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Unravelling the Role of *Piriformospora indica* in Combating Water Deficiency by Modulating Physiological Performance and Chlorophyll Metabolism-Related Genes in *Cucumis sativus*

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Abstract: Water stress is the most critical aspect restricting the development of agriculture in regions with scarce water resources, which requires enhancing irrigation water-saving strategies. The current work discusses the potential application of the plant-strengthening root endophyte *Piriformospora indica* against moderate (25% less irrigation water) and severe (50% less irrigation water) water stress in comparison to the optimum irrigation conditions of greenhouse cucumbers. *P. indica* improved growth, nutrient content, and photosynthesis apparatus under normal or water-stress conditions. On the other hand, moderate and severe water stress reduced yield up to 47% and 83%, respectively, in non-colonized cucumber plants, while up to 28 and 78%, respectively, in *P. indica*-colonized plants. In terms of water-use efficiency (WUE), *P. indica* improved the WUE of colonized cucumber plants grown under moderate (26 L/kg) or severe stress (73 L/kg) by supporting colonized plants in producing higher yield per unit volume of water consumed by the crop in comparison to non-colonized plants under the same level of moderate (43 L/kg) or severe (81 L/kg) water stress. Furthermore, *P. indica* increased the indole-3-acetic acid (IAA) content, activity levels of catalase (CAT) and peroxidase (POD) with an apparent clear reduction in the abscisic acid (ABA), ethylene, malondialdehyde (MDA), proline contents and stomatal closure compared to non-stressed plants under both water-stress levels. In addition, chlorophyll *a*, *b*, *a* + *b* contents were increased in the leaves of the colonized plants under water-stress conditions. This improvement in chlorophyll content could be correlated with a significant increment in the transcripts of chlorophyll biosynthesis genes (protochlorophyllide oxidoreductase [POR], chlorophyll a oxygenase [CAO]) and a reduction in the chlorophyll degradation genes (PPH, pheophorbide a oxygenase [PAO], and red chlorophyll catabolite reductase [RCCR]). In conclusion, *P. indica* has the potential to enhance the cucumber yield grown under moderate water stress rather than severe water stress by improving WUE and altering the activity levels of antioxidant enzymes and chlorophyll metabolism-related genes.

Keywords: *Piriformospora indica*; cucumber; WUE; chlorophyll; water stress; qRT-PCR

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1. Introduction

Cucumber is one of the main protected culture crops grown in the Mediterranean region because it has potential health benefits and is quite well adapted to grow under different protected culture conditions [1–3]. Furthermore, the greenhouse industry has

rapidly expanded worldwide, together with demanding new strategies to solve specific biotic and abiotic stress-limiting production factors [4–7]. Water stress is one of the most important factors restricting the development of agricultural extension in countries and regions with scarce water resources. The problem of irrigation water shortage and its low quality has become a concern for semi-arid zones [8,9] and is considered a real challenge to development and progress in food security [10,11]. Hence, the competition for water resources is increasing dramatically nowadays, which requires enhancing water-use efficiency strategies [12,13]. Consequently, water deficiency alters a suite of modifications that obstruct several biochemical and metabolic processes of the plant [14], reduce plant photosynthesis through chlorophyll degradation, disrupt electron transfer [15], and decrease growth and yield [16,17]. Plant strategies to cope with water shortage include several adaptive changes, such as developing larger and deeper root systems [18,19], stomatal closure mechanisms [20], osmotic pressure adjustment [21], and generation of reactive oxygen species (ROS) scavenging systems [22]. A leaf is the first plant organ to reflect the harmful effects of drought [23]. Accordingly, water stress is specified to destruct and decrease chlorophyll content in plant fresh leaves, which is considered an indicator of photosynthetic incapability of plant machinery [24–26]. Besides, water stress triggers the leaf senescence programme as an adaptative technique in plants subjected to water shortage to reduce the water demand cumulated over the whole plant cycle [27]. Mainly, chlorophyll *a* and *b* are essential for the primary photosynthetic reaction [28]. In consequence, chlorophyll degradation is a necessary integral process of leaf senescence, which is catalyzed sequentially by a series of chlorophyll catabolic enzymes [29]. Therefore, to alleviate the adverse effects caused by water stress, symbiotic fungi have been declared to improve colonized plants' tolerance to various biotic and abiotic stresses [30–32]. The root endophyte fungi enhance the physical and chemical properties of the soil, nutrient absorption, and translocation [33]. The mutualistic root fungus *Piriformospora indica* has been reported to colonize a wide range of plants [34]. *P. indica* promotes plant growth [35], stress tolerance [36–38], and production of IAA, gibberellin, cytokinin [39,40], and secondary metabolites [41]. Several reports have indicated the potential of *P. indica* application in commercial production practices due to its ability to propagate axenically in different media, which makes its use more practically feasible than mycorrhiza [42–44]. The current work aims to study the potential of *P. indica* utilization on greenhouse cucumber plants grown under three irrigation levels to improve plant growth, production, and resistance to irrigation water stress. In this respect, morphophysiological traits, photosynthetic activity, hormone quantification, the activity levels of antioxidant enzymes, and specific chlorophyll metabolism related genes were evaluated under normal irrigation and moderate and severe water-stress conditions.

