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The Effect of Plant Growth-Promoting Rhizobacteria on Soil Properties and the Physiological and Anatomical Characteristics of Wheat under Water-Deficit Stress Conditions

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Abstract: This study aimed to evaluate the effects of plant growth-promoting Rhizobacteria (PGPR) treatments, B1, *Azosprillium lipoferum* Sp2 and B2, *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12, as well as inorganic nitrogen doses (60, 100, 140 and 180 kg N ha^{-1}) on some soil physical characters, physiological, anatomical and yield parameters as well as nitrogen use efficiency (NUE) of wheat under water deficit stress. Results showed that water stress significantly decreased physiological characters such as chlorophyll content (6.7 and 9.8%) and relative water content (13.7 and 11.2%) in both seasons, respectively. Nevertheless, proline and malondialdehyde (MDA) were increased significantly (26.9, 12.3% and 90.2, 96.4%) in both seasons, respectively, as signals for water stress. The anatomical characteristics of flag leaves were negatively affected. Inoculation of wheat grains with PGPR significantly increased field capacity and RWC, adjusted enzymes' activity and thus improved the physiological and yield traits and NUE as well as improving the anatomical features of flag leaves. Moreover, the combination of integrated PGPR and 140 kg N ha^{-1} significantly improved grain yield and its components as well as grain N uptake in comparison to control treatments. In conclusion, PGPR improved wheat productivity and NUE; they are an eco-friendly and cost-effective approach for improving plant production, and reducing nutrient leaching hazards and the negative impact of water stress.

Keywords: wheat; drought stress; PGPR; physiological traits; anatomical parameters

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1. Introduction

Wheat (*Triticum aestivum* L.) is one of the most significant crops worldwide. In Egypt, grain wheat production (9.0 Mt) is not sufficient for grain wheat consumption (19.0 Mt), therefore, Egypt is considered one of the largest importers of wheat [1]. Wheat productivity can be substantially increased by using sustainable technologies, such as reducing the usage of contaminants and input of inorganic fertilizers [2], because such inputs can have injurious impacts on the environment, and consequently on animals and humans [3]. In addition, PGPR [4–7] and water stress-resistant wheat cultivars [8] can be used for improving soil traits and wheat yield under environmental stress conditions. Furthermore, salt-tolerant wheat lines and cultivars can be useful genetic resources for increasing grain yield and salinity tolerance of wheat crop [9]. Plants suffer from many stress factors that affect production and yield, among the most dangerous of these factors are drought [10–12], salinity [13–15] and plant diseases [16,17]. Water stress is considered one of the most main threats globally and is predicted to double in arable lands by 2050 [18] and extremely affect the crop growth during various periods [19]. Growth parameters such as stem length, leaves number and chlorophyll content were significantly reduced under drought stress [19]. Additionally, nutrient uptake, yield, and productivity were adversely affected under drought conditions [20]. Plant water status is associated with some morphological and physiological characteristics [21]. Reductions in RWC and chlorophyll are thought to lessen the photosynthesis rate and damage cell membranes [22,23]. Exposing plants to water stress can cause nutrient imbalance and disrupt photoinorganic systems via the stomatal control of CO_2 supply and disturbance of water balance, resulting in reduced plant growth performance and yield production [24]. Oil management strategies that rely on inorganic fertilizers may be hazardous to human health, cause environmental degradation, and can negatively affect the complex system of biogeochemical cycles. Annually, more than 143.88 Mt of inorganic fertilizers are used worldwide to increase crop yields [3]. Despite their efficiency in promoting crop yield, they had serious adverse effects on soil health because of the leaching and run-off of most important nutrients [25]. At present, studies have been focusing on the sustainable production of nutrient–rich, high-quality foods to ensure biosafety [2], and promote alternative options of soil fertilization via the amelioration of nutrient application by using PGPRs [26]. A promising approach for improving nitrogen use efficiency (NUE) and reducing the application of inorganic fertilizers is utilizing PGPR, which confer helpful impacts to plant species [27–29]. Microbial inoculants can be used as an alternative strategy to enhance N fixation, improve photosynthesis, and increase nitrogen and water absorption under water and salinity stress [2,7,30]. Numerous bacterial species are known to have nitrogen-fixing ability, such as *Azospirillum* sp., *Bacillus* sp., *Beijerinckia* sp., *Enterobacter* sp., and *Pseudomonas* sp. [3,7,28,31]. The highest yield achieved in inoculated plants might be attributed to the plant growth materials that are produced by the root-colonizing bacteria. In general, 50 to 80% of applied N is lost through leaching or volatilization, and the remaining 20 to 50% is absorbed by plant roots. Therefore, the aim of this research was to assess if reducing the levels of inorganic fertilizer integrated with PGPR (sole *Azosprillium* or coupling of *Azosprillium* plus *Pseudomonas*) will improve growth and yield parameters.

2. Materials and Methods

2.1. Plant Materials and Soil

Two field experiments were carried out during two winter growing seasons (2020/2021 and 2021/2022). A split-split-plot design with three replicates was used to investigate the effects of PGPR inoculation and inorganic nitrogen fertilization on soil, physiological properties, yield attributes, yield, and NUE of wheat plants grown under water stress. Water stress [water deficit stress (WS), well-watered, WW (control)] was arranged in main plots, inorganic N fertilization (60, 100, 140 and 180 kg N ha^{-1}) in sub-plots, and three PGPR treatments B1, *Azosprillium lipoferum* Sp2 and B2, *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12 were placed into sub-sub-plots. The water deficit treatment (WS) was achieved after two irrigations (at sowing stage and stem elongation) by withholding the water supply after the stem elongation until physiological maturity. The WW condition was achieved by supplying water for four irrigations (sowing, stem elongation, booting, and reproductive stages). Nitrogen fertilizer as urea (46%) (Delta company for chemicals and fertilizers, Mansoura, Egypt) was divided into two doses, the first was added with the first irrigation and the second dose was added after 50 days from sowing. PGPR treatments (2.5 kg inoculant 140 kg^{-1} of grains ha^{-1}) as nitrogen fixers included B1 (*Azosprillium lipoferum* Sp2), B2 (mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12), and pure cultures volumes of the test strains (108–109 colony-forming units (CFU mL^{-1}) and of individual strain or mixtures of strains (1/1 *v/v*) (mixed with the peat carrier), which were kept at room temperature for one day and stored under refrigeration until use. The counts were 108–109 CFU g^{-1} according to Guarda et al. [32]. During seed bed preparation, phosphorous was applied at the level of 240 kg ha^{-1}, whereas potassium was at 125 kg ha^{-1}. Meteorological information such as temperature, relative humidity, and precipitation in the research area were presented in Table 1.

Table 1. The meteorological data for the two seasons.

	2020/2021				2021/2022			
	Temperature (°C)		Precipitation (mm)	RH (%)	Temperature (°C)		Precipitation (mm)	RH (%)
	max	min			max	min		
December	25.8	10.4	0.86	31.3	26.9	11.4	0.80	30.7
January	22.6	9.4	3.54	42.3	23.5	9.4	3.50	41.4
February	21.2	10.2	7.10	46.3	22.0	11.0	7.00	45.1
March	23.3	15.1	0.60	39.5	24.0	16.2	0.30	37.2
April	26.5	16.1	0.50	38.0	27.4	17.2	0.20	40.2
May	30.8	16.7	0.00	36.9	31.9	17.8	0.00	37.9

Data were provided by Central Lab. for Agricultural Climate, ARC, Ministry of Agriculture and Land Reclamation, Egypt. RH = relative humidity.

Wheat grains were sown in plots of 10.5 m^2 (3 m $\times$ 3.5 m, spacing of 0.15 m between rows). The subplots were separated using small bund blocks and 1.5 m unplanted distances to maintain isolation from each other and decrease the lateral movement of water. A locally adopted wheat cultivar (Masr-1) was sown (after maize) on December 10 during the 2020/2021 season, and on December 5 during the 2021/2022 season. The seeding rate of the wheat cultivar was 140 kg grains ha^{-1}.

Soil samples were collected using an auger and analyzed for physical and chemical traits before cultivation [33,34]. The soil texture was clayey, and the bulk density was 1.2 and 1.3 g cm^{-3} before the both seasons. Electrical conductivity was 1.01 and 1.12 dS m^{-1} (1:5), and soil pH was 8.1 and 7.8 (1:2.5) before the two seasons. Organic matter was 1.43% and 1.38% in the two seasons, respectively. Nitrogen, available phosphorus, and exchangeable potassium were 1.62, 36.01, and 0.25 g kg^{-1} before the first season, and 1.79, 38.02, and 0.27 mg kg^{-1} before the second season, respectively.

