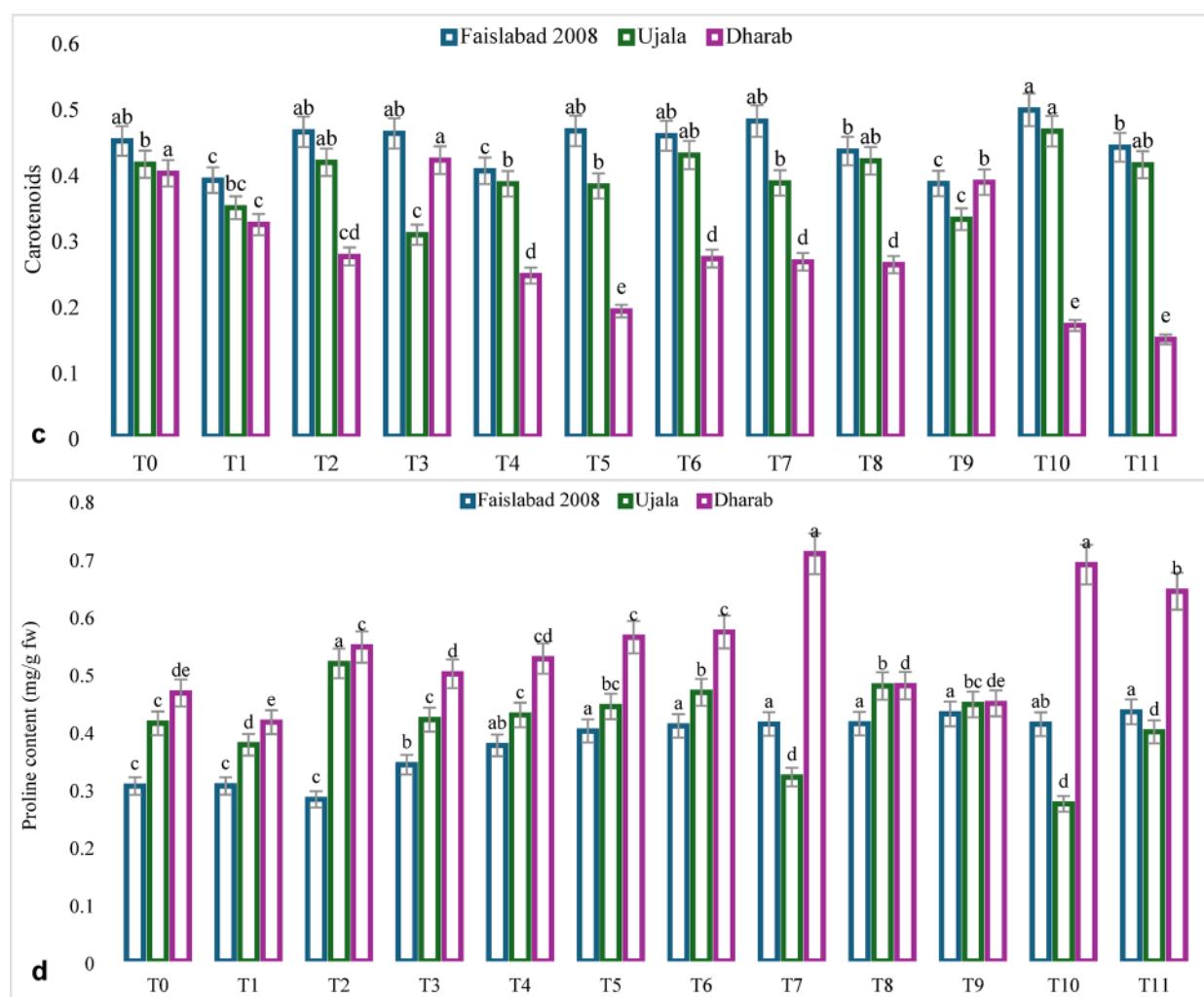


Fig. 2 (continued)

Shoot dry weight was affected by treatment in some cultivars (Fig. 3e). In Faisalabad 2008, T10 recorded 0.06 g and T11 recorded 0.04 g. In Ujala, both T10 and T11 recorded 0.02 g. In Dharab, T10 recorded 0.10 g and T11 recorded 0.09 g.

Root dry weight showed limited treatment variation (Fig. 3f). In Faisalabad 2008, T10 and T11 recorded 0.0367 and 0.0333 g, respectively. In Ujala and Dharab, T10 and T11 each recorded 0.02 g.