2. Materials and Methods

2.1. Plant Materials, Greenhouse Conditions, and Experimental Design

Cucumber seeds (*Cucumis sativus* cv. Sama) were surface sterilized with sodium hypochlorite (1.5%) for 15 min, rinsed three times with distilled water, and then sown in cell trays filled with a sterilized mixture of peat moss, sand, and vermiculite (1:1:1). Three-week old cucumber seedlings were transplanted in 40-L plastic pots filled with sandy soil and grown in a 560 m^2 fiberglass greenhouse at the Faculty of Agriculture, Cairo University, Egypt, during the two spring seasons of 2019 and 2020. Each treatment had 16 pots with two grown plants per pot and two and a half plants per square meter. The greenhouse was equipped with a pad and fan cooling system. The fan "on-off" switch was connected to a sensor located in the middle of the greenhouse, one meter above the plants, to keep cold air flow among crop lines and keep day/night temperatures between 26/18 °C. Each compartment was connected to a drip irrigation system serving the individual planting rows with two drippers per pot; each dripper provided 4 L of irrigation water. One week post transplanting, the cucumber plants were subjected to three irrigation levels, applied as

follows: W (normal irrigation), W-25 (moderate water stress, equal to 25% less than normal irrigation), and W-50 (severe water stress, equal to 50% less than normal irrigation).

2.2. *P. indica* Propagation and Inoculation

Piriformospora indica was propagated on a solid KM medium and incubated at 24 °C for two weeks [45]. Briefly, 250 mL liquid KM medium was supplied with five fungal plugs and incubated at 26 °C/130 rpm in a rotary shaker for ten days. Pure white mycelium was collected and washed three times with sterilized water. For root inoculation, 3 g of *P. indica* mycelium was mixed with 100 mL sterilized water to produce a 3% *P. indica* suspension ($\pm 2.3 \times 10^6$ spores/mL). Later, 10 mL of *P. indica* suspension was inoculated into the root zone of cucumber plants at 3, 7, and 15 days after transplanting. Based on *P. indica* inoculation, three other treatments were conducted as follows: PI (inoculation with *P. indica* under normal irrigation), PI-25 (inoculation with *P. indica* under 25% less than normal irrigation), and PI-50 (inoculation with *P. indica* under 50% less than normal irrigation), as shown in Figure 1.

