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Rhizobium-Based Seed Priming Enhances Drought Tolerance and Yield Performance of Spring Bread Wheat (*Triticum aestivum* L.) Under Controlled Water Deficit Conditions

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Abstract

Bread wheat (*Triticum aestivum* L.) production is increasingly threatened by water scarcity, particularly in drought-prone regions. Rhizobial inoculation offers a potential complementary strategy to improve wheat performance under water-limited conditions. This study evaluated the effects of three native Moroccan rhizobial strains (1159N11, 1159N32, and 686N5), two spring bread wheat varieties (Malika (drought-sensitive) and Qafedu (drought-tolerant)), and four irrigation levels (100%, 75%, 50%, and 25% field capacity) under controlled greenhouse conditions. Irrigation was the primary factor influencing phenotypic variation, significantly affecting a broad range of vegetative, yield-related, physiological, and biochemical traits, while genotype also contributed significantly to several traits. Despite the predominant influence of water availability, rhizobial inoculation produced significant effects on total tiller number, seed number, thousand-kernel weight, and CAT activity, with responses varying according to genotype and irrigation level. Under severe drought, inoculation with strain 686N5 increased total tiller number by 46.4% and thousand kernel weight by 16.1% in the drought-sensitive genotype Malika. In the drought-tolerant genotype Qafedu, the same strain was associated with a 72.8% increase in seed number per plant at 75% field capacity. Changes in catalase activity further indicated that antioxidant responses may be involved in the effects of inoculation under water deficit, although the underlying mechanisms require further investigation. No strain consistently outperformed the others across conditions, highlighting the context-dependent nature of rhizobial effects and the need for multi-environment field validation before practical application.

Keywords: bread wheat; drought stress; *Rhizobium*; rhizobial inoculation; tolerance

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1. Introduction

Wheat (*Triticum aestivum* L.) is one of the world's most important cereal crops and a major contributor to global food security, providing approximately 20% of the calories and protein consumed by the global population [1]. However, sustaining wheat productivity is becoming increasingly challenging under climate change, particularly in arid and

semi-arid regions where recurrent drought events and increasing water scarcity threaten crop production.

Drought is among the most important abiotic constraints limiting wheat productivity. Water deficit adversely affects plant growth, photosynthesis, nutrient uptake, cellular metabolism, and biomass accumulation, ultimately leading to substantial yield losses. The severity of these effects depends on drought intensity, duration, developmental stage, and plant genotype [2–4]. Improving drought tolerance has therefore become a major objective of wheat breeding programs. Although considerable progress has been achieved through conventional breeding, molecular breeding, and genomic approaches, drought tolerance remains a highly complex quantitative trait controlled by multiple interacting physiological, genetic, and environmental factors [4]. Furthermore, developing drought-tolerant cultivars requires considerable time and resources, highlighting the need for complementary approaches that could improve crop performance under water-limited conditions.

Among these complementary strategies, the use of beneficial soil microorganisms has attracted increasing attention. Plant growth-promoting rhizobacteria (PGPR) enhance plant performance through multiple mechanisms, including improved nutrient mobilization, phytohormone production, stimulation of root development, and regulation of physiological, biochemical, and antioxidant responses [5–7]. Under drought conditions, PGPR inoculation has been associated with improved plant water status, photosynthetic efficiency, nutrient acquisition, biomass production, and stress tolerance. A recent meta-analysis across major cereal and legume crops further confirmed that PGPR generally improve plant performance under water deficit, although the magnitude of these benefits depends on crop species, bacterial taxa, environmental conditions, and the traits evaluated [8]. Thus, microbial inoculation represents a promising but highly context-dependent strategy for improving crop tolerance to drought. Several mechanisms may contribute to PGPR-mediated responses under drought, including phytohormone production, nutrient mobilization, modulation of osmotic adjustment, and regulation of antioxidant defenses. In wheat, *Azospirillum, Bacillus*, *Paenibacillus*, and *Pseudomonas* have been reported as microbial plant biostimulants under drought conditions [9]. The contribution of individual mechanisms, however, depends on the microorganism–host combination and the conditions under which plants are grown. Indole-3-acetic acid (IAA), for example, can influence root architecture and thereby improve water and nutrient acquisition. Drought stress also triggers physiological and biochemical responses, including osmotic adjustment and antioxidant activity [10]. Proline may contribute to osmotic regulation and cellular protection under stress, although its accumulation can also reflect the severity of stress experienced by the plant [11]. Measurement of physiological and biochemical traits can therefore help characterize plant responses to inoculation, but changes in these indicators do not, by themselves, establish the mechanisms responsible for improved growth or yield. Although *Rhizobium* is best known for symbiotic nitrogen fixation in legumes, rhizobia can also promote plant growth through phosphorus solubilization and the production of siderophores, phytohormones, exopolysaccharides, enzymes, and secondary metabolites [12]. Their beneficial effects may also extend to non-leguminous crops, including cereals [13], although their role in alleviating drought stress in cereals remains insufficiently understood. Other phosphate-solubilizing microorganisms, including *Mesorhizobium ciceri* in chickpea [14], *Streptomyces laurentii* and *Penicillium* sp. in great millet [15], and *Pantoea alhagi* in wheat [16], have also been associated with improved plant performance under drought. Reported benefits include improved growth, nutrient acquisition, chlorophyll content, and plant water status; however, these effects are often inconsistent and strongly dependent on bacterial strain, host genotype, and environmental conditions. For example, Barquero et al. [9] demonstrated that only selected *Rhizobium leguminosarum* strains significantly improved wheat growth and physiological per-

formance under water stress, whereas other strains produced little or no benefit. Similarly, genotype × *Rhizobium* × environment interactions have been shown to influence plant performance, emphasizing that successful inoculation depends on the compatibility among bacterial strains, crop genotypes, and environmental conditions [17].

This context dependency is particularly relevant when evaluating rhizobial isolates originating from drought-prone environments. Such native strains may possess adaptive traits that enhance their survival under osmotic stress and potentially improve plant performance under limited water availability. Moreover, drought tolerance demonstrated by bacterial isolates under laboratory conditions does not necessarily translate into consistent beneficial effects under greenhouse or field conditions, because inoculation outcomes are influenced by complex interactions among bacterial identity, plant genotype, and water availability. Consequently, evaluating candidate strains across contrasting host genotypes and irrigation regimes is essential for identifying inoculants with stable and reproducible effects. Despite growing interest in microbial inoculation as a strategy for enhancing drought tolerance in wheat, important knowledge gaps remain. Many previous studies have evaluated only a limited number of bacterial strains or water regimes, while relatively few have simultaneously examined the combined effects of rhizobial treatment, wheat genotype, and water availability on a range of growth, yield-related, physiological, and biochemical traits. In addition, native drought-adapted *Rhizobium* strains isolated from semi-arid environments remain insufficiently characterized with respect to their potential use as bioinoculants in wheat. A better understanding of these interactions is therefore needed to identify effective microbial inoculants and determine the environmental conditions under which they provide consistent agronomic benefits.