Yield-contributing traits under grain-filling drought in the pot experiment

Plant height varied among treatments and cultivars under grain-filling drought (Fig. 4a). In Faisalabad 2008, plant height was 23.00 cm under drought-only treatment and increased to 60.33 cm under T10, compared with 53.00 cm under T11. In Ujala, plant height was 72.00 cm under T1, 65.67 cm under T10, and 53.00 cm under T11. In Dharab, T10 produced the highest plant height (101.00 cm), followed by T11 (98.00 cm).

The number of spikes per plant differed among treatments (Fig. 4b). In Faisalabad 2008, T10 produced 3.33 spikes plant⁻¹, compared with 1.33 spikes plant⁻¹ under T11. In Ujala, T10 produced 4.00 spikes plant⁻¹, compared with 3.00 spikes plant⁻¹ under T11. In Dharab, T10 produced 6.00 spikes plant⁻¹, compared with 4.00 spikes plant⁻¹ under T11.

Spike length varied among treatments and cultivars (Fig. 4c). In Faisalabad 2008, spike length was 7.60 cm under T10 and 5.60 cm under T11. In Ujala, T10 recorded 5.50 cm, while T11 recorded 5.83 cm. In Dharab, T10 recorded the highest spike length (12.83 cm), compared with 11.50 cm under T11.

Grain number per plant showed treatment-dependent variation (Fig. 4d). In Faisalabad 2008, T10 recorded the highest grain number among treatments (125.67 grains plant⁻¹), compared with 70.00 grains plant⁻¹ under T11. In Ujala, T5 recorded the highest grain number (168.00 grains plant⁻¹), while T10 and T11 recorded 60.00 and 84.00 grains plant⁻¹, respectively. In Dharab,