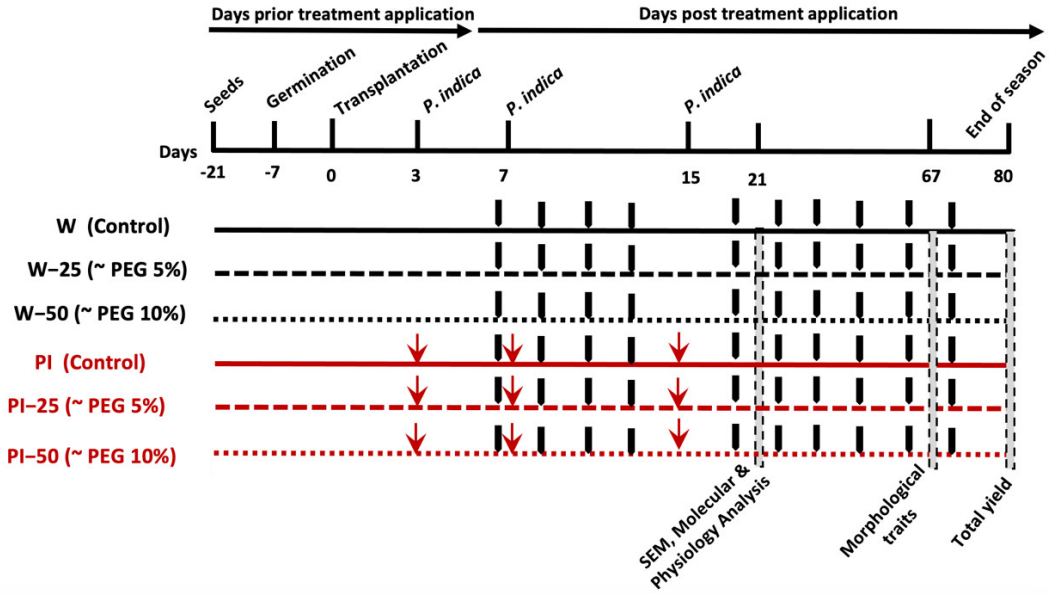

Figure 1. Scheme of the experimental setup. Horizontal lines characterize different plant treatments as follow: W (normal irrigation), W-25 (moderate water stress; equal 25% less than normal irrigation; equivalent to PEG 5%), W-50 (severe water stress; equal 50% less than normal irrigation; equivalent to PEG 10%), PI (inoculation with *P. indica* under normal irrigation), PI-25 (inoculation with *P. indica* under 25% less than normal irrigation; equivalent to PEG 5%), and PI-50 (inoculation with *P. indica* under 50% less than normal irrigation; equivalent to PEG 10%).

2.3. *P. indica* Staining and Spore Monitoring

One month post inoculation, root samples of the cucumber plants were monitored for *P. indica* colonization under a light microscope (Figure S1). Roots were washed, cut into segments (1–2 cm length), bleached in 10% KOH for 30 min at 90 °C, and submerged in 2 N HCl for 2 min. Samples were stained with trypan blue (0.05%) in lactoglycerol for 15 min at 90 °C in a water bath and bleached in lactic acid according to Johnson et al. [46].

2.4. Morphological Traits

Sixty-seven days post transplantation, the leaf and root samples were randomly evaluated for growth performance. Plant height, plant fresh weight, root fresh and dry weight, root length and leaf area of the fifth fully expanded leaf from the top were assessed for 24 plants either inoculated or non-inoculated with *P. indica* under two different irrigation water-stress conditions in comparison to normal irrigation.

2.5. Yield and WUE

The cucumber fruits were harvested 2–3 times per week and the average fruit weight, number of fruits per plant, and total yield were recorded for the two successive growing seasons. WUE was estimated as the ratio of the total fruit yield and the total water applied using the following equation: WUE (L/kg) = water applied (L/m^2)/total yield (kg/m^2) as described by Zipadelli et al. [47].

2.6. Macronutrient Quantification

Dry leaves were digested in acidic solution as described by Linder [48] to determine nitrogen content using the Micro-Keelhaul and phosphorus content using molybdophosphoric blue according to Chapman and Parker [49], while potassium, calcium, and magnesium were measured using a flame photometer [50].

2.7. Chlorophyll Content and Photosynthesis Parameters

One gram of fresh cucumber leaves was ground in 80% acetone (v/v), the homogenate was filtered on Whatman paper, and the extraction was adjusted to 100 mL. Chlorophyll *a*, *b*, and *a* + *b* were determined using the spectrophotometer at 645, 663, and 652 nm, respectively [51]. Photosynthetic measurements were recorded at 8–10 am, under a constant greenhouse temperature (26–28 °C), with 65% relative humidity, through the two growing seasons. The infrared gas analyzer Li-COR 6400 (Lincoln, NE, USA) was used to determine the photosynthesis and transpiration rates in the fifth fresh leaf of the cucumber plants.