2.2. Soil Analysis

Soil samples were collected from the plots after harvesting, air-dried, and passed through a 5 mm sieve. Wilting point, field capacity, available soil moisture and bulk density were determined using a pressure plate and pressure cooker machine. Moisture as gravimetric form was determined using a cylindrical sharp-edged core, as described by Klute [25].

2.3. Plant Growth and Physiological Traits

The leaf area index (LAI) expresses the ratio of leaf area to the ground area occupied by the crop. The total plant leaves' area was determined following the procedure of Daughtry and Hollinger [35] using a leaf area meter (Protable Leaf Area Meter, BLAM-1 BLAM-2, Lincoln, NE, USA). The flag leaf area (cm^2) was measured using eight fully expanded flag leaves from each plot 105 days after sowing using a leaf area meter.

Chlorophyll was determined using SPAD-502, Konica Minolta Sensing, Inc., Sakai Osaka, Japan [36].

Relative water content (RWC) was estimated by collecting fresh tissue of flag leaves and measuring them after detachment. Next, the turgid weight was recorded after incubation in distilled water for 24 h. Finally, the leaves were oven dried for 72 h at +60 °C, and the dry weight was recorded. The RWC was calculated according to Sanchez et al. [37] as follows:

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

where FW: fresh weight, DW: dry weight, and TW: turgid weight.

Stomatal conductance was recorded using a Porometer AP4, Delta-T Devices Ltd., Cambridge, UK. Stomatal conductance was measured from four flag leaves for each treatment [38].

Free proline content was analyzed according to Bates et al. [39]. Fresh leaves (0.5 g) were homogenized in sulfosalicylic acid 5 mL (3%) using a mortar and pestle. Next, 2 mL of the extract was placed in a tube with 2 mL of ninhydrin reagent and 2 mL of glacial acetic acid. The mixture was boiled for 30 min at 100 °C in a water bath. After cooling, 6 mL of toluene was added to each tube. Subsequently, samples were transferred to a separating funnel. The chromophore-containing toluene was separated, the absorbance was recorded at 520 nm using a spectrophotometer (model: UV-160A, Shimadzu, Japan) against a toluene blank. Proline was expressed as µmol g^{-1} fresh weight.

The activity of CAT and POX enzymes was determined in leaves samples as follows. Catalase activity (CAT) was assayed spectrophotometrically according to Aebi [40]. Peroxidase activity (POX) was assayed in the crude enzyme extract according to Hammerschmidt et al. [41]. The absorbance was measured at every 30 s interval for 3 min at 470 nm.

According to the method of Davenport et al. [42] malondialdehyde (MDA) was determined using a spectrophotometer, as follows: MDA (nmol g^{-1}FW) = [6.45 × (A532 − A600) − (0.56 × A450)] × V^{-1}W, V = volume (mL), W = weight (g).

2.4. Anatomical Structure of Flag Leaf

Wheat flag leaves (0.5 cm) were taken during the season 2021/2022 at 50 days from the sowing date. The killing and fixing were carried out using formalin, alcohol, and acetic acid, and the samples were dehydrated in normal butyl alcohol. Samples were studied and photomicrographed using a light microscope [43–45].

2.5. Yield and Yield Parameters

At maturity, spike length, plant height, number of grains per spike, number of spikes per square meter, and 1000-grain weight were measured and recorded by randomly collecting twelve plants from the middle rows within each plot. In addition, wheat plants from one m^2 area were harvested and threshed for determining grain, straw, and biological yields.

Grain nitrogen uptake and nitrogen use efficiency (NUE):

Grain N uptake (kg N ha^{-1}) was calculated according to Guarda et al. [32] as follows:

$$\text{Grain N uptake}\left(\text{kg ha}^{-1}\right) = \frac{\text{Grain N content}\left(\text{g kg}^{-1}\right) \times \text{Grain yield}\left(\text{kg ha}^{-1}\right)}{1000}$$

NUE was calculated as described by Foulkes et al. [33].

$$\text{NUE}\ (\text{kg kg}_{\text{N}}{}^{-1}) = \frac{\text{GY}_{\text{t}} - \text{GY}_{\text{c}}\left(\text{kg ha}^{-1}\right)}{\text{NF}_{\text{t}} - \text{NF}_{\text{c}}\left(\text{kg ha}^{-1}\right)}$$

GYt and GYc are the grain yield at different N treatments and control, respectively, whereas NFt and NFc are the N application rates for different N treatments and control, respectively.

2.6. Statistical Analysis

The recorded data from the effects of PGPR, inorganic fertilizer, and water stress on soil physical traits after harvest, and growth, physiological and yield characteristics of wheat were analyzed according to ANOVA procedures, using a general linear model from the SPSS Statistical Software package (version 22, IBM Inc., Chicago, IL, USA). Means were compared using Tukey's multiple range test when the ANOVA showed significant differences ($p \leq 0.05$).

3. Results

3.1. Impact of PGPR on Soil Characters

The application of different Rhizobacteria types resulted in improved physical characteristics of the soil (Table 2). Compared to those of the B1 and B2, the grains before sowing had an increased field capacity and availability of water. However, the application of B2 reduced the wilting point.

Table 2. Physical soil traits before sowing and at harvest after the application of plant growth-promoting Rhizobacteria during both seasons.

Time \ Parameters	FC (%)		WP (%)		BD (g cm^{-3})		Available H_2O (Gravimetric, %)	
	S1	S2	S1	S2	S1	S2	S1	S2
Before sowing	42.2	44.0	22.9	21.7	1.2	1.3	19.2	25.7
At harvest (B1)	52.1	62.7	19.6	18.4	1.0	1.1	37.2	41.6
At harvest (B2)	58.7	63.7	20.6	19.9	1.0	1.2	38.1	43.8
S.E.M.	1.02	1.06	0.43	0.32	0.04	0.05	1.00	0.93

S1 = 2020/2021, S2 = 2021/2022, B1 (*Azosprillium lipoferum* Sp2, control), B2 (mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12), FC = Field capacity, WP = Wilting point, BD = Bulk density, S.E.M. = Standard Error of Means.

3.2. Effect of Water Stress, Inorganic N Fertilization, and PGPR on Wheat Growth

Water stress treatments, inorganic N fertilization, and PGPR inoculation had significant effects on physiological and morphological parameters such as flag leaf area, LAI and chlorophyll content (Table 3). The LAI and flag leaf area were decreased in the water stress (WS) treatment compared to the well-watered (WW) treatment. In addition, these parameters were lower in N-deficient plants than in N-sufficient plants, particularly when plants were fertilized with 140 or 180 kg N ha^{-1}. However, PGPR inoculation with B2 resulted in a significant increase ($p \leq 0.05$) in the LAI and flag leaf area compared to those in the B1 plants, especially in the first seasons (Table 3).

Table 3. Effect of water stress, inorganic nitrogen (N), and PGPR applications on leaf area index (LAI), flag leaf area (cm^2) and chlorophyll content (SPAD).

Treatments	LAI		Flag Leaf Area (cm^2)		Chlorophyll Content (SPAD)	
	S1	S2	S1	S2	S1	S2
Water treatments (W)						
WW	5.34 ^{a}	3.72 ^{a}	40.42 ^{a}	38.48 ^{a}	40.10 ^{a}	41.92 ^{a}
WS	5.11 ^{b}	3.23 ^{b}	37.78 ^{b}	36.81 ^{b}	37.41 ^{b}	37.80 ^{b}
N-levels (N) (kg ha^{-1})						
60	4.51 ^{d}	3.32 ^{d}	30.14 ^{d}	26.19 ^{d}	31.92 ^{d}	33.79 ^{d}
100	5.09 ^{c}	3.49 ^{c}	36.37 ^{c}	35.15 ^{c}	36.24 ^{c}	38.23 ^{c}
140	5.42 ^{b}	3.72 ^{b}	42.42 ^{b}	43.70 ^{b}	41.27 ^{b}	41.94 ^{b}
180	6.08 ^{a}	3.96 ^{a}	47.36 ^{a}	45.54 ^{a}	45.59 ^{a}	47.45 ^{a}
PGPR (B)						
B1	4.19 ^{c}	3.04 ^{b}	36.14 ^{c}	36.45 ^{b}	36.24 ^{c}	37.15 ^{b}
B2	5.23 ^{b}	3.62 ^{ab}	39.05 ^{b}	38.10 ^{a}	38.93 ^{b}	40.84 ^{a}
ANOVA						
W	*	**	**	*	**	**
N	**	**	**	**	**	**
B	**	**	**	*	**	**
W × N	ns	ns	ns	ns	ns	ns
W × B	ns	ns	ns	ns	ns	ns
N × B	ns	ns	ns	ns	ns	ns
W × N × B	ns	ns	ns	ns	ns	ns

S1 = 2020/2021, S2 = 2021/2022, Data within columns followed by different letters are significantly different at $p \leq 0.05$, $p \geq 0.05$ = ns (not significant), * = $p \leq 0.05$, ** = $p \leq 0.01$. WW = well-watered, WS = water stress. B1 (*Azosprillium lipoferum* Sp2, control) and B2 (mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12).