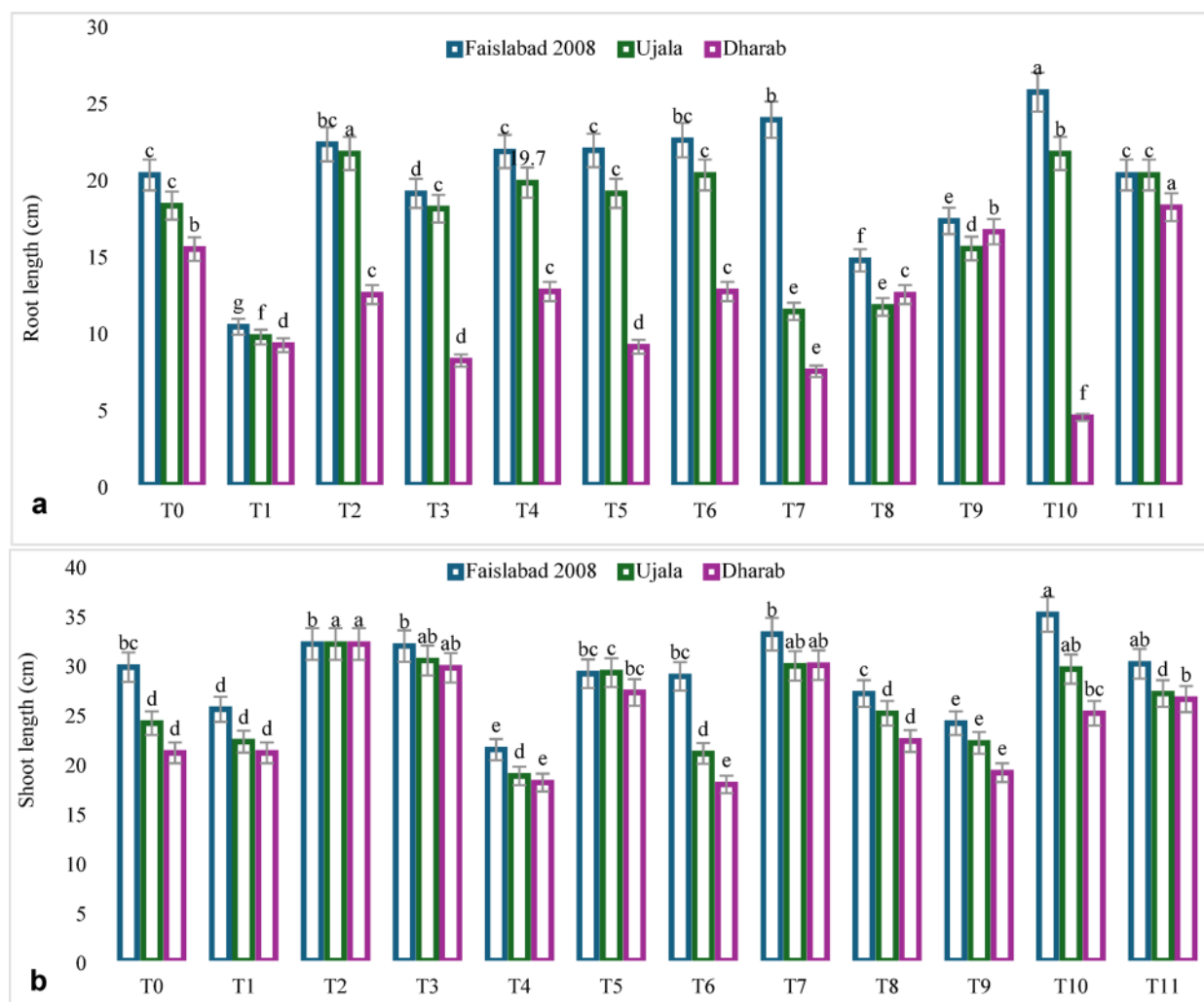


Fig. 3 Effects of PGPR and nutrient treatments on (a) root length, (b) shoot length, (c) root fresh weight, (d) shoot fresh weight, (e) shoot dry weight, and (f) root dry weight of wheat seedlings exposed to 20% PEG-6000-induced osmotic drought stress. Length values are expressed in cm, and biomass values are expressed in g plant⁻¹. Bars represent mean $\pm$ SE ($n=3$). Different letters above bars indicate significant differences among treatments within each cultivar at $p \leq 0.05$ according to Tukey's HSD test

T10 produced the highest grain number (206.67 grains plant⁻¹), compared with 160.00 grains plant⁻¹ under T11.

Across the pot experiment, T10 recorded higher plant height, spike number, spike length, and grain number than T11 in Faisalabad 2008 and Dharab. In Ujala, T10 recorded higher plant height and spike number than T11, while T11 recorded higher spike length and grain number than T10.

Discussion

This study evaluated whether *Bacillus subtilis* improves wheat drought tolerance more effectively when combined with phosphorus (P) or potassium (K), and whether these effects differ between seedling-stage PEG-induced osmotic stress and grain-filling soil-moisture drought. Overall, drought reduced water status, photosynthetic pigments, growth, biomass, and yield-related traits, while

integrated PGPR–nutrient treatments partly reduced these losses. Among the drought-applied combined treatments, *B. subtilis* + P under drought (T10) produced the most consistent improvement across both experimental systems, although responses varied by cultivar and trait.

The clearest evidence of drought mitigation was observed in relative water content, pigment retention, osmolyte accumulation, growth, and yield components. Under PEG-induced drought, T10 improved RWC compared with drought-only treatment in all cultivars. Faisalabad 2008 showed the strongest seedling-stage water-status response, with RWC increasing from 32.22% under drought-only treatment to 76.00% under T10. Ujala increased from 14.44 to 41.00%, and Dharab increased from 28.00 to 50.07%. Compared with *B. subtilis* + K under drought (T11), T10 produced higher RWC in Faisalabad 2008 and Dharab, whereas Ujala showed