2.8. ABA, IAA, and Ethylene Assays

Twenty grams of the fifth cucumber leaves were kept in liquid nitrogen to quantify the ABA and IAA hormones. Ten mg of freeze-dried cucumber leaves were milled into a fine powder using a mortar and pestle. The powdered samples were washed three times (once for three hours and twice for one hour) with 80% methanol (v/v) and 2,6-bis (1,1-dimethyl ethyl)-4-methylphenol at 4 °C in darkness. The extract was centrifuged at 4000 rpm, the supernatant was poured and adjusted to pH 8.6, and then the residues were extracted twice with an equal volume of pure ethyl acetate. The combined supernatant with ethyl acetate extracts was dehydrated over anhydrous sodium sulphate then filtered. The filtrated supernatant was evaporated under vacuum at 35 °C and redissolved in 1 mL absolute methanol. The quantification of the ABA and IAA was performed with Ati-Unicum gas-liquid chromatography, 610 Series, equipped with a flame ionization detector, according to the method stated by Furniss [52].

For ethylene hormone, fresh samples of cucumber leaves were cut into 1-cm pieces (1 cm) and incubated in a sealed 9 mL glass vial containing water (50 µL) overnight. After incubation, a 0.5 mL sample was taken from the headspace, and ethylene was quantified as described by Iwai et al. [53].

2.9. Antioxidant Enzymes, Malondialdehyde, and Proline Assays

The preparation method of leaf samples to assess the activity levels of antioxidant enzymes was described by Polle et al. [54] and Abdelaziz et al. [43], where 500 mg of the fresh cucumber leaves was ground in liquid nitrogen and mixed with 5 mL of potassium phosphate buffer (100 mM, pH 7.0) containing 0.5% Triton X-100, 2% (w/v) N-vinylpyrrolidinone, 5 mM ethylene diaminete traacetic acid disodium salt dehydrates, and 1 mM ascorbic acid. The resulted mixture was centrifuged at 15,000 rpm for 17 min, and the supernatants were used to measure the activity levels of peroxidase (POD) according to Nakano and Asada [55], catalase (CAT) by Aebi [56] and superoxide dismutase (SOD) by Beauchamp and Fridovich [57].

Malondialdehyde (MDA) was determined according to Heath and Packer [58], with some modifications. Briefly, samples were homogenized with trichloroacetic acid (TCA) and then centrifuged for 5 min at 20 °C. Next, the supernatant was mixed with TCA and thiobarbituric acid. Then, the absorbance of the supernatants was then detected at 532 nm.

For proline quantification, fresh leaves were ground in 5 mL of 3% aqueous sulphosalicylic acid, the filtrate was mixed with 2% (*v*/*v*) ninhydrin and determined at 546 nm [59].

2.10. Polyethylene Glycol Assay (PEG 6000)

To pattern how *P. indica* affects stomatal closure, we grow cucumbers in an experiment with separate pots filled with sterilized peatmoss and sand mixer (1:1 *v*:*v*) for seed germination. Seedlings were irrigated with two levels of polyethylene glycol, 5% and 10%, to stimulate moderate and severe water stress in comparison to non-colonized plants. For *P. indica* co-cultivation, seedlings were supplied with 3% *P. indica* suspension at 3, 7, and 15 days post-germination. One week post-germination, seedlings were treated with full-strength Hoagland's nutrient solution as a single treatment or mixed with 5% or 10% polyethylene glycol (PEG-6000) for water-stress treatments as described by Fan et al. [60]. Thirty-six plants received each treatment, while leaf samples were collected 21 days post-germination and kept in liquid nitrogen for RNA extraction.