3.3. Effect of Water Stress, Inorganic N Fertilization, and PGPR on Some Physiological Traits of Wheat

Unlike WW treatment, WS treatment reduced leaf chlorophyll content, the RWC (Figure 1) and stomatal conductance (Figure 2) of wheat leaves in the two seasons. The magnitude of decline was superior ($p \leq 0.05$) for 60 kg N ha^{-1} than for higher levels, particular with 140 or 180 kg N ha^{-1} in both seasons (Figures 1 and 2). Conversely, plants grown under different WS treatments were well recovered when they were inoculated with PGPR (B1 or B2), unlike the single-inoculated plants. The analysis of data in (Figure 3) showed significantly ($p \leq 0.05$) higher leaf proline content after WS treatment than after WW treatment.

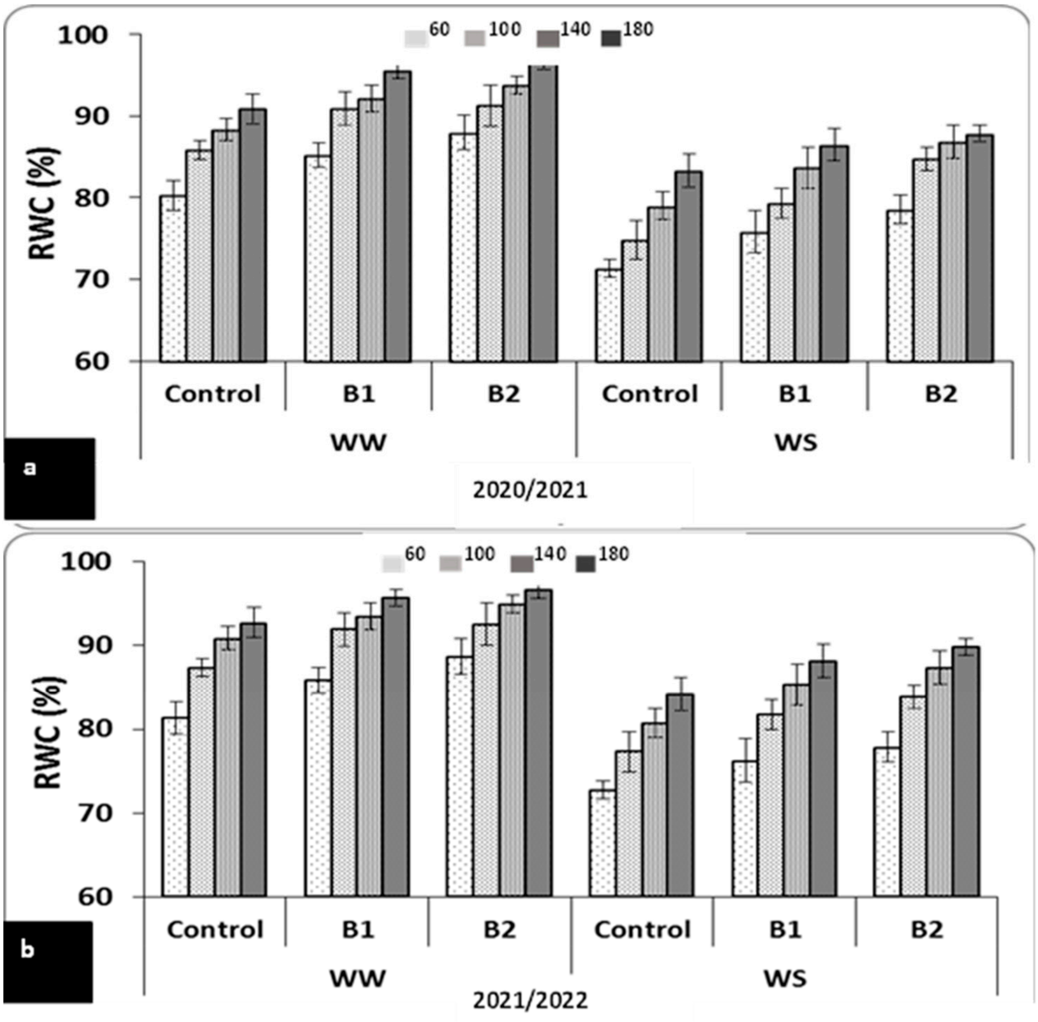


Figure 1. Effect of PGPR and inorganic N applications on RWC of wheat leaves under water stress (WS) and well-watered (WW) treatments at the heading stage during the 2020/2021 (**a**) and 2021/2022 (**b**) seasons. The data are means ± SE of three replicates. B1 (*A. lipoferum* Sp2, control) and B2 (mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12). WS = Water stress, WW = Well-watered.