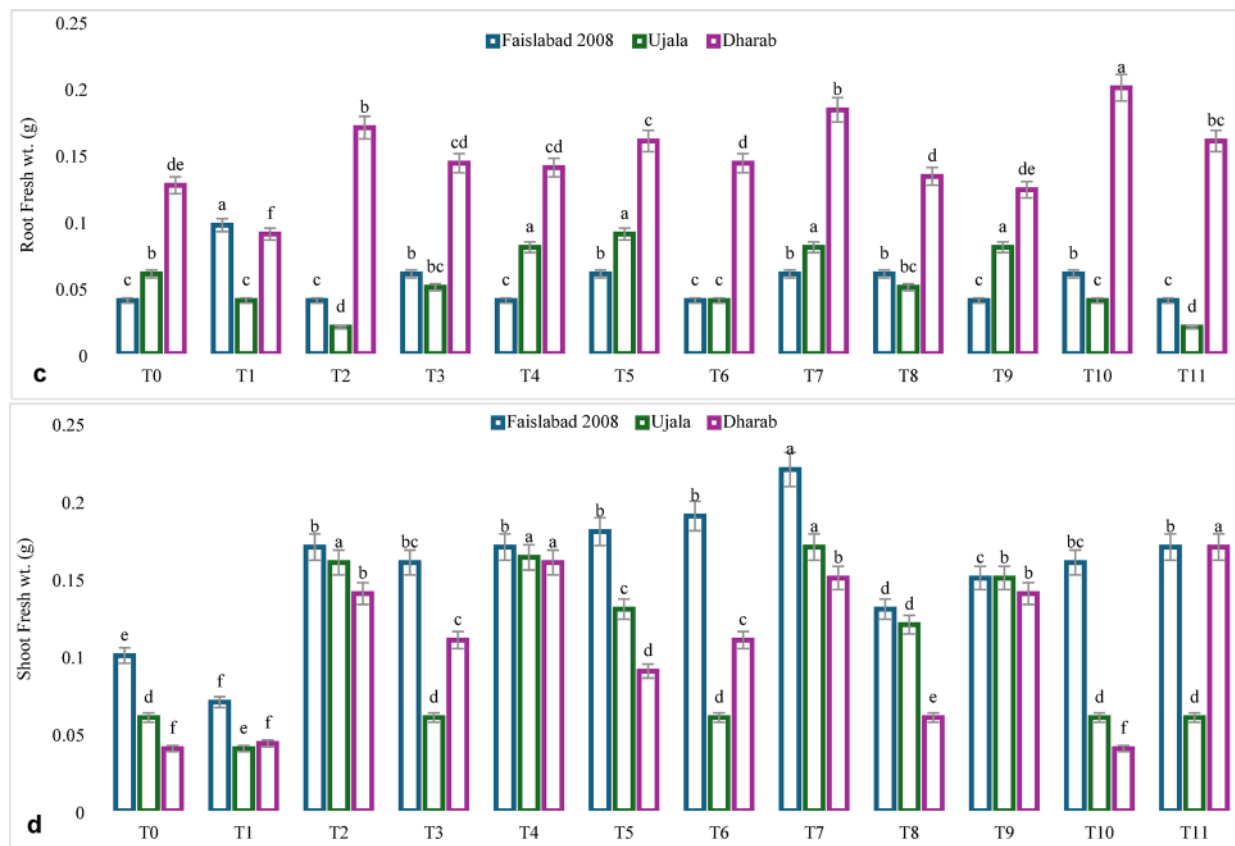


Fig. 3 (continued)

higher RWC under T11. These results show that PGPR–nutrient effects on water relations were cultivar dependent. Similar PGPR-mediated improvements in plant water relations under drought have been reported previously and are often associated with improved root function, rhizosphere water availability, and stress signalling [23, 24].

Photosynthetic pigment responses also indicated treatment-dependent drought mitigation. T10 maintained higher chlorophyll b and carotenoid contents than drought-only treatment in several cultivar comparisons, particularly in Faisalabad 2008 and Ujala. Maintenance of chlorophyll and carotenoid contents under drought is important because pigment degradation is commonly linked with stress-induced disruption of photosynthetic capacity [25, 26, 21]. However, antioxidant enzymes and oxidative damage indicators such as SOD, CAT, POD, and MDA were not measured in the present study. Therefore, pigment retention should be interpreted as evidence of improved photosynthetic pigment stability, not as direct proof of enhanced antioxidant activity or reduced lipid peroxidation. Antioxidant protection remains a possible explanation, but it requires direct biochemical confirmation.

Osmotic adjustment differed strongly among cultivars. Dharab generally showed higher proline accumulation under drought-combination treatments, including T10 and T11, while Faisalabad 2008 showed stronger improvement in water status and selected pigment traits. Ujala showed intermediate or treatment-specific responses. These patterns suggest that cultivars may differ in their dominant drought-response strategies: Faisalabad 2008 appeared more responsive in hydration and pigment stability at the seedling stage, whereas Dharab showed stronger osmolyte accumulation and reproductive-stage performance. Such variation agrees with previous evidence that drought tolerance is not controlled by a single trait, but by coordinated physiological, biochemical, and morphological responses [5, 27].