2.11. Scanning Electron Microscopy (SEM)

Fresh samples of cucumber leaves were prepared for scanning electron microscopy (SEM), using the method described by Daud et al. [61]. First, leaf disks were fixed with 2% paraformaldehyde and 2% glutaraldehyde in a 0.1 mM sodium cacodylate buffer (pH 7.2), and then dehydrated in an ethanol isoamyl series (50, 70, 80, 90, 95, and 100%) using a critical point dryer and liquid carbon dioxide. The dehydrated samples were mounted on metal stubs coated with gold palladium and examined by SEM (JEOL-JSM 35, Tokyo, Japan).

2.12. Real-Time PCR Analysis of Chlorophyll Synthesis and Degradation Genes

The cucumber leaves were stored in liquid nitrogen for RNA extraction using Trizol and DNase I, while cDNA was synthesized for the three biological replicates using the SuperScript^{TM}II according to the manufacturer's manual (Invitrogen, Carlsbad, CA, USA, Cat.#: 18064014). The sequences of the primers of genes related to the chlorophyll pathway—glutamyl-tRNA reductase (Glu-TR), protochlorophyllide oxidoreductase (POR), chlorophyll a oxygenase (CAO), pheophytinase (PPH), pheophorbide a oxygenase (PAO), red chlorophyll catabolite reductase (RCCR) and the reference gene (Actin)—are listed in Supplementary Table S1. In addition, the qRT-PCR assessment was performed according to Beaubois et al. [62] using the Mx3000P QPCR System (Agilent Technologies, Inc., Santa Clara, CA, USA).

2.13. Statistical Analysis

The data for the two successive seasons, 2019 and 2020, were subjected to combined analysis after performing the normality distribution test [63] and homogeneity test [64]. In addition, the obtained data from the combined analysis were subjected to the statistical analysis of variance, and means were compared at the 0.05 level according to Duncan's test using the Statistica 7 program. Each experiment was conducted in three replicates, and differences among gene expression datasets were assessed using a paired student t-test based on three biological replicates.

3. Results

3.1. P. indica Colonization Improves the Growth of Water-Stressed Cucumber Plants

Water stress significantly decreased plant height, plant fresh weight, and average leaf area in colonized or non-colonized cucumber plants when subjected to 25% (W-25, as moderate stress) and 50% (W-50, as severe stress) reduction in irrigation water (Figure 2A–C). On the other hand, gradual increases were observed in root length, combined with a significant decrease in fresh and dry weight of water-stressed plants compared to non-stressed plants (Figure 2D–F). Colonizing cucumber with *P. indica* improved the above growth traits under normal or stress conditions compared to non-colonized plants.