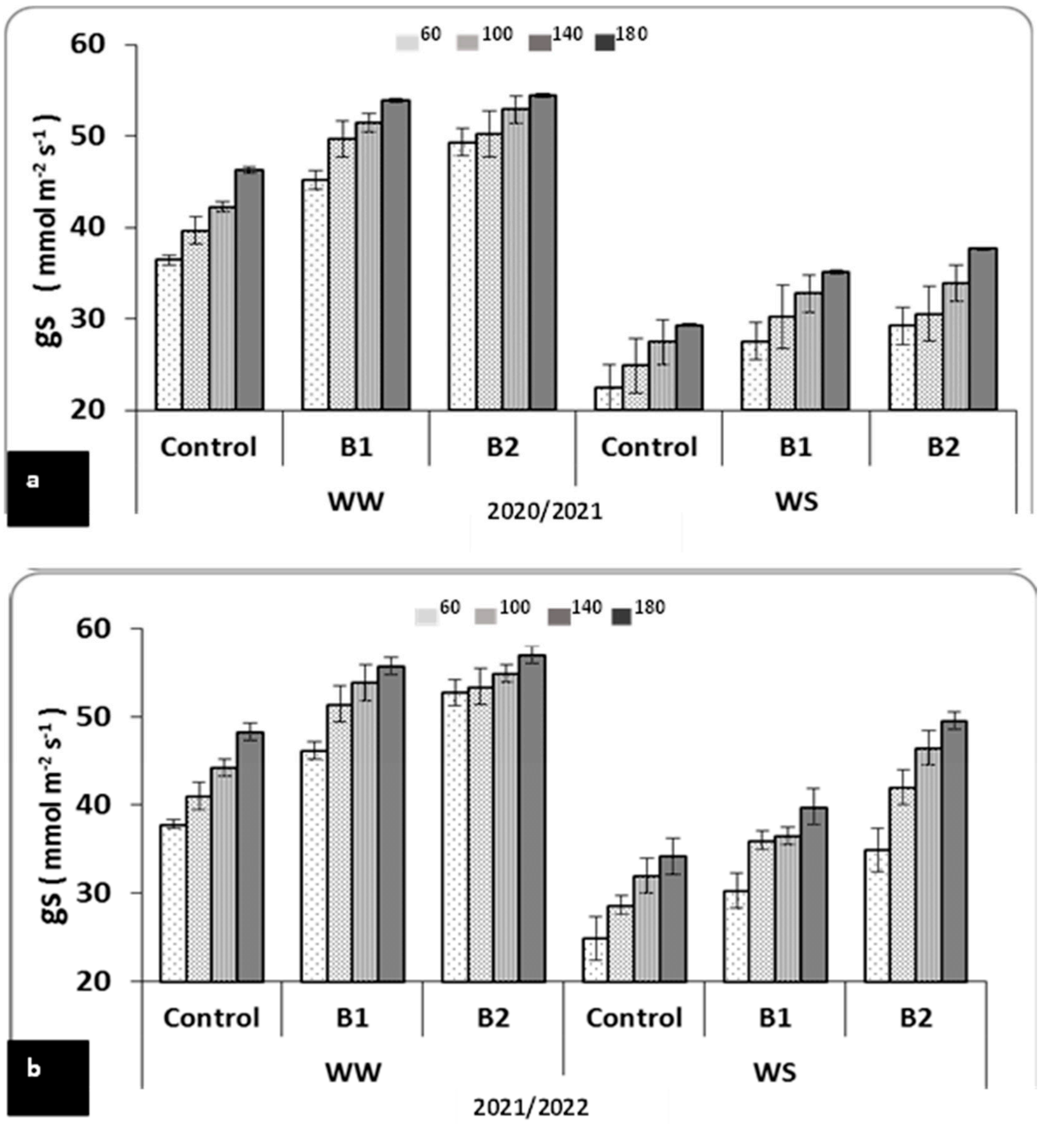


Figure 2. Effect of PGPR and inorganic N applications on stomatal conductance (gs) of wheat under water stress at the heading stage during the 2020/2021 (**a**) and 2021/2022 (**b**) seasons. The data are means ± SE of three replicates. B1 (*A. lipoferum* Sp2, control) and B2 (mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12(. WS = Water stress, WW = Well-watered.

A slight increase in leaf proline content was noted at higher level of inorganic N application compared to that at lower inorganic N levels under water treatments in both seasons. Wheat plants treated with PGPR showed marked reduction in leaf proline content and increase chlorophyll content. Irrespective of water treatments, plots that did not receive PGPR showed significantly ($p \leq 0.05$) enhanced leaf proline content by 35% and 28% in 2020/2021 and 2021/2022, respectively. Contrariwise, the proline content of wheat plants inoculated with B1 or B2 was less affected by water stress and was reduced after water treatment (Figure 3).

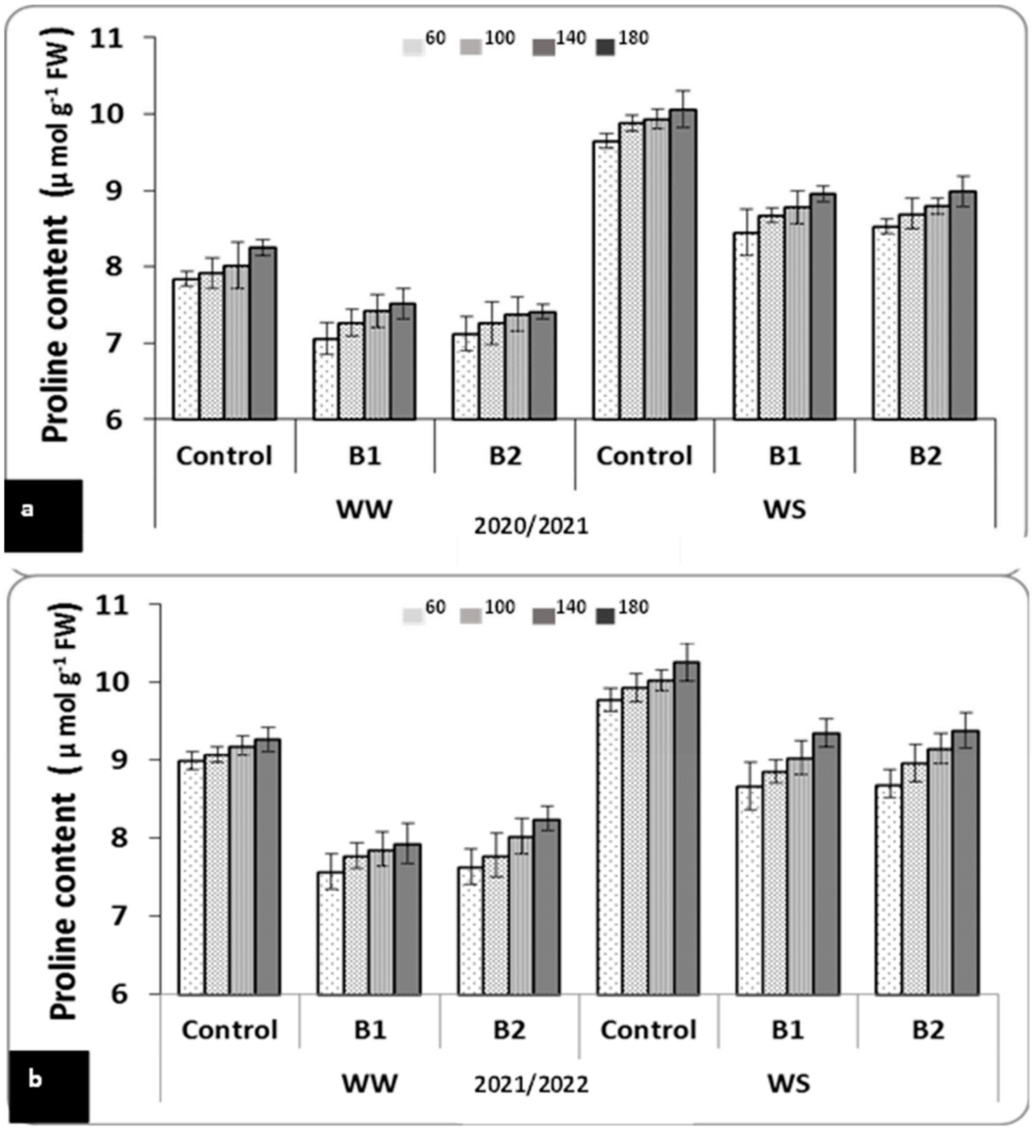


Figure 3. Effect of PGPR and inorganic N applications on proline content (μ mol proline g^{-1} FW) of wheat leaves grown under water stress at the heading stage during the 2020/2021 (**a**) and 2021/2022 (**b**) seasons. The data are means ± SE of three replicates. B1 (*A. lipoferum* Sp2, control) and B2 (mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12). WS = Water stress, WW = Well-watered.

3.4. Effect of Water Stress, Inorganic N Fertilization, and PGPR on Yield and Related Traits of Wheat

In general, water stress significantly ($p \leq 0.05$) reduced the grain yield and related traits, whereas application of inorganic N and PGPR improved these traits (Tables 4 and 5). No significant differences in most yield and yield component parameters were noted between the two highest inorganic N applications (140 and 180 kg N ha^{-1}) as well as between B1 and B2, although they were significantly increased ($p \leq 0.05$) in comparison with those in the lowest dose of N application and B1 treatment (Tables 4 and 5). Moreover, no significant differences were noted for the interaction of water, inorganic fertilizer, and PGPR on yield and its related traits. Effect of water stress treatments and inorganic N as well as PGPR applications were significant on spike length and plant height in both seasons (Table 4). WS significantly decreased spike length and the plant height compared to that in WW treatment. Application of 100, 140, and 180 kg N ha^{-1} significantly ($p \leq 0.01$) increased the spike length by 10.1, 37.3, and 42.2% compared to that in 60 kg N ha^{-1}, respectively. In addition, inoculation of wheat grains with B1 and B2 at sowing significantly

($p \leq 0.05$) enhanced and improved spike length by 25.6 and 28.1%, respectively, compared with that in B1 treatment.

WS significantly ($p \leq 0.01$) reduced the number of spikes per square meter, the 1000-grain weight, and the number of grains per spike compared to those of WW plants (Table 4). Nevertheless, application of inorganic nitrogen fertilizer and PGPR significantly increased these traits. Compared to those of 60 kg N ha^{-1}, spikes number per square meter and number of grains per spike were significantly ($p \leq 0.01$) increased by 11.0 and 7.0% with 100 kg N ha^{-1}, by 30.7 and 16.2% with 140 kg N ha^{-1}, and by 34.3 and 17.7% with 180 kg N ha^{-1}, respectively. Conversely, plants treated with B1 and B2 showed an 18.2 and 20.9% higher number of spikes per square meter than those that underwent the B1 treatment. WS treatments significantly ($p \leq 0.05$) reduced grain (−18.3%), straw (−17.3%), and biological yields (−17.7%) as well as decreasing the HI compared to those of WW plants (Table 5). Nevertheless, application of PGPR (B1 or B2) and inorganic nitrogen fertilizer (140 and 180 kg N ha^{-1}) significantly ($p \leq 0.01$) improved these traits and minimized the negative impact of WS treatments. Application of B1 and B2 as PGPR enhanced grain yield by 48.6 and 52.2%, and biological yield by 42.8 and 45.6%, respectively, compared to those in B1 treatment (Table 5). In addition, increasing inorganic fertilizers from 60 to 100, 140, and 180 kg N ha^{-1} improved the grain yield of wheat by +56.1, +87.7, and +99.0%, respectively. Nevertheless, the differences between the effect of 140 and 180 kg N ha^{-1} on grain, straw, and biological yields were not significant.