Growth and biomass responses further supported the positive effect of integrated PGPR–nutrient treatments under drought, but these responses were not uniform across cultivars. In Faisalabad 2008, T10 increased root length from 10.30 cm under drought-only treatment to 25.60 cm and exceeded T11, which recorded 20.20 cm. In Ujala, T10 and T11 produced similar root-length responses, while in Dharab, T11 exceeded T10 for root length. Root traits are important for drought adaptation because improved root development can support water

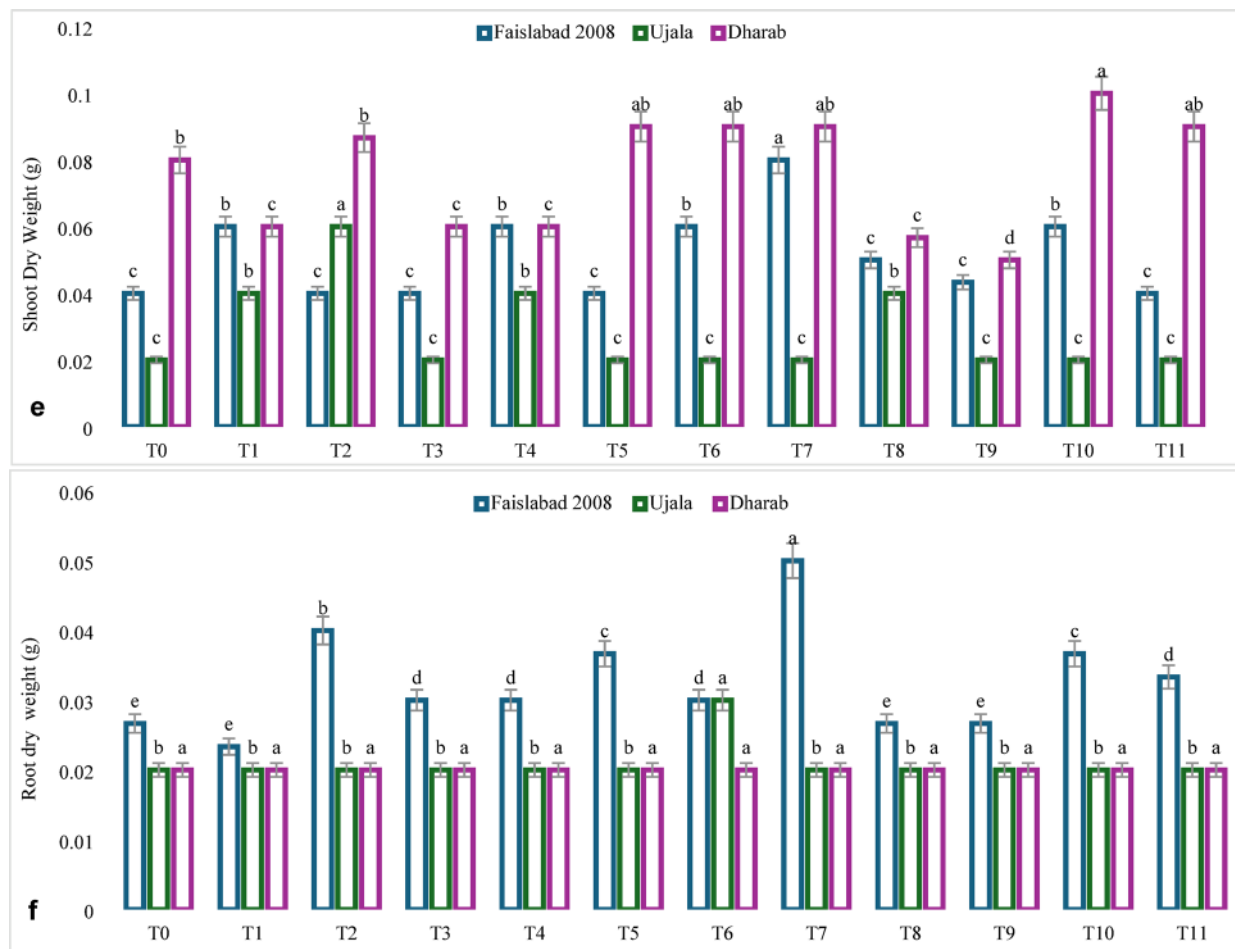


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and nutrient acquisition under limited moisture [27]. However, only basic root and shoot traits were measured here, so explanations involving improved root system architecture should remain inferential. More detailed traits such as root volume, root surface area, lateral root density, and root hair development would be needed to confirm root-architecture mechanisms.