To address these knowledge gaps, the present study evaluated three Moroccan *Rhizobium* strains isolated from lentil (*Lens culinaris* Medik.) root nodules in semi-arid environment [18]. The strains were applied individually and as a mixed inoculum in two spring bread wheat genotypes differing in their drought response, Malika (drought-sensitive) and Qafedu (drought-tolerant), under four irrigation regimes (100%, 75%, 50%, 25% field capacity). The experiment was designed to examine the effects of inoculation in combination with host genotype and water availability under controlled greenhouse conditions. Agronomic, physiological, and biochemical traits were assessed to characterize plant responses to these treatments. Specifically, the objectives were to (i) assess the effects of selected rhizobial treatments on growth and yield-related traits under contrasting irrigation regimes; (ii) evaluate physiological and biochemical responses associated with rhizobial inoculation and water deficit; (iii) determine whether inoculation responses depend on wheat genotype and irrigation regime; and (iv) identify promising rhizobial treatments for further evaluation as potential wheat bioinoculants under water-limited conditions.

2. Materials and Methods

2.1. The Experimental Design

A greenhouse pot experiment was conducted to evaluate the effects of three *Rhizobium* strains on the growth, physiological performance, and yield of spring bread wheat (*Triticum aestivum* L.) under different levels of water deficit. The experiment followed a completely randomized design with three biological replicates. Each pot was considered an independent experimental unit and contained five plants at the time of measurement. Individual plants within a pot were treated as subsamples rather than independent replicates. Where plant-level measurements were recorded, the mean per pot was used for statistical analysis.

Two spring bread wheat varieties with contrasting drought responses, Malika (drought-sensitive, G1) and Qafedu (drought-tolerant, G2), were evaluated. Seeds were inoculated

with three native rhizobial strains (1159N11, 1159N32, and 686N5) either individually or as a combined inoculum (Mixt), while uninoculated seeds served as the controls.

Four irrigation regimes corresponding to 100%, 75%, 50%, and 25% of field capacity (FC) were applied to simulate well-watered, mild, moderate, and severe drought stress conditions, respectively.

The experiment consisted of a factorial arrangement of 2 wheat genotypes × 5 bacterial treatments × 4 irrigation regimes, resulting in 40 treatment combinations. Each treatment combination was replicated three times, giving a total of 120 experimental units. Pots were randomly distributed within the greenhouse, and their positions were periodically rearranged throughout the experiment to minimize potential environmental gradients and positional effects. The experimental factors are summarized in Table 1.

Table 1. Experimental factors and treatment levels.

Factor	Levels
Genotype	Malika, Qafedu
Bacterial treatment	B1, B2, B3, Mixt, Control
Irrigation regime	100%, 75%, 50%, 25% FC

Footnote: B1 = *Rhizobium* strain 1159N11; B2 = *Rhizobium* strain 1159N32; B3 = *Rhizobium* strain 686N5; Mixt = combined inoculum of B1, B2, and B3.

2.2. Soil and Seed Preparation

The experiment was conducted using a clay-rich Vertisol collected from the Merchouch Experimental Station, Morocco. The soil consisted of 51% clay, 37% silt, and 12% sand, as previously characterized by El Mekkaoui et al. [19]. To eliminate indigenous microorganisms and ensure that observed plant responses resulted primarily from the introduced rhizobial inoculants, the soil was sterilized by autoclaving at 120 °C for 20 min twice with a 24-h interval between treatments, following the procedures described by Van Schreven and Dietrich et al. [20,21].

Seeds of both wheat genotypes were surface-sterilized by immersion in 70% (*v*/*v*) ethanol for 3 min, followed by treatment with a sodium hypochlorite solution containing 2% Tween-80. After sterilization, the seeds were rinsed six times with sterile distilled water to remove residual disinfectants and soaked in sterile distilled water for 4 h before bacterial inoculation, following previously established protocols [22–24].

2.3. Rhizobial Treatments and Seed Inoculation

Three rhizobial strains (1159N11, 1159N32, and 686N5) previously selected for their performance in drought-tolerance screening and their plant growth-promoting traits were obtained from a collection of 68 bacterial isolates previously recovered from root nodules of lentil (*Lens culinaris* Medik.) grown at the Merchouch Experimental Station, Morocco. These strains were selected based on their superior performance in previous screenings for drought tolerance and plant growth-promoting (PGP) traits, including phosphate-solubilizing activity (PSA), indole-3-acetic acid (IAA) production, and growth under osmotic stress conditions [18].

Among the selected isolates, strains 1159N11 and 1159N32 exhibited high phosphate-solubilizing activity, releasing 144.6–205.6 µg P_2O_5 mL^{-1} under non-stress conditions. Notably, strain 1159N11 maintained phosphate solubilization above 100 µg P_2O_5 mL^{-1} under osmotic stress ($\Psi = -0.21$ and -0.53 MPa), indicating strong functional stability under water-limiting conditions. In contrast, strain 686N5 was distinguished by its high IAA production and moderate phosphate-solubilizing capacity. Molecular characterization based on 16S rRNA gene sequencing identified strains 1159N11 and 686N5 as *Rhizobium*

leguminosarum, whereas strain 1159N32 showed close phylogenetic affinity to either *R. laguerreae* or *R. leguminosarum* [18]. These complementary physiological characteristics justified their selection as candidate bioinoculants for improving wheat performance under drought stress.

For inoculum preparation, each strain was cultured separately in 100 mL of sterile yeast extract mannitol (YEM) broth in 250-mL Erlenmeyer flasks and incubated at 28 ± 1 °C on an orbital shaker at 100 rpm for 48–72 h until the culture reached the exponential growth phase. Bacterial cells were harvested by centrifugation at 4000× *g* for 15 min at 4 °C, washed with sterile distilled water, and resuspended to a final concentration of approximately 10^8 CFU mL^{-1}, corresponding to a 0.5 McFarland standard, as determined spectrophotometrically.

Seed inoculation was performed using a seed-priming approach. Surface-sterilized wheat seeds were immersed in the respective bacterial suspensions (10^8 CFU mL^{-1}) for 12 h at 28 °C with gentle agitation (22 rpm) to facilitate bacterial attachment and colonization of the seed surface. For the mixed-inoculum treatment, equal volumes of the three bacterial suspensions were combined immediately before seed priming to obtain the same final cell concentration. Control seeds were treated identically using sterile YEM broth without bacterial cells. After inoculation, all seeds were air-dried under sterile conditions overnight at 28 °C to promote bacterial adhesion before sowing.

2.4. Plant Growth Conditions and Drought Treatments

Eight inoculated seeds were sown in 25-cm-diameter pots filled with sterilized Vertisol (pH 6.5). The experiment was conducted in a controlled-environment greenhouse at the International Center for Agricultural Research in the Dry Areas (ICARDA), Rabat, Morocco. Plants were grown under a 16-h light/8-h dark photoperiod, day/night temperatures of 30 ± 2/22 ± 2 °C, and a relative humidity of 60–80%.

At the three-leaf stage (Zadoks GS13), seedlings were thinned to five uniform plants per pot. All pots were maintained under well-watered conditions until the onset of stem elongation (GS30–45 according to the Zadoks growth scale), after which four irrigation regimes corresponding to 100%, 75%, 50%, and 25% FC were imposed to simulate well-watered, mild, moderate, and severe drought stress, respectively. The target soil moisture levels were maintained until physiological maturity throughout the experimental period.