Table 4. Effect of water stress, inorganic N, and PGPR applications on plant height (cm) and yield components of wheat.

Treatments	Plant Height (cm)		Spike Length (cm)		No. Spikes m^{-2}		No. Grains Spike^{-1}		1000-Grain Weight (g)	
	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
Water treatments (W)										
WW	107.99 ^{a}	107.61 ^{a}	13.92 ^{a}	13.83 ^{a}	392.78 ^{a}	379.76 ^{a}	57.37 ^{a}	59.51 ^{a}	52.54 ^{a}	53.30 ^{a}
WS	96.01 ^{b}	93.85 ^{b}	10.07 ^{b}	10.03 ^{b}	376.14 ^{b}	364.91 ^{b}	50.90 ^{b}	53.73 ^{b}	47.61 ^{b}	46.89 ^{b}
Nitrogen (N) (kg ha^{-1})										
60	91.22 ^{d}	88.20 ^{d}	9.85 ^{c}	9.70 ^{b}	333.83 ^{c}	302.01 ^{c}	49.39 ^{c}	51.11 ^{c}	48.11 ^{d}	47.31 ^{d}
100	98.52 ^{c}	97.35 ^{c}	10.79 ^{b}	9.93 ^{b}	358.00 ^{b}	348.03 ^{b}	53.63 ^{b}	53.87 ^{b}	49.02 ^{c}	48.45 ^{c}
140	106.57 ^{b}	105.91 ^{b}	13.43 ^{a}	13.42 ^{a}	418.02 ^{a}	413.00 ^{a}	56.13 ^{a}	60.64 ^{a}	50.23 ^{b}	51.47 ^{b}
180	111.69 ^{a}	111.4 ^{a}	13.92 ^{a}	13.88 ^{a}	428.03 ^{a}	426.00 ^{a}	57.39 ^{a}	60.86 ^{a}	52.94 ^{a}	51.15 ^{a}
PGPR (B)	99.57 ^{c}	99.31 ^{b}	10.26 ^{b}	10.03 ^{b}	359.71 ^{b}	309.09 ^{b}	50.19 ^{b}	54.32 ^{b}	47.90 ^{b}	47.88 ^{b}
B1	102.32 ^{b}	101.14 ^{a}	12.71 ^{a}	12.78 ^{a}	396.00 ^{a}	395.60 ^{a}	55.89 ^{a}	57.73 ^{a}	51.12 ^{a}	49.82 ^{a}
B2	104.10 ^{a}	101.75 ^{a}	13.02 ^{a}	12.98 ^{a}	398.00 ^{a}	411.81 ^{a}	56.33 ^{a}	57.81 ^{a}	51.20 ^{a}	51.09 ^{a}
ANOVA										
W	**	**	**	**	**	**	**	**	**	**
N	**	**	**	**	**	**	**	**	**	**
B	**	*	*	*	**	**	**	*	ns	ns
W N	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
W B	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
NB	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
WNB	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

S1 = 2020/2021, S2 = 2021/2022, Data within columns followed by different letters are significantly different at $p \leq 0.05$, $p \geq 0.05$ = ns (not significant), * = $p \leq 0.05$, ** = $p \leq 0.01$. WW = well-watered, WS = water stress. B1 (*A. lipoferum* Sp2, control) and B2 (mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12). Water treatments (W); N-levels (N); (kg ha^{-1}) PGPR (B).

Table 5. Effect of water stress, inorganic N, and PGPR applications on wheat yield.

Treatments	Plant Height (cm)		Spike Length (cm)		No. Spikes m^{-2}		No. Grains Spike^{-1}		1000-Grain Weight (g)	
	S1	S2	S1	S2	S1	S2	S1	S2	S1	S2
Water treatments (W)										
WW	107.99 ^{a}	107.61 ^{a}	13.92 ^{a}	13.83 ^{a}	392.78 ^{a}	379.76 ^{a}	57.37 ^{a}	59.51 ^{a}	52.54 ^{a}	53.30 ^{a}
WS	96.01 ^{b}	93.85 ^{b}	10.07 ^{b}	10.03 ^{b}	376.14 ^{b}	364.91 ^{b}	50.90 ^{b}	53.73 ^{b}	47.61 ^{b}	46.89 ^{b}
Nitrogen (N) (kg ha^{-1})										
60	91.22 ^{d}	88.20 ^{d}	9.85 ^{c}	9.70 ^{b}	333.83 ^{c}	302.01 ^{c}	49.39 ^{c}	51.11 ^{c}	48.11 ^{d}	47.31 ^{d}
100	98.52 ^{c}	97.35 ^{c}	10.79 ^{b}	9.93 ^{b}	358.00 ^{b}	348.03 ^{b}	53.63 ^{b}	53.87 ^{b}	49.02 ^{c}	48.45 ^{c}
140	106.57 ^{b}	105.91 ^{b}	13.43 ^{a}	13.42 ^{a}	418.02 ^{a}	413.00 ^{a}	56.13 ^{a}	60.64 ^{a}	50.23 ^{b}	51.47 ^{b}
180	111.69 ^{a}	111.4 ^{a}	13.92 ^{a}	13.88 ^{a}	428.03 ^{a}	426.00 ^{a}	57.39 ^{a}	60.86 ^{a}	52.94 ^{a}	51.15 ^{a}
PGPR (B)	99.57 ^{c}	99.31 ^{b}	10.26 ^{b}	10.03 ^{b}	359.71 ^{b}	309.09 ^{b}	50.19 ^{b}	54.32 ^{b}	47.90 ^{b}	47.88 ^{b}
B1	102.32 ^{b}	101.14 ^{a}	12.71 ^{a}	12.78 ^{a}	396.00 ^{a}	395.60 ^{a}	55.89 ^{a}	57.73 ^{a}	51.12 ^{a}	49.82 ^{a}
B2	104.10 ^{a}	101.75 ^{a}	13.02 ^{a}	12.98 ^{a}	398.00 ^{a}	411.81 ^{a}	56.33 ^{a}	57.81 ^{a}	51.20 ^{a}	51.09 ^{a}
ANOVA										
W	**	**	**	**	**	**	**	**	**	**
N	**	**	**	**	**	**	**	**	**	**
B	**	*	*	*	**	**	**	*	ns	ns
W N	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
W B	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
NB	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
WNB	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

S1 = 2020/2021, S2 = 2021/2022, Data within columns followed by different letters are significantly different at $p \leq 0.05$, $p \geq 0.05$ = ns (not significant), * = $p \leq 0.05$, ** = $p \leq 0.01$. WW = well-watered, WS = water stress. B1 (*A. lipoferum* Sp2, control) and B2 (mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12). Water treatments (W); N-levels (N); (kg ha^{-1}) PGPR (B).

3.5. Effect of Water Stress, N Levels, and PGPR on CAT, POX and Malondialdehyde (MDA) in Wheat Plants

Water stress, inorganic N, and PGPR application significantly ($p \leq 0.05$) affected CAT, POX and malondialdehyde (MDA) (Figure 4). Our findings showed that MDA as a stress signal was increased significantly in stressed wheat plants (WS) (Figure 4c), compared with well-watered plants (WW), however, application of PGPR (B1 or B2) led to significant decrease in MDA in the treated plants (WW and WS). POX and CAT activities significantly increased in stressed wheat plants (WS) compared with well-watered plants (WW). Inoculation of wheat under sufficient water with B1 or B2 led to unregulated enzyme activity without significant deference when compared with the control; nevertheless, application of B1 or B2 led to a significant decrease in CAT and POX activity (Figure 4a,b) when compared with stressed plants (WS). Additionally, inoculation of wheat under water stress with B1 or B2 caused a significant difference compared with other plants subjected to water stress (WS). The application of B1 or B2 with 140 kg N ha^{-1} gave the best results in the stressed wheat plants, compared with wheat under water stress conditions without treatment.