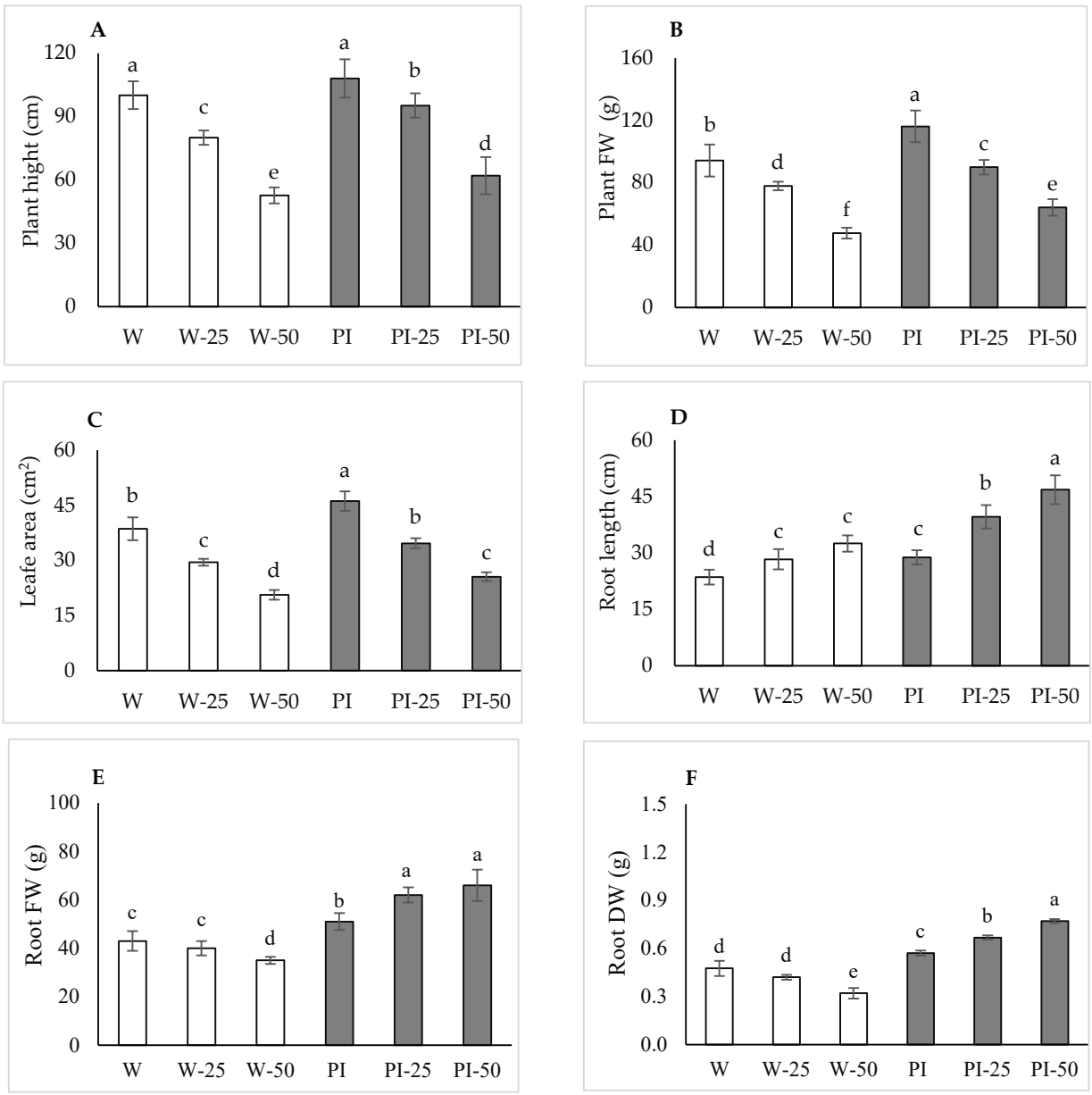

Figure 2. Impact of *P. indica* on growth performance of cucumber plants grown under well and limited water irrigation regimes: W (normal irrigation), W-25 (moderate water stress; equal 25% less than normal irrigation), W-50 (severe water stress; equal 50% less than normal irrigation), PI (inoculation with *P. indica* under normal irrigation), PI-25 (inoculation with *P. indica* under 25% less than normal irrigation), and PI-50 (inoculation with *P. indica* under 50% less than normal irrigation). (**A**) Plant height, (**B**) plant fresh weight, (**C**) leaf area, (**D**) root length, (**E**) root fresh weight, and (**F**) root dry weight. Data are means of combined analysis of two growing seasons. Means marked by the same lower-case letter are not significantly different ($p < 0.05$). Error bars represent standard error of the means (SE), $n = 24$.

3.2. P. indica Colonization Enhances Macronutrients Content and Photosynthesis Rate

The data in Table 1 reveal that *P. indica* colonization increased the N, P, K, Ca, and Mg content and photosynthesis apparatus of cucumber leaves grown in normal (W), moderate (W-25), or severe (W-50) water stress more so than non-colonized plants. Notably, moderate water stress caused slight decreases in the values of the tested parameters compared to the nonstress plants. Meanwhile, decreasing irrigation water up to 50% (severe stress) resulted in a significant reduction in all traits of colonized or non-colonized plants.

Table 1. Macronutrient content, transpiration rate and photosynthesis rate of cucumber leaves at 60 days post *P. indica* inoculation, under well and limited water irrigation regimes.