All plants received identical crop management practices, and no nutrient deficiencies or pest infestations were observed during the experiment. At physiological maturity, plants were harvested for the assessment of agronomic, physiological, biochemical, and grain quality traits.

2.5. Morphological and Yield Measurements

Agronomic and yield-related traits were evaluated at physiological maturity following standard wheat phenotyping protocols. Shoot and root biomass were determined after oven-drying plant samples at 70 °C for 48 h or until constant weight was reached.

The following traits were recorded on individual plants and averaged across the five plants within each pot to obtain a mean per-plant value for each pot (Table 2): shoot dry biomass (SDB), root dry weight (RDW), root length (RL), plant height (PLH), flag leaf length (FL), productive tiller number (PT), total tiller number (NT), spike length (SL), number of seeds per plant (NS), seed weight per plant (WS), spike weight (SW), thousand-kernel weight (TKW), grain length (GL), grain width (GW), grain projected area (GPA), grain protein content (GPC), and grain moisture content (GMC).

Table 2. *p*-values from three-way ANOVA showing the effects of rhizobial treatment, irrigation regime, genotype, and their interactions on vegetative, yield, grain, physiological and biochemical traits of wheat.

Trait	Genotype (G)	Strain (S)	Irrigation (I)	G × S	G × I	I × S	G × I × S
			Vegetative traits				
SDB	3.31 † (0.076)	0.34 (0.849)	58.55 *** (<0.001)	0.98 (0.432)	1.26 (0.301)	0.60 (0.828)	1.16 (0.346)
RDW	0.27 (0.609)	0.81 (0.526)	13.33 *** (<0.001)	0.19 (0.943)	0.47 (0.704)	0.82 (0.631)	0.17 (0.999)
RL	5.35 * (0.026)	0.75 (0.565)	0.91 (0.444)	1.40 (0.252)	0.49 (0.692)	0.36 (0.972)	0.44 (0.935)
PLH	27.97 *** (<0.001)	0.34 (0.849)	55.18 *** (<0.001)	2.93 * (0.033)	0.81 (0.494)	1.18 (0.327)	1.53 (0.153)
FL	7.74 ** (0.008)	1.03 (0.406)	0.18 (0.907)	0.56 (0.696)	2.63 † (0.063)	0.49 (0.908)	2.08 * (0.041)
			Yield traits				
PT	105.42 *** (<0.001)	2.40 † (0.066)	144.26 *** (<0.001)	1.18 (0.336)	0.63 (0.599)	1.35 (0.228)	1.49 (0.167)
NT	1.52 (0.226)	3.71 * (0.012)	36.30 *** (<0.001)	0.89 (0.480)	1.15 (0.339)	2.15 * (0.035)	1.90 † (0.064)
SL	97.26 *** (<0.001)	1.94 (0.123)	3.30 * (0.030)	1.25 (0.305)	0.21 (0.887)	0.41 (0.952)	1.40 (0.209)
NS	0.03 (0.869)	2.54 † (0.055)	88.25 *** (<0.001)	3.38 * (0.018)	5.58 ** (0.003)	1.47 (0.176)	2.11 * (0.039)
WS	0.53 (0.470)	0.55 (0.701)	45.04 *** (<0.001)	2.10 † (0.099)	4.41 ** (0.009)	1.08 (0.403)	1.33 (0.243)
SW	0.25 (0.618)	0.39 (0.812)	59.74 *** (<0.001)	1.95 (0.120)	4.41 ** (0.009)	1.34 (0.236)	1.18 (0.328)
TKW	0.01 (0.938)	2.92 * (0.033)	12.08 *** (<0.001)	1.15 (0.347)	3.16 * (0.035)	1.61 (0.127)	1.86 † (0.071)
			Grain traits				
GL	1.31 (0.259)	1.28 (0.293)	1.04 (0.386)	0.31 (0.871)	0.47 (0.707)	1.74 † (0.095)	0.39 (0.961)
GW	0.05 (0.820)	1.57 (0.202)	1.68 (0.186)	0.39 (0.815)	0.80 (0.503)	1.77 † (0.087)	0.38 (0.963)
GPA	2.13 (0.152)	1.58 (0.199)	2.14 (0.110)	0.40 (0.811)	1.09 (0.365)	1.70 (0.104)	0.43 (0.943)
GPC	20.34 *** (<0.001)	1.80 (0.147)	0.82 (0.492)	1.02 (0.408)	0.20 (0.897)	0.49 (0.909)	0.45 (0.930)
GMC	68.36 *** (<0.001)	2.27 † (0.079)	1.35 (0.272)	1.39 (0.256)	0.05 (0.984)	0.84 (0.608)	0.68 (0.762)
			Physiological and Biochemical traits				
CCBS	39.62 *** (<0.001)	1.33 (0.27)	4.03 * (0.013)	0.75 (0.55)	1.85 (0.15)	0.92 (0.52)	1.36 (0.22)
CCAS	158.87 *** (<0.001)	1.07 (0.38)	6.74 *** (<0.001)	0.04 (0.99)	0.61 (0.6)	1.45 (0.18)	0.97 (0.48)
Chl	3.72 ** (0.007)	0.46 (0.8)	1.8 (0.11)	1.99 † (0.07)	0.54 (0.89)	0.62 (0.8)	0.8 (0.7)
Anth	0.78 (0.56)	1.56 (0.19)	0.33 (0.93)	1.52 (0.18)	0.38 (0.97)	0.51 (0.92)	0.48 (0.96)
MDA	1.83 (0.12)	0.48 (0.78)	0.58 (0.76)	0.94 (0.48)	0.5 (0.92)	0.61 (0.84)	0.66 (0.85)
POD	0.79 (0.55)	0.24 (0.93)	0.86 (0.53)	0.76 (0.62)	0.55 (0.88)	0.75 (0.72)	0.47 (0.97)
Phenol	27.99 *** (<0.001)	0.99 (0.43)	0.24 (0.97)	0.42 (0.88)	0.96 (0.51)	0.94 (0.52)	1.55 (0.1)
Sugar	0.7 (0.62)	0.96 (0.44)	1.81 (0.11)	0.89 (0.52)	1.63 (0.1)	0.72 (0.75)	0.92 (0.57)
Protein	11.77 *** (<0.001)	0.43 (0.82)	5.26 *** (<0.001)	2.65 * (0.02)	4.11 *** (<0.001)	3.05 ** (0.002)	3.87 *** (<0.001)
Proline	0.25 (0.93)	0.66 (0.65)	1.81 (0.11)	0.13 (0.99)	0.16 (0.99)	0.62 (0.84)	0.22 (0.99)
CAT	0.0025 (0.9)	2.69 * (0.03)	0.65 (0.58)	0.37 (0.83)	2.73 * (0.04)	1.75 † (0.07)	1.88 * (0.04)

Footnote: Values are F-statistics, with corresponding *p*-values shown in parentheses, obtained from a three-way ANOVA. Significance levels are indicated as follows: †, $0.05 \leq p < 0.10$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Degrees of freedom (df) were as follows: Genotype = 1, Irrigation = 3, Strain = 4, Genotype × Irrigation = 3, Genotype × Strain = 4, Irrigation × Strain = 12, and Genotype × Irrigation × Strain = 12. Abbreviations: shoot dry biomass (SDB), root dry weight (RDW), root length (RL), plant height (PLH), flag leaf length (FL), productive tiller number (PT), total tiller number (NT), spike length (SL), number of seeds per plant (NS), seed weight per plant (WS), spike weight (SW), thousand-kernel weight (TKW), grain length (GL), grain width (GW), grain projected area (GPA), grain protein content (GPC), grain moisture content (GMC), chlorophyll content before stress (CCBS), chlorophyll content after stress (CCAS), chlorophyll content (Chl), anthocyanins content (Anth), malondialdehyde concentration (MDA), peroxidase activity (POD), total phenolic content, total soluble sugar content, protein, proline content, and catalase activity (CAT).