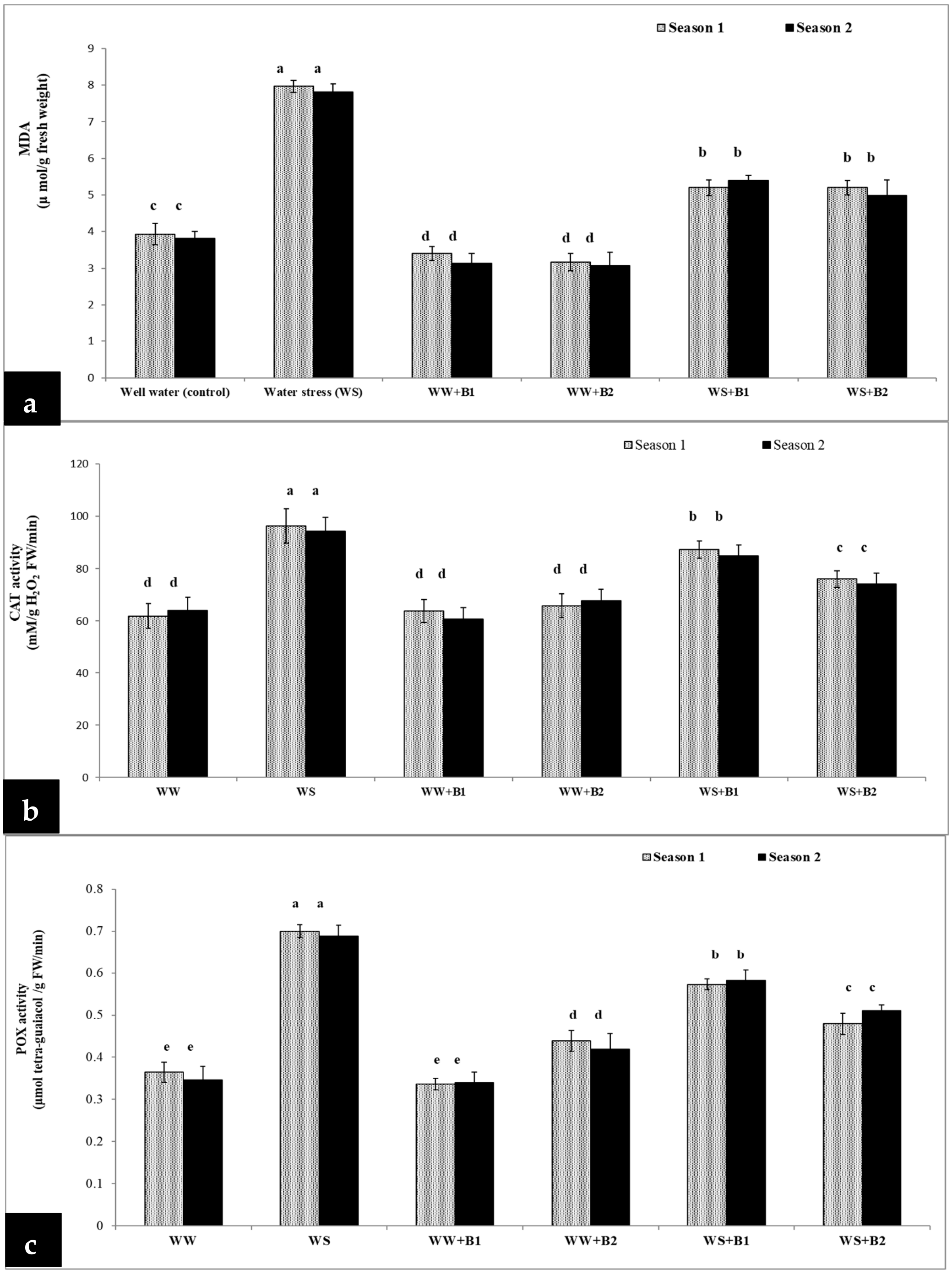


Figure 4. Effect of PGPR and inorganic N applications on MDA (**a**), CAT (**b**) and POX (**c**) of wheat grown under water stress during the 2020/2021 and 2021/2022 seasons. The data are the means ± SE of three replicates. B1 (*A. lipoferum* Sp2, control) and B2 (mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12). Different letters are significantly different at $p \leq 0.05$.

3.6. Effect of Water Stress, N Levels, and PGPR on Anatomical Leaf Structure

The presented results in (Figure 5) showed the anatomical changes in the structure of flag leaves of wheat under water stress. The upper and lower epidermis thickness, the lamina thickness and the diameter of vascular bundle significantly decreased in water stressed wheat plants (WS) because of water stress compared with plants subjected to the well-watered treatment (WW). However, the applications of B1 or B2 had a helpful impact and increased the upper and lower epidermis thickness and vascular bundle diameter under water deficit stress; the best findings were obtained in the well-watered plants inoculated with B2 (WW + B2), followed by the well-watered plants inoculated with B2 (WW + B2) compared with the control plants (WW). Application of PGPR (B1 or B2) led to improvements in the anatomical characters of wheat flag leaves under water stress conditions (Figure 5c–f).

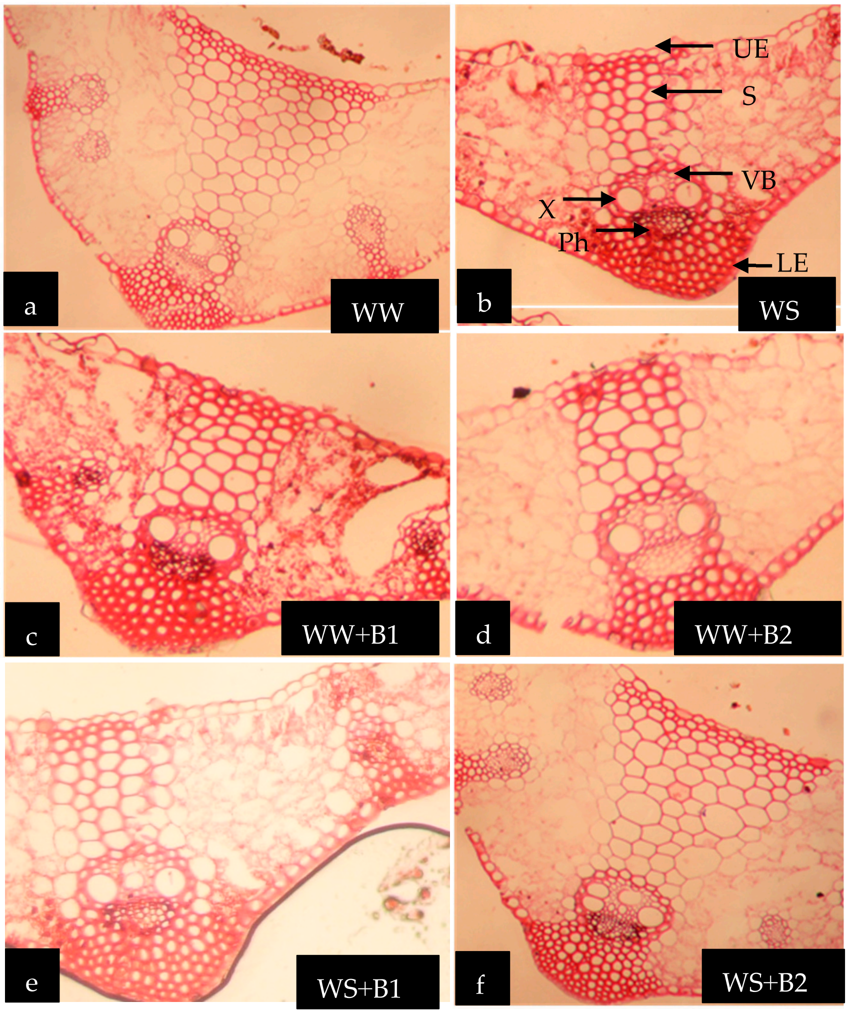
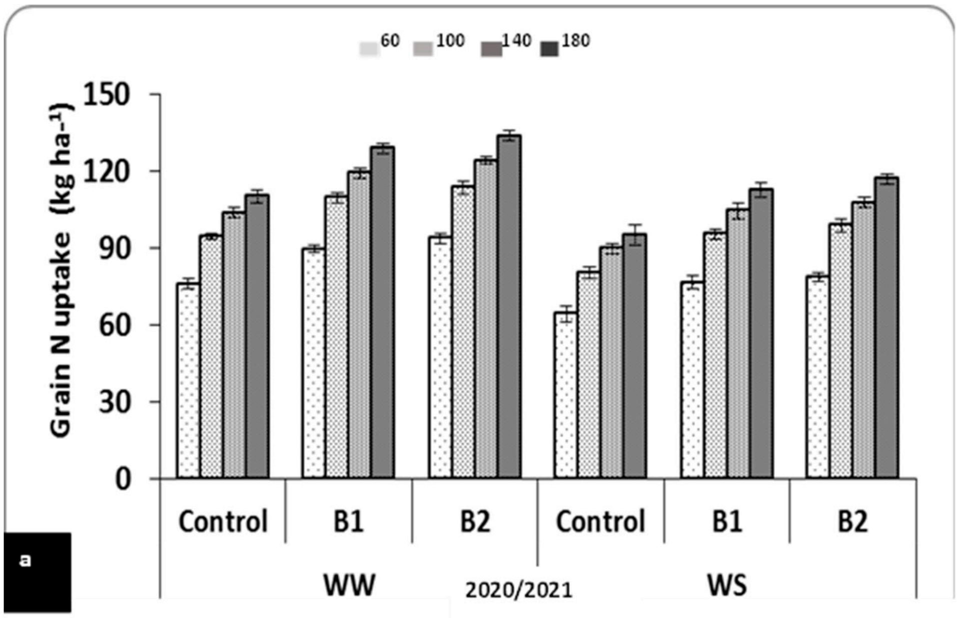