At grain filling, T10 improved several yield-contributing traits compared with drought-only treatment and often exceeded T11. In Faisalabad 2008, grain number increased from 46.67 grains plant⁻¹ under drought-only treatment to 125.67 grains plant⁻¹ under T10, compared with 70.00 grains plant⁻¹ under T11. In Dharab, T10 produced the highest grain number, 206.67 grains plant⁻¹, compared with 160.00 grains plant⁻¹ under T11. Dharab also showed higher plant height, spike number, and spike length under T10 than T11. However, Ujala showed a different pattern for grain number, where T11 exceeded T10. These findings indicate that the advantage of *B. subtilis* + P was generally stronger in Faisalabad 2008 and Dharab, but not universal across all cultivars. Since grain filling is highly sensitive to water deficit, improved yield

components under integrated treatments may reflect better maintenance of physiological function during reproductive development [21].

The comparatively stronger performance of *B. subtilis* + P may be related to the physiological role of phosphorus in drought-stress recovery. Phosphorus is essential for ATP production, energy transfer, nucleic acid synthesis, membrane function, root activity, and post-stress growth recovery [12]. Under drought, these processes are important for maintaining metabolism, supporting repair, and sustaining growth. Therefore, P supplementation may have supported drought recovery more effectively than K in several measured traits. In addition, *Bacillus* spp. are often associated with improved P availability through phosphate solubilization and other rhizosphere processes [28, 29]. However, phosphate-solubilizing activity was not directly measured in this study. Thus, improved P availability is a plausible explanation for the stronger T10 response, but it should not be presented as a confirmed mechanism.

Potassium remains physiologically important for drought tolerance because it contributes to stomatal