Grain morphometric characteristics, including seed length, seed width, and projected seed area, were determined using the Opto-Agri Grain Scanner System, an image-based phenotyping platform that enables rapid, accurate, and non-destructive measurement of grain dimensions.

2.6. Chlorophyll Determination

Leaf chlorophyll status was evaluated using both non-destructive and destructive approaches. Relative chlorophyll content was measured at the heading stage on the flag leaf using a SPAD-502 chlorophyll meter (Konica Minolta, Tokyo, Japan), with measurements taken from the central portion of the leaf blade to minimize positional variation.

For pigment quantification, fresh leaf tissue was homogenized in 80% (v/v) acetone and centrifuged to obtain a clear extract, as described in Supplementary Material S1. Absorbance was measured at 645 and 663 nm using a FLUOstar Omega UV–visible spectrophotometer

(BMG LABTECH, Offenburg, Germany), and chlorophyll a (Chl a) and chlorophyll b (Chl b) were calculated according to the method described by Bassa et al. [25], using the following equations:

$$\text{Chl a} = 0.999 \text{ x A663} - 0.0989 \text{ x A645}$$

$$\text{Chl b} = -0.328 \text{ x A663} + 1.77 \text{ x A645}$$

2.7. Protein and Moisture Determination

Grain protein and moisture contents were determined using near-infrared reflectance (NIR) spectroscopy, a rapid and non-destructive analytical technique widely used for cereal quality assessment. Measurements were performed according to the manufacturer's calibration procedures, and the results were expressed as percentages on a grain weight basis.

Total soluble protein concentration in leaf tissues was determined using the Bradford colorimetric assay described in Supplementary Material S1 [26], with bovine serum albumin (BSA) used to generate the standard calibration curve. Protein concentrations were quantified spectrophotometrically and expressed on a fresh-weight basis.

2.8. Biochemical Analyses

To characterize biochemical responses to water deficit and rhizobial inoculation, flag leaf samples were collected at anthesis from plants within each experimental unit.

The samples were analyzed for anthocyanin content, malondialdehyde (MDA) concentration, peroxidase (POD) activity, total phenolic content, total soluble sugar content, proteins, proline content, and catalase activity (CAT) (Table 2). Proline concentration, MDA content, and the activities of CAT and POD were determined according to the protocols described by Chen et al. [27]. Anthocyanin content was quantified following Ticconi et al. [28], total phenolic content was determined using the method of Velioglu et al. [29], and total soluble sugars were measured according to the phenol–sulfuric acid method of DuBois et al. [30]. Spectrophotometric measurements were performed at the wavelengths specified in the corresponding protocols provided in the Supplementary Material S1.

2.9. Soil Physicochemical Analysis

At the end of the experiment, bulk soil samples were collected from each experimental treatment to evaluate the effects of genotype, irrigation regime, and rhizobial inoculation on soil physicochemical properties. The analyzed parameters included soil pH (measured in H_2O and KCl), electrical conductivity (EC), organic matter (OM) content, available phosphorus (P_2O_5), and exchangeable potassium (K_2O). The uninoculated treatment (control) served as the control and was compared with the individual rhizobial treatments and the mixed-inoculum treatment. All analyses were performed at the Research Unit for Environment and Natural Resource Conservation, Regional Center for Agricultural Research (INRA-Rabat), Morocco, following standard soil analytical procedures [31–35] as described in Supplementary Material S2.

2.10. Statistical Analysis

All statistical analyses were performed in R (version 4.4.1; R Core Team, Vienna, Austria). For each trait, the pot was considered the experimental unit, and pot-level observations were analyzed using a three-way factorial analysis of variance (ANOVA) to evaluate the effects of genotype (G), rhizobial treatment (S), irrigation regime (I), and their two-way and three-way interactions. When significant effects or interactions were detected, treatment means were compared using Fisher's least significant difference (LSD) test at the 5% probability level. Mean comparisons were conducted separately within each irrigation regime using genotype × rhizobial treatment combinations. Principal component

analysis (PCA) was performed on standardized trait values to investigate multivariate relationships among treatments, and Pearson correlation coefficients were calculated to quantify associations among agronomic, physiological, biochemical, and yield-related traits. Data visualization was performed using the ggplot2 package, while mean separation was conducted using the agricolae package, and data manipulation was carried out using dplyr.

3. Results

3.1. Effects of Irrigation, Genotype, Rhizobial Treatment, and Their Interactions on Wheat Traits

Three-way ANOVA revealed that irrigation regime was the main source of variation across the evaluated traits (Table 2). Significant irrigation effects were detected for CCBS, CCAS, SDB, RDW, PLH, PT, NT, SL, NS, WS, SW, TKW, and protein content. These results demonstrate the strong influence of water availability on wheat growth, productivity, and physiological performance.

Genotype significantly affected several traits, including CCBS, CCAS, SL, PT, PLH, GPC, GMC, phenolic content, and protein content ($p < 0.001$), while SDB, RL, and FL were also significantly affected by genotype ($0.1 < p < 0.05$). These findings indicate substantial genetic differences between the drought-sensitive genotype Malika and the drought-tolerant genotype Qafedu in their responses to varying water regimes.

Rhizobial treatment significantly affected NT ($p = 0.012$), TKW ($p = 0.033$), and CAT activity ($p = 0.03$), with marginal effects observed for PT ($p = 0.066$), NS ($p = 0.055$), and GMC ($p = 0.079$), suggesting that rhizobial inoculation influenced selected yield-related and biochemical traits.

Several significant interactions revealed context-dependent responses. The irrigation × strain interaction significantly affected NT ($p = 0.035$), suggesting that the effect of rhizobial inoculation depended on moisture availability. The genotype × strain interaction affected PLH ($p = 0.033$) and NS ($p = 0.018$), indicating differential responses of wheat genotypes to individual rhizobial strains. Genotype × irrigation interactions were significant for NS ($p = 0.003$), WS ($p = 0.009$), SW ($p = 0.009$), TKW ($p = 0.035$) and CAT activity ($p = 0.040$). The three-way genotype × irrigation × strain interaction was significant for FL ($p = 0.041$) and NS ($p = 0.039$), with marginal effects for NT ($p = 0.064$), TKW ($p = 0.071$) and CAT ($p = 0.04$), highlighting the complexity of plant–microbe–environment interactions under drought conditions.