Figure 5. Effect of PGPR and inorganic N applications on the anatomical structure of wheat leaves under water stress conditions. (**a**): Well-watered (WW); (**b**): Water stress (WS); (**c**): WW + B1; (**d**): WW + B2; (**e**): WS + B1; (**f**): WS + B2; B1 (*A. lipoferum* Sp2, control) and B2 (mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12). WS = Water deficit stress, WW = Well-watered. UE: Upper epidermis S: Sclerenchyma tissue VB: Vascular bundle X: Xylem Ph: Phloem LE: Lower epidermis.

3.7. Effect of Water Stress, N Levels, and PGPR on Grain N Uptake and NUE in Wheat

Water stress, inorganic N, and PGPR application significantly ($p \leq 0.05$) affected grain N uptake and NUE (Figures 6 and 7). Inoculation of wheat with B1 or B2 distinctly induced higher grain N uptake and NUE than those in B1 treatment (Figure 6). As the N level increased from 60 to 180 kg N ha^{-1}, grain N uptake was significantly ($p \leq 0.01$) increased, whereas NUE was significantly ($p \leq 0.05$) decreased. In addition, application of 140 kg N ha^{-1} had a relatively similar effect on grain N uptake to 180 kg N ha^{-1} application under water treatments.

The application of PGPR with a high N level (140 or 180 kg N ha^{-1}) increased grain N uptake compared to that in B1 treatment under water stress, but reduced the adverse impact on NUE compared to that in B1 treatment under water treatments.



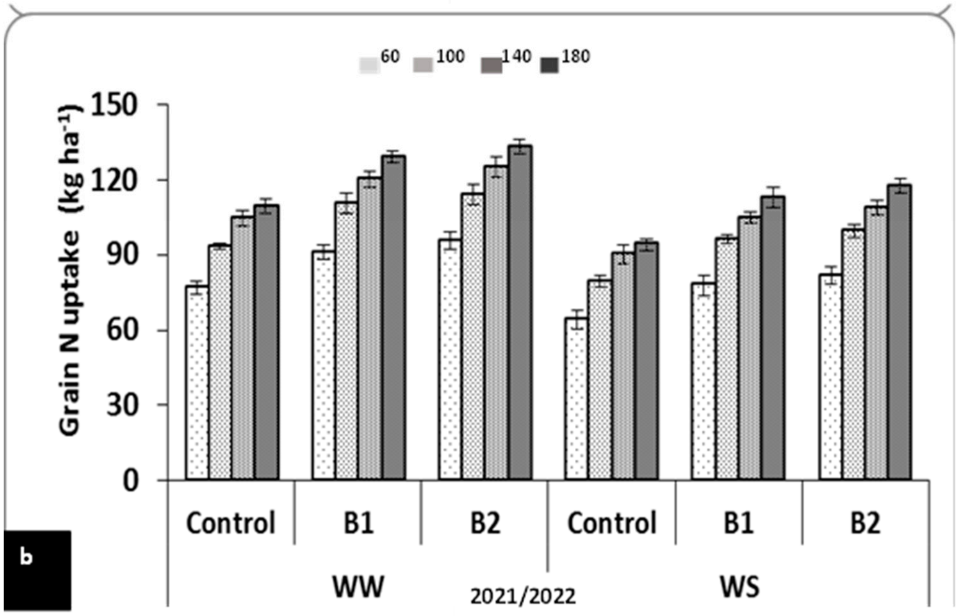


Figure 6. Effect of PGPR and inorganic N applications on grain N uptake of wheat grown under water stress during the 2020/2021 (**a**) and 2021/2022 (**b**) seasons. The data are means ± SE of three replicates. B1 (*A. lipoferum* Sp2, control) and B2 (mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12).

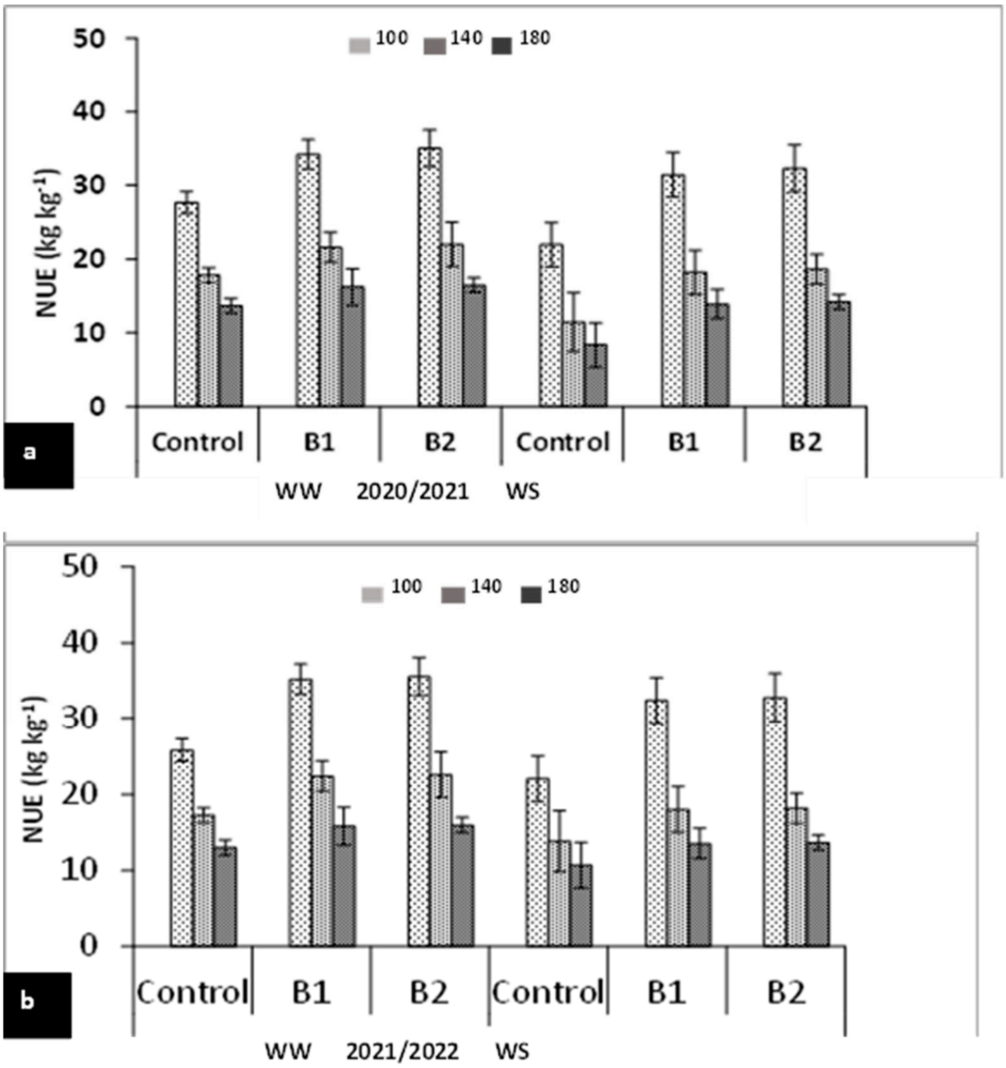


Figure 7. Effect of PGPR and inorganic N applications on nitrogen use efficiency of wheat grown under water stress during the 2020/2021 (**a**) and 2021/2022 (**b**) seasons. The data are the means ± SE of three replicates. B1 (*A. lipoferum* Sp2, control) and B2 (mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12).