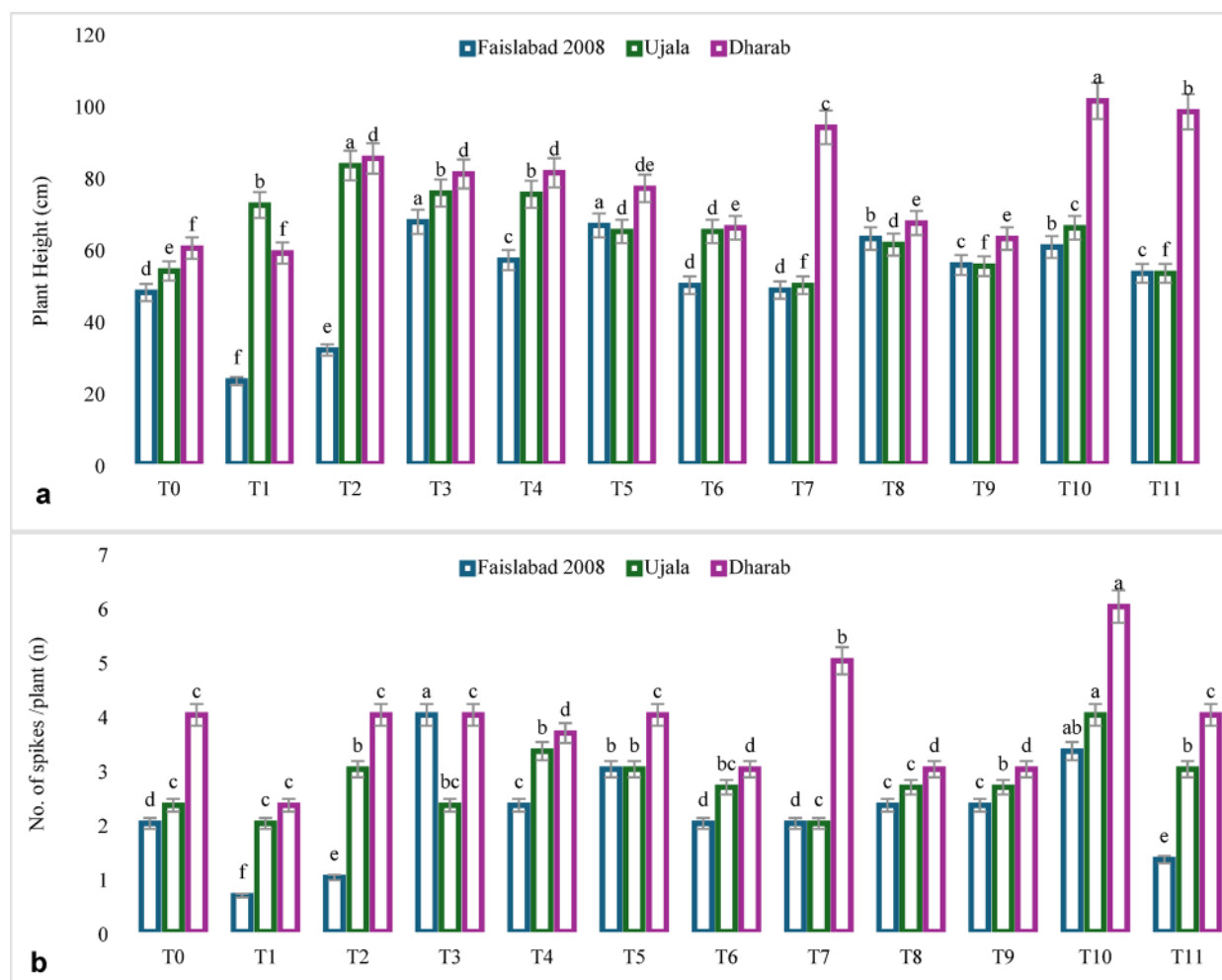


Fig. 4 Effects of PGPR and nutrient treatments on (a) plant height, (b) number of spikes per plant, (c) spike length, and (d) number of grains per plant in wheat under grain-filling soil-moisture drought. Plant height and spike length are expressed in cm; spike number and grain number are expressed as counts plant⁻¹. Bars represent mean $\pm$ SE ($n=3$). Different letters above bars indicate significant differences among treatments within each cultivar at $p \leq 0.05$ according to Tukey's HSD test

regulation, enzyme activation, osmotic balance, and photosynthetic performance [30–32]. The less consistent performance of T11 does not mean that K was unimportant. Instead, the response may have depended on cultivar, baseline soil K availability, fertilizer rate, drought timing, or growth stage. Because tissue P and K concentrations were not measured, the study cannot confirm whether T10 superiority resulted from greater nutrient uptake, improved nutrient-use efficiency, or another physiological response. Nutrient uptake effects should therefore be discussed only as possible explanations.

Similarly, the contribution of *B. subtilis* should be interpreted cautiously. The study measured plant responses but did not directly quantify rhizosphere colonization, bacterial survival, bacterial activity, or functional traits such as phosphate solubilization, ACC deaminase activity, siderophore production, or exopolysaccharide production. Although *Bacillus* spp. have been associated

with drought mitigation through phytohormonal modulation, induced systemic tolerance, EPS production, and improved rhizosphere function [33–35], these processes were not directly tested here. Therefore, bacterial colonization efficiency and rhizosphere activity cannot be claimed directly. The observed improvements under PGPR treatments indicate plant-level responses associated with inoculation, while the underlying microbial mechanisms remain inferential.

Overall, the data directly show that *B. subtilis* combined with phosphorus improved several drought-response traits, including water status, pigment stability, osmotic adjustment, growth, and yield components. The results also show clear cultivar-specific response patterns, with Faisalabad 2008 showing stronger seedling-stage water-status and pigment responses, and Dharab showing stronger osmolyte accumulation and grain-filling performance. Mechanistic explanations involving