Grain morphometric traits (GL, GW, and GPA) were not significantly affected by genotype, irrigation, or rhizobial treatment, nor by their interactions, although irrigation × strain effects were marginal for GL ($p = 0.095$) and GW ($p = 0.087$). Overall, rhizobial treatment influenced a subset of traits, with significant interactions with genotype and water availability.

3.2. Principal Component Analysis and Trait Associations

PCA was performed to summarize multivariate relationships among the physiological, vegetative, yield-related, grain quality, and biochemical traits evaluated under contrasting irrigation regimes and rhizobial treatments (Figure 1). The first two principal components accounted for 36.3% of the total variation, with PC1 and PC2 explaining 22.7% and 13.6%, respectively. PC1 primarily contrasted traits associated with plant growth, biomass accumulation, and yield formation from grain-size-related and selected biochemical traits. PLH, PT, Chl, RL, TKW and several biochemical traits were generally positioned on the positive side of PC1, whereas SDB, RDW, NS, WS, and SW were oriented toward the negative side.

NS, WS, and SW showed closely aligned vectors, indicating similar patterns of variation and positive associations among these yield-related traits. SDB was similarly oriented with NS and WS, suggesting a positive association between biomass production and yield-related traits. PLH and PT also showed comparable orientations, indicating a positive association

between plant stature and productive tillering. TKW was oriented in the opposite direction to several traits associated with NS and SDB, consistent with its negative correlation with NS ($r = -0.33$). In contrast, GL, GW, and GPA showed similar patterns of variation and strong positive correlations with one another (Figure 2).

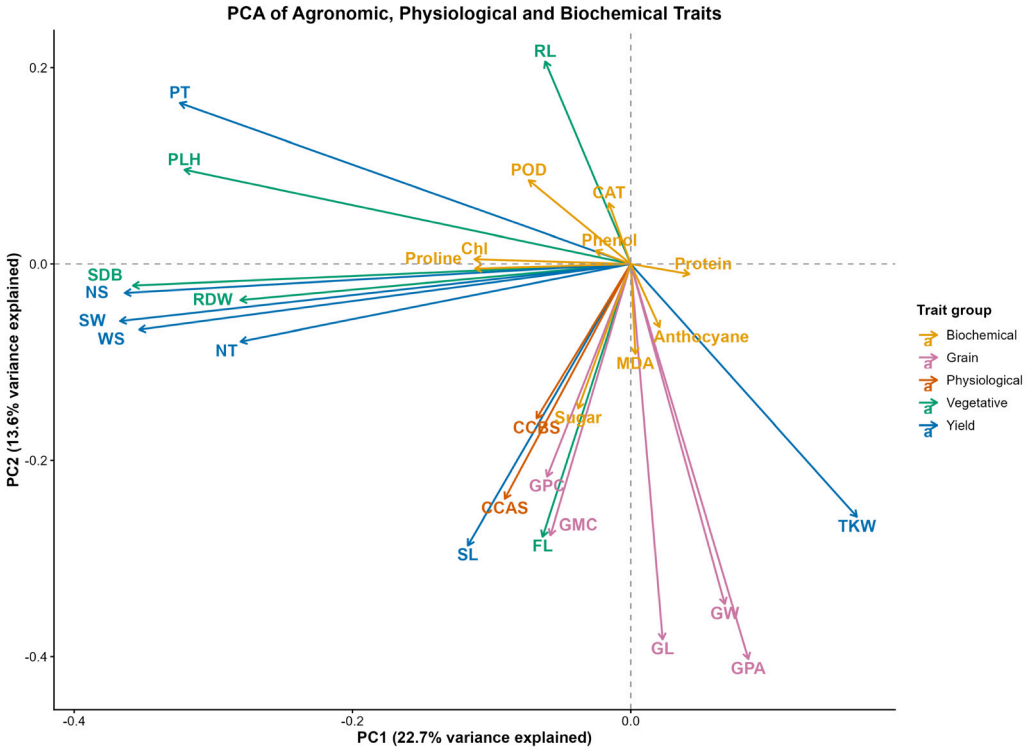


Figure 1. PCA biplot showing the relationships among the measured physiological, vegetative, yield, and grain quality traits.

Among the physiological and biochemical traits, RL contributed strongly to PC2, whereas RDW was positioned in the opposite direction. POD and proline were associated with the positive side of PC2, while anthocyanins and MDA contributed strongly to the positive side of PC1.

3.3. Correlation Among Agronomic, Physiological, and Yield Traits

Pearson correlation analysis revealed several significant associations among the measured traits (Figure 2). SDB showed strong positive correlations with SW ($r = 0.89$), NS ($r = 0.78$), and WS ($r = 0.84$). PLH was positively correlated with PT ($r = 0.73$), SDB ($r = 0.81$), NS ($r = 0.74$), and WS ($r = 0.78$). PT was also positively correlated with SDB ($r = 0.77$), NS ($r = 0.74$), and WS ($r = 0.67$). NT was positively associated with NS ($r = 0.60$) and WS ($r = 0.56$).

TKW was negatively correlated with NS ($r = -0.33$), suggesting a trade-off between grain size and grain number. RL showed negative correlations with GPC ($r = -0.30$) and GMC ($r = -0.25$), whereas CCAS was positively correlated with GPC ($r = 0.53$) and GMC ($r = 0.68$). Grain morphometric traits were strongly and positively correlated with GW, showing high correlations with GPA ($r = 0.97$) and GL ($r = 0.97$), while GL was also strongly intercorrelated with GPA ($r = 0.95$).