4. Discussion

Our results showed that LAI, chlorophyll content, flag leaf area, RWC, and stomatal conductance were significantly decreased under water deficit stress. The harmful effect of water deficit stress on the aforementioned characters could be due to the decrease in water availability and water uptake from soil under water deficit stress [22,46,47]. Inoculation of wheat grains with PGPR by B1 (*A. lipoferum* Sp2, control), B2 (mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12) was very effective and beneficial for soil traits, wheat growth, and wheat yield under different water treatments (Tables 2–5). The positive trend was recorded when inorganic nitrogen fertilizer (140 kg N ha^{-1}) was applied. PGPR application led to improved bulk density and available soil water at the end of the current investigation compared to those before sowing (Table 2). This effect can be attributed to the role of PGPR in improving soil water availability and aggregates, and thereby alleviating water stress negative impacts [46,48]. Improved root system and soil traits can directly enhance plant growth and physiological traits [49]. *Azospirillum* sp. have the capacity to create plant hormones, for example, indole acetic acid [2]; *Azotobacter* can produce indole acetic acid and cytokinin [50], which can enhance lateral root formation and increase the uptake of water and nutrients under drought stress in wheat plants. Moreover, *Pseudomonas* sp. can produce cytokinin, which can elevate abscisic acid in the shoots and enhance stomatal conductance. Thus, the application of mixture of *A. lipoferum* Sp2 + *Pseudomonas* can increase the photo inorganic capacity of plants [51].

The improvement of physiological characters such as LAI, flag leaf area, RWC, and total chlorophyll (Table 3, Figure 1) could be due to the role of PGPR in reducing the negative effects of water deficit stress [48] and NUE. Application of a mixture of *A. lipoferum* Sp2 + *Pseudomonas* sp. can enhance soil fertility in terms of micro- and macro-nutrients through different activities and processes such as N fixation, P and K solubalization or mineralization, organic matter biodegradation and root exudation [8]. The application of mixture of *A. lipoferum* Sp2 + *Pseudomonas* can produce exo-polysaccharides, which play a significant role in the enhancement of soil structure, consequently improve soil permeability and characteristics. This can have a positive effect of upholding more water in the soil, consequently maintaining plant growth under water stress. Additionally, PGPR can improve soil microbial biomass and can lead to increase the soil water-holding capacity under water stress. The positive impact of PGPR was recorded also in sugar beet and wheat plants [52,53]. Application of the higher levels of inorganic N (140 and 180 kg N ha^{-1}) resulted in the highest LAI, flag leaf area, chlorophyll content, RWC, and stomatal conductance. Additionally, nitrogen fertilization could alleviate the harmful impacts of heat stress on the grain yield and quality of waxy maize [54]. Furthermore, the higher levels of inorganic N fertilization gave the highest yield and its components and grain N uptake compared to the unfertilized plants under WW and WS treatments. Combination of 140 or 180 kg N ha^{-1} with PGPR also improved these traits. Normally, a high percentage (i.e., 60–90%) of the total applied inorganic fertilizers can be easily lost via leaching or volatilization, and the rest (i.e., 10–40%) is taken up by plant roots [8]. Thus, treating grains with microbial inoculants can have a significant impact on the integration of different nutrients to sustain crop productivity and the agricultural environment [55]. These mechanisms influence the improvement of soil physiochemical properties and mineral uptake.

Application of mixture of *A. lipoferum* Sp2 + *Pseudomonas* and increasing inorganic N fertilization significantly ($p \leq 0.05$) increased grain N uptake under both WW and WS treatments (Figure 4). The combination of N fertilizers and PGPR not only enhanced plant growth [28], but also improved nutrient uptake, leading to an increase in the NUE. PGPR immobilize N to make it available for absorption by plant roots, and prohibit gaseous losses of N; thus, the application of PGPR can reduce the need for inorganic fertilizers and alleviate the harmful effects of water stress. The highest NUE was obtained when plants were fertilized with 100 kg N ha^{-1}, followed by that with 140 kg N ha^{-1}, whereas the lowest NUE was obtained for plants fertilized with 180 kg N ha^{-1} (Figure 5). Moreover, the highest NUE was obtained for plants treated with PGPR (either B1 or B2) under WW and WS conditions. The efficient use of N mainly resulted from the remobilization of absorbed N to the grains. The increase in NUE was more pronounced in the integrated chemical application with PGPR compared to that with the individual inorganic N fertilization [55]. PGPR play an important role in improvement plant growth and increase NUE under normal and stress conditions [55]. The negative effects of water deficit on wheat were shown in physiological changes such as the incrementation of malondialdehyde, reduction in RWC, and increase in enzyme activity. This effect may be due to the decrease in water availability and water flow from the soil to root, and consequently, decreased water availability and RWC% [56–58].

The harmful effects of water deficit stress on MDA may be due to the oxidative stress in organelles such as chloroplasts and mitochondria; this increase in MDA is indicative of many stress factors in several crops [59–62]. The increase in CAT and POX activity is an important approach in plants that can be used to overcome water stress and minimize its damaging impact on wheat. The same result was recorded under salinity [14,44,63] and drought stress [64–66]. Contrariwise, PGPR application with inorganic N (140 kg N ha^{-1}) led to improvements in the growth characters of wheat under drought conditions, resulting in the regulation of the activity of CAT and POX enzymes, and significantly increased

RWC; however, MDA was decreased significantly. This positive impact might be due to the promoting role of PGPR in increasing root growth, enhancing water absorption, and increasing the growth characteristics of wheat plants under drought. With our findings, Rehman et al. [67] found a positive effect of PGPR on wheat plants. According to the cross sections in wheat flag leaves, water stress led to anatomical changes in the upper epidermis and lower epidermis, lamina thickness, and vascular tissues diameter. This adverse impact of water deficit may be due to a decrease in water in the soil and a reduction in nutrient uptake, resulting in leaf growth inhibition, a decreased photoinorganic rate, and consequently, reduced anatomical features in wheat leaves. Nonetheless, the lower epidermis, upper epidermis thickness and blade (lamina) thickness, and diameter of vascular bundles were improved with the application of PGPR (B1 or B2) in stressed wheat plants (WS + B1) and (WS + B2). The promoting effect of PGPR on the anatomical features of wheat flag leaves may be due to the role of PGPR in increments in some nutrients and phytohormones, which enhance anatomical features such as the epidermis, mesophyll tissue, xylem vessels, and phloem tissue. The damaging impacts of drought and salinity on anatomical characteristics have been recorded in various plants [10,13].

5. Conclusions

Water deficit stress negatively affected wheat plants and led to decreased chlorophyll and relative water contents; the anatomical characteristics of flag leaves were also negatively affected. However, malondialdehyde (MDA) and proline were increased under water stress conditions. Application of plant growth-promoting Rhizobacteria (PGPR) (*Azosprillium lipoferum* Sp2, *A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12(effectively modified the morphological and physiological characteristics, yield, and anatomical characteristics of wheat plants. These treatments upregulated the plant growth, physiological, anatomical, and yield traits, as well as wheat productivity under water stress conditions. Moreover, the combination of PGPR (*A. lipoferum* Sp2 + *Pseudomonas* sp. SARS12) and 140 kg N ha^{-1} considerably increased grain yield and its components as well as grain N uptake in comparison with inorganic fertilizer or control treatments. We concluded that application of PGPR as an eco-friendly and cost-effective approach leads to increased NUE and wheat productivity, and decreased nutrient leaching hazards and negative impacts from water stress.

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