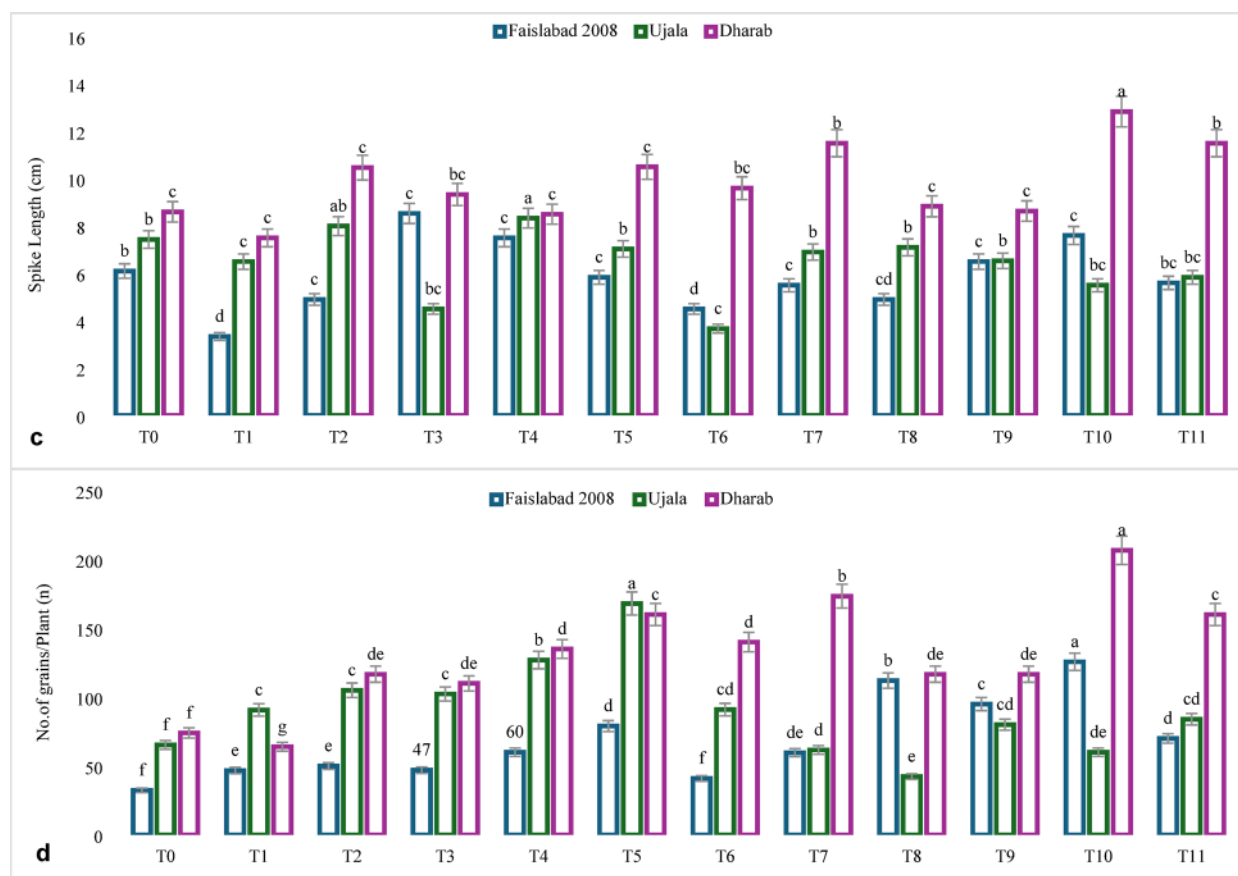


Fig. 4 (continued)

antioxidant activity, bacterial colonization, nutrient uptake, and rhizosphere functioning remain plausible but unconfirmed because these parameters were not directly measured [36–38].

The main limitations of the study include the use of PEG-induced osmotic stress, which does not fully represent soil drought; the pot-based nature of the reproductive-stage experiment; the absence of direct rhizosphere colonization or bacterial activity measurements; lack of tissue P and K uptake data; and absence of antioxidant and oxidative damage markers such as SOD, CAT, POD, and MDA. Previous work has also shown that PEG-induced osmotic stress and soil drought may produce different physiological and molecular responses in wheat [39]. Future studies should include microbial colonization assays, tissue nutrient profiling, antioxidant enzyme activity, lipid peroxidation measurements, and multi-location field validation to confirm the mechanisms suggested by the present physiological and yield responses.

Conclusion

This study showed that drought stress substantially reduced wheat physiological performance, growth, and yield-related traits, while integrated application

of *Bacillus subtilis* with mineral nutrition helped mitigate these effects. Among the tested combinations, *B. subtilis* + phosphorus was generally the most effective treatment under drought, producing more consistent improvements in relative water content, photosynthetic pigment stability, root–shoot growth, biomass accumulation, and grain-filling traits than drought-only treatment and most other nutrient combinations. The response was cultivar dependent, with Faisalabad 2008 showing stronger seedling-stage water-status and pigment responses, while Dharab showed stronger reproductive-stage yield recovery. Overall, the findings suggest that combining *B. subtilis* inoculation with phosphorus supplementation may be a promising strategy for improving wheat drought resilience, although field validation and direct measurements of bacterial colonization, nutrient uptake, and oxidative stress markers are needed to confirm the mechanisms involved.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40538-026-00977-1>.

Supplementary Material 1

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Author contributions

WI, SF, MF, HQ, TA: supervision, experimentation, data curation, methodology, data collection, writing, drafting, research design, investigation; HSEB, IMA, KHA, NYR, SB: validation, software, writing, drafting, statistical analysis, validation; JB, RMA, SAA, FMA, AS: writing, software, resource, research design, validation, drafting, statistical analysis.

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Data availability

All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate

This article does not contain any studies on human or animal subjects. The current experimental research and field study including the collection of plant material, is complying with relevant institutional, national, and international guidelines and legislation and used for research and development.

Consent for publication

Not applicable.

Clinical Trial Number

Not applicable.

Study protocol must comply with relevant institutional, national, and international guidelines and legislation

Our experiment follows the relevant institutional, national, and international guidelines and legislation.

Competing interests

The authors declare no competing interests.

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