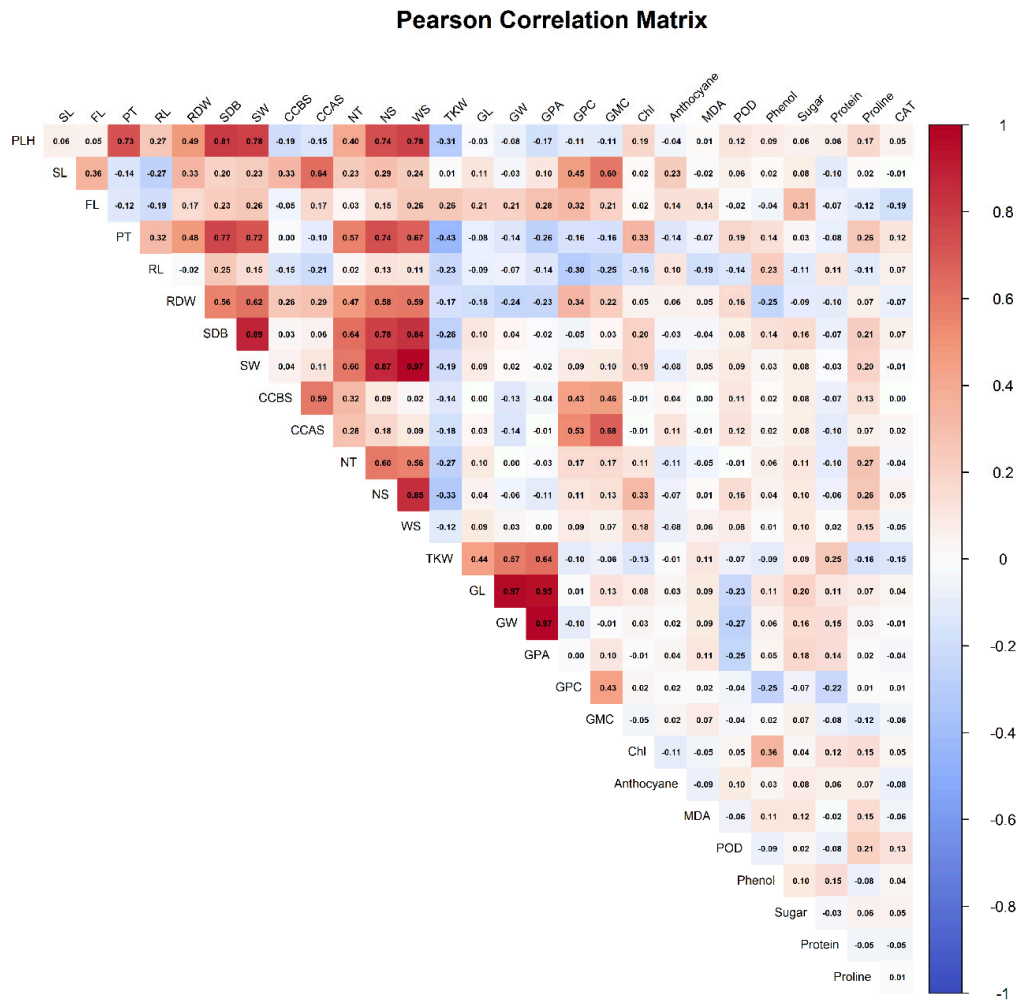


Figure 2. Pearson correlation heatmap of the measured physiological, vegetative, yield, and grain quality traits in wheat.

3.4. Effects of Genotype, Irrigation Regime, and Rhizobial Treatment on Selected Agronomic, Physiological, and Biochemical Traits

The effects of genotype, irrigation regime, and rhizobial treatment on selected traits are shown in Figure 3 and Table 3. Traits were selected based on significant or marginal three-way interactions and significant interactions involving rhizobial treatment, with an emphasis on responses that varied according to genotype and irrigation regime. The rhizobial treatments comprised *Rhizobium* strains 1159N11 (B1), 1159N32 (B2), and 686N5 (B3), a mixture of the three strains (Mixt), and an uninoculated control.

Seed number per plant (NS) was significantly affected by genotype × irrigation ($p = 0.003$), genotype × strain ($p = 0.018$), and genotype × irrigation × strain ($p = 0.039$) interactions (Figure 3b). NS generally increased with increasing irrigation levels, although inoculation responses varied substantially between genotypes and irrigation regimes. The largest increases relative to uninoculated controls reached 75% in G1 under 25% FC with Mixt and 72.8% in G2 under 75% FC with 686N5 (Table 3). Other notable increases were observed with B2 in G1 and G2 under 25% FC (+71.0% and +65.3%, respectively) and with B3 in G1 at the same irrigation level (+62.2%).

WS was significantly affected by irrigation ($p < 0.001$) and genotype × irrigation interaction ($p = 0.009$), indicating genotype-dependent responses to water availability (Figure 3c). WS generally increased with increasing irrigation levels and was strongly positively correlated with NS (r = 0.85), reflecting similar patterns of variation in these two

yield-related traits. Although rhizobial treatment and its interactions did not significantly affect WS, positive responses relative to uninoculated controls were observed under specific genotype × irrigation combinations. The largest increases were recorded with B3 reaching 88.28% in G1 under 25% FC and +72.45% in G2 under 75% FC. Additional increases with B2 under 25% FC reached 72.77% in G1 and +39.94% in G2 (Table 3).

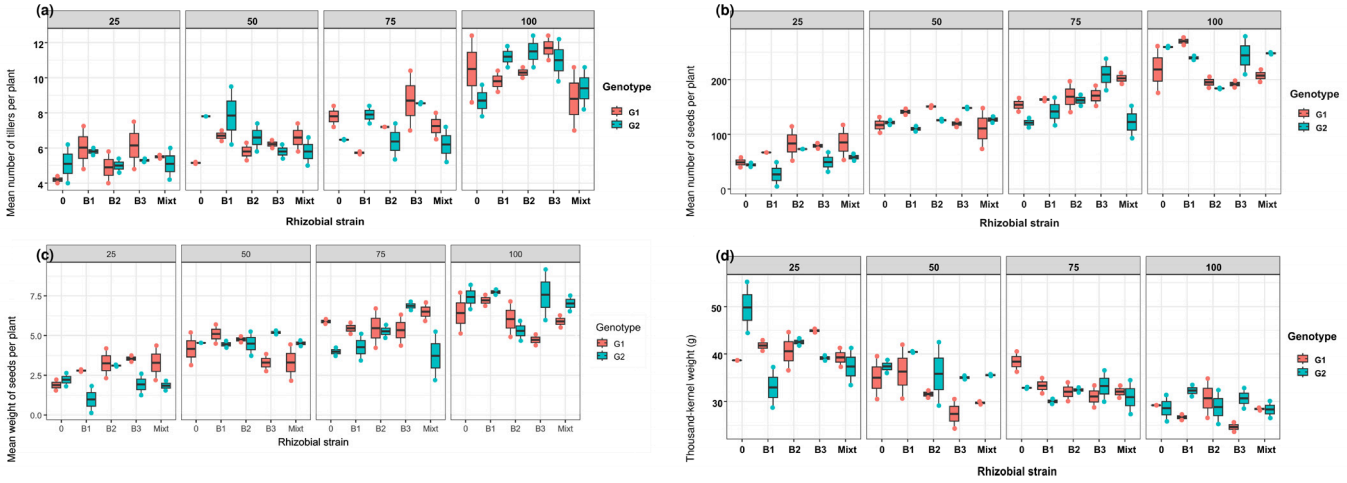


Figure 3. Effects of genotype, irrigation regime, and rhizobial treatment on selected agronomic and yield-related traits of wheat: (**a**) number of tillers per plant (NT), (**b**) number of seeds (NS), (**c**) weight of seeds per plant (WS), and (**d**) thousand kernel weight (TKW). Boxplots show the distribution of observations for each genotype × rhizobial treatment combination under four irrigation regimes (25, 50, 75, and 100% field capacity). G1 and G2 represent the two wheat genotypes, while 0, B1, B2, B3, and Mixt represent the uninoculated control, individual rhizobial treatments, and mixed inoculum, respectively.

Table 3. Selected positive percentage changes in yield-related traits following rhizobial inoculation relative to the corresponding uninoculated controls under specific genotype × irrigation combinations.

Strain	Genotype × Irrigation Condition	NT	NS	WS	TKW	CAT
B1 (1159N11)	G1, 25% FC	43.50%	37.10%	48.12%	8.00%	—
	G2, 100% FC	28.70%	—	—	12.90%	—
	G1, 50% FC	30.10%	20.70%	22.35%	3.60%	>100%
B2 (1159N32)	G1, 25% FC	16.70%	71.00%	72.77%	4.90%	—
	G2, 25% FC	—	65.30%	39.94%	—	—
	G2, 75% FC	—	33.90%	32.24%	—	9.76%
B3 (686N5)	G1, 25% FC	46.40%	62.20%	88.28%	16.10%	—
	G2, 75% FC	32.00%	72.80%	72.45%	1.10%	>100%
	G2, 100% FC	26.40%	—	—	7.30%	—
Mixt	G1, 25% FC	31.00%	75.00%	74.32%	1.60%	87.46%
	G2, 25% FC	—	31.60%	—	—	—
	G1, 75% FC	—	31.40%	10.47%	—	—

Footnote: This table summarizes representative positive responses observed for the different rhizobial treatments. Total tiller number (NT), number of seeds per plant (NS), seed weight per plant (WS), thousand-kernel weight (TKW), catalase activity (CAT).

NT was significantly affected by irrigation ($p < 0.001$) and rhizobial treatment ($p = 0.012$), with a significant irrigation × strain interaction ($p = 0.035$; Figure 3a). Positive responses were particularly evident under reduced irrigation. Relative to uninoculated controls, NT increased with B3 by up to 46.4% in G1 under 25% FC and by 32.0% in G2 under 75% FC (Table 3). B1 increased NT by 43.5% in G1 under 25% FC and by 30.1% under 50% FC, while B2 increased

NT by 16.7% in G1 under 25% FC. The marginal three-way interaction ($p = 0.064$) suggests variation in strain responses across genotype × irrigation combinations.

TKW was significantly affected by irrigation ($p < 0.001$), rhizobial treatment ($p = 0.033$), and the genotype × irrigation interaction ($p = 0.035$), whereas the genotype × irrigation × strain interaction was marginal ($p = 0.071$; Figure 3d). TKW generally decreased under reduced irrigation, but responses to inoculation varied between genotypes and irrigation regimes. In G1 under 25% FC, all rhizobial treatments increased TKW relative to the uninoculated control, with increases ranging from +1.6% with Mixt to +16.1% with B3 (Table 3). Under 100% FC, B1 and B3 increased TKW in G2 by 12.9% and 7.3%, respectively.

GPC and GMC were significantly affected by genotype ($p < 0.001$), while rhizobial treatment showed a marginal effect ($p = 0.07$). However, GL, GW, and GPA showed no significant effects of genotype, irrigation, rhizobial treatment, or their interactions. CCBS and CCAS were significantly affected by genotype and irrigation, while Chl was primarily affected by genotype ($p = 0.007$). CAT activity was significantly affected by rhizobial treatment ($p = 0.03$). Anthocyanins, MDA, POD, and proline showed no significant main effects or interaction effects.

3.5. Effects of Genotype, Irrigation Regime, and Rhizobial Treatment on Soil Physicochemical Properties

The effects of genotype, irrigation regime, and rhizobial treatment on soil physicochemical properties are presented in Table 4. Irrigation significantly affected EC, K_2O and P_2O_5 ($p < 0.001$), and OM ($p = 0.003$), whereas effects on pH (KCl) ($p = 0.085$) and pH (water) ($p = 0.075$) were marginal. Genotype significantly affected K_2O ($p < 0.001$), with a marginal effect on pH (water) ($p = 0.078$). Rhizobial treatment had no significant main effect on any soil property, while the genotype × strain interaction was marginal for OM ($p = 0.092$), and the irrigation × strain interaction significantly affected pH (water) ($p = 0.027$). The genotype × irrigation × strain interaction was marginally significant for pH (water) ($p = 0.095$).

Table 4. Three-way ANOVA of the effects of genotype, irrigation regime, rhizobial treatment, and their interactions on soil physicochemical properties of wheat.

Trait	Genotype (G)	Irrigation (I)	Strain (S)	G × I	G × S	I × S	G × I × S
Soil chemical traits							
EC	0.005 (0.946)	30.740 *** (<0.001)	0.900 (0.468)	0.236 (0.871)	0.909 (0.463)	0.517 (0.898)	0.368 (0.971)
K_2O	13.169 *** (<0.001)	36.369 *** (<0.001)	0.695 (0.597)	0.116 (0.950)	0.730 (0.574)	0.559 (0.868)	0.503 (0.907)
OM	0.002 (0.965)	5.015 ** (0.003)	0.225 (0.924)	1.784 (0.157)	2.074 † (0.092)	0.627 (0.813)	0.823 (0.627)
P_2O_5	0.125 (0.725)	15.028 *** (<0.001)	0.771 (0.547)	1.240 (0.301)	0.356 (0.839)	0.456 (0.934)	0.643 (0.799)
pH (KCl)	0.622 (0.433)	2.288 † (0.085)	0.282 (0.889)	0.791 (0.503)	0.202 (0.937)	0.898 (0.552)	0.847 (0.603)
pH (water)	3.192 † (0.078)	2.395 † (0.075)	0.649 (0.630)	0.692 (0.560)	0.473 (0.755)	2.087 * (0.027)	1.653 † (0.095)

Footnote: Values are F-statistics, with corresponding *p*-values in parentheses, obtained from a three-way ANOVA. Significance levels are indicated as follows: †, $0.05 \leq p < 0.10$; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Degrees of freedom were: Genotype = 1, Irrigation = 3, Strain = 4, Genotype × Irrigation = 3, Genotype × Strain = 4, Irrigation × Strain = 12, and Genotype × Irrigation × Strain = 12. Electrical conductivity (EC), exchangeable potassium (K_2O), organic matter (OM) content, available phosphorus (P_2O_5), and pH (measured in H_2O and KCl).

4. Discussion

Water deficit is a major constraint to wheat productivity, affecting plant growth, photosynthetic activity, biomass accumulation, and yield formation. The magnitude of these effects depends on stress intensity, duration, developmental stage, and genotype [3,4,36]. Wheat genotypes also differ substantially in their physiological and molecular responses to drought, including variation in relative water content, water loss, proline accumulation, and the expression of stress-responsive genes [3]. Moreover, the timing and severity of water limitation can influence the effectiveness of microbial inoculation, as demonstrated by studies in which bacterial effects on plant growth, physiology, nutrient uptake, and yield may vary according to the developmental stage at which drought stress occurs [37].

Consistent with these observations, irrigation regime was the dominant experimental factor in the present study, significantly influencing numerous vegetative, yield-related, physiological, biochemical, and soil traits. However, the absence of significant irrigation effects on several root, grain, and biochemical traits highlights the trait-specific nature of wheat responses to drought. Genotype also contributed significantly to phenotypic variation, particularly for physiological, vegetative, and grain-quality traits, while significant genotype × irrigation interactions for several yield-related traits confirmed that the two wheat genotypes responded differently to water availability. Similar genotype-dependent responses have been reported for plant height, tillering, spike characteristics, thousand-kernel weight [38,39], and broader physiological responses to drought [4].

Against this strong influence of irrigation and genotype, rhizobial inoculation produced significant responses in seed number, number of tillers, thousand-kernel weight, and catalase activity, together with significant interactions involving several growth and yield-related traits. In particular, the significant genotype × irrigation × strain interactions for NS and NT demonstrate that the effectiveness of inoculation may also depend on the combination of bacterial strain, wheat genotype, and water availability. These findings are consistent with previous studies showing that bacterial inoculation outcomes can be influenced by bacterial identity, host genotype, environmental conditions, and their interactions [9,17,40]. Likewise, genotype × *Rhizobium* × environment interactions have previously been reported to influence plant productivity, emphasizing the importance of evaluating specific plant–microbe combinations under defined environmental conditions [17].

The present results therefore complement previous evidence that bacterial inoculation can improve wheat performance under drought but that these benefits are not universal. Gontia-Mishra et al. [40] reported that bacterial strains differed in their ability to influence wheat growth, water status, membrane stability, osmolyte accumulation, and stress-responsive gene expression during drought. Similarly, Barquero et al. [9] found that *Rhizobium leguminosarum* strains modified physiological and molecular drought responses in wheat, although the magnitude and direction of these responses differed among strains. At a broader scale, meta-analyses have demonstrated that bacterial inoculation effects vary according to bacterial taxon, drought severity and duration, crop species, and the plant traits evaluated [8,41]. Together, these findings support the interpretation that microbial inoculation should be regarded as a context-dependent strategy whose effectiveness depends on the interaction between microbial, plant, and environmental factors.

Multivariate analyses provided additional insight into the relationships among measured traits. Principal component analysis revealed that vegetative growth, biomass accumulation, and yield formation represented the major sources of phenotypic variation across treatments. Correlation analysis supported this pattern by showing strong positive associations between shoot dry biomass and spike weight, number of seeds, and weight of seeds, together with positive relationships among plant height, productive tillers, and several yield components. These relationships may partly reflect the plant-growth-promoting

capacity of the *Rhizobium* strains used in this study. Differences among rhizobial treatments may partly reflect strain-specific functional traits. Previous screening identified high indole-3-acetic acid (IAA) production in 686N5, whereas 1159N11 and 1159N32 showed high phosphate-solubilizing activity; 1159N11 also maintained substantial activity under osmotic stress [18]. IAA may promote root development and support water and nutrient acquisition, while phosphate solubilization may improve phosphorus availability under water-limited conditions [42]. Consistent with this potential, 686N5 was associated with descriptive increases in total tiller number (46.4%), weight of seeds (+88.28%), and thousand-kernel weight (16.1%) in the drought-sensitive genotype Malika under severe water deficit (25% field capacity). These responses highlight 686N5 as a candidate for further evaluation under severe drought. Similarly, the maintenance of phosphate-solubilizing activity by 1159N11 under osmotic stress provides a rationale for investigating its performance under moderate drought. The strain-dependent responses observed here are consistent with previous studies reporting effects of rhizobial inoculation on wheat under contrasting water regimes [4,38]. The modest negative correlation between seed number and thousand-kernel weight suggests a possible trade-off between grain number and grain weight [38], while the strong positive correlations among grain length, grain width, and grain projected area indicate coordinated variation in grain dimensions.

The biochemical responses further emphasized the predominant influence of genotype over rhizobial inoculation. Genotype significantly affected chlorophyll content, phenols, and soluble proteins, whereas rhizobial treatment affected catalase.

Among the biochemical traits, CAT was the parameter showing a significant main effect of rhizobial treatment. Catalase is a key antioxidant enzyme involved in detoxifying hydrogen peroxide generated during oxidative stress, suggesting that selected rhizobial strains may have influenced antioxidant regulation under specific experimental conditions [2,4]. The observed variation among strain × genotype × irrigation combinations suggests that these responses were context-dependent. Increased phenolic content under water deficit may also contribute to cellular protection during drought. In Malika and Qafedu, biochemical responses varied across strains and irrigation levels, indicating that inoculation did not uniformly affect all measured indicators. This pattern agrees with meta-analytic evidence indicating that the effects of plant growth-promoting rhizobacteria on biochemical traits, including proline, soluble sugars, phenolic compounds, malondialdehyde (MDA), and antioxidant enzyme activities, vary considerably among bacterial taxa, host species, drought severity, and experimental conditions [8,41]. Likewise, not all biochemical parameters respond uniformly to bacterial inoculation, and some traits may remain unchanged depending on the specific plant–microbe–environment combination. Consequently, not all rhizobial strains possess equal potential for promoting drought tolerance in non-leguminous crops.

The soil analyses highlighted the predominant influence of irrigation. Water availability significantly affected several soil chemical properties, whereas rhizobial inoculation produced no consistent main effects on bulk soil chemistry. Although irrigation × strain interactions were detected for soil pH (water), the absence of an overall inoculation effect indicates that rhizobial treatments did not consistently modify soil chemical properties under the experimental conditions. These results are consistent with the observation that changes in soil moisture strongly influence nutrient mobility, solute concentrations, and physicochemical processes independently of microbial inoculation. The absence of significant effects on bulk soil properties should not be interpreted as evidence that rhizobial inoculation had no influence on the rhizosphere environment. Bulk soil analyses may not detect localized changes occurring around roots, where microbial colonization, root exudation, nutrient mobilization, and plant–microbe signaling are concentrated [6,24].

Although the precise mechanisms responsible for rhizobial-mediated drought tolerance remain unresolved, strain-specific differences in IAA production and phosphate solubilization provide plausible pathways for influencing plant growth and nutrient acquisition [18,42]. The present findings indicate that multiple mechanisms are likely involved, including improved nutrient availability, phytohormone production, enhanced shoot and root development, osmotic adjustment, and protection against oxidative stress. These mechanisms are not mutually exclusive and may act synergistically to improve plant performance under drought. As emphasized by Timmusk et al. [43], understanding the complex interactions between plants and beneficial rhizosphere microorganisms remains a major research challenge.

Overall, this study indicates that selected native rhizobial strains can enhance specific growth and yield-related traits in wheat under water-limited conditions. The results reinforce the potential of rhizobia as microbial bio-stimulants for non-leguminous crops and support further investigation of their potential sustainable drought management strategies. Further field-scale validation, assessment of root colonization dynamics, and molecular analyses are needed to elucidate genetic mechanisms governing wheat–*Rhizobium* interactions under drought stress.

5. Conclusions

Water availability was the primary factor influencing wheat performance, while genotype and rhizobial inoculation contributed to trait-specific responses. Rhizobial treatment significantly affected yield- and antioxidant-related traits, and a significant three-way interaction indicated that its effects depended jointly on irrigation regime, wheat genotype and inoculation treatment. Under severe water deficit, strain 686N5 showed promising responses in the drought-sensitive genotype Malika, including descriptive increases in total tiller number and thousand-kernel weight, while mixed inoculation showed a stable, broad-spectrum growth-promoting effect across the conditions tested. The functional traits identified during preliminary screening, particularly IAA and siderophore production, provide a basis for further investigation of these native rhizobia as candidate wheat biostimulants. Nevertheless, multi-environment field validation, together with studies of root colonization, rhizosphere processes and molecular responses, is needed before their application can be recommended.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijpb17110131/s1, Supplementary Methods S1: Biochemical and Physiological Analyses; Supplementary Methods S2: Physico-Chemical Characterization of Soil.

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