Treatment	N	P	K	Transpiration Rate	Photosynthesis Rate
	(g.kg^{-1})	(g.kg^{-1})	(g.kg^{-1})	(mmol H_2O m^{-2} s^{-1})	(µmol CO_2 m^{-2} s^{-2})
W	29.89 ± 3.9 b	2.8 ± 0.34 b	26.4 ± 1.6 b	6.10 ± 0.23 c	15.21 ± 0.12 b
W-25	25.50 ± 2.7 c	2.4 ± 0.09 c	27.3 ± 2.9 b	6.06 ± 0.31c	15.10 ± 0.11 c
W-50	19.70 ± 1.4 d	1.9 ± 0.18 d	20.1 ± 2.7 c	3.64 ± 0.13 e	9.08 ± 0.07 e
PI	35.81 ± 2.4 a	3.5 ± 0.08 a	31.01 ± 3.2 a	8.14 ± 0.22 a	20.30 ± 0.16 a
PI-25	30.02 ± 3.3 b	3.4 ± 0.40 a	32.3 ± 3.5 a	6.45 ± 0.24 b	16.07 ± 0.13 b
PI-50	26.71 ± 1.7 c	2.5 ± 0.23 bc	26.06 ± 2.3 c	5.39 ± 0.23 d	13.45 ± 0.10 d

W (normal irrigation), W-25 (moderate water stress; equal 25% less than normal irrigation), W-50 (severe water stress; equal 50% less than normal irrigation), PI (inoculation with *P. indica* under normal irrigation), PI-25 (inoculation with *P. indica* under 25% less than normal irrigation) and PI-50 (inoculation with *P. indica* under 50% less than normal irrigation). Data are means of combined analysis of two growing seasons. Means marked by the same lower-case letter are not significantly different ($p < 0.05$). Error bars represent standard error of the means (SE), $n = 12$.

3.3. P. indica Augments Fruit Production and WUE

Limiting irrigation water applications decreased yield and the components of colonized and non-colonized cucumber plants (Figure 3A–C). In comparison to normal irrigation, moderate (W-25) and severe (W-50) water stress reduced yield down to 47% and 83% with non-colonized plants, and 28% and 78% with *P. indica*-colonized plants, respectively (Figure 3A). However, no significant differences were observed among colonized and non-colonized plants under normal conditions regarding the average fruit weight or the number of fruits per plant (Figure 3B,C). On the contrary, *P. indica* improved fruit weight and increased the number of fruits per colonized plant under moderate stress (W-25). However, *P. indica* did not affect fruit characteristics under normal or high-water stress (W-50). In terms of WUE (Figure 3D), irrigation water reduction (W-25 and W-50) caused a significant demand for required irrigation water (43 and 81 L) to produce 1 kg of fresh cucumber compared to normal irrigation (27 L/kg). Markedly, with *P. indica* colonization, WUE at moderate stress (W-25) resulted in lower values (26 L/kg) in comparison to non-colonized plants (43 L). Meanwhile, under severe stress (W-50), *P. indica* reduced WUE value up to seven liters less than noncolonized, despite colonized and non-colonized production of a similar yield (~4 kg.m^{-2}).

3.4. P. indica Alters Antioxidant Enzymes, Proline, and Chlorophyll Content in Water-Stressed Plants

Water stress increased the level of the CAT, POD, and SOD enzyme activates and the contents of MDA and proline in the leaves of W-25 and W-50 water-stressed cucumber plants when compared to non-stressed plants (Figure 4A–E). Remarkably, colonization with *P. indica* increased the activity levels of CAT and POD enzymes (Figure 4A,B), while significant decreases were observed in the accumulation of MDA and proline in colonized plants compared to non-colonized plants (Figure 4D–E) under both water-stress conditions. In addition, Chl. *a*, *b*, *a* + *b* tended to decrease in plants exposed to water stress, while *P. indica* improved chlorophyll accumulation in leaves of colonized plants under W-25 or W-50 stress conditions (Figure 4F).

3.5. P. indica Alters Phytohormone Content under Moderate and Severe Water Stress

Leaves of non-colonized cucumber plants revealed a lower content of IAA with increasing levels of water stress compared to colonized plants (Figure 5A). On the contrary, water stress increased levels of ABA and ethylene up to two-fold and four-fold in comparison to non-stressed plants. Notably, *P. indica* colonization caused a similar pattern, although the levels of ABA and ethylene were lower than in non-colonized plants (Figure 5B,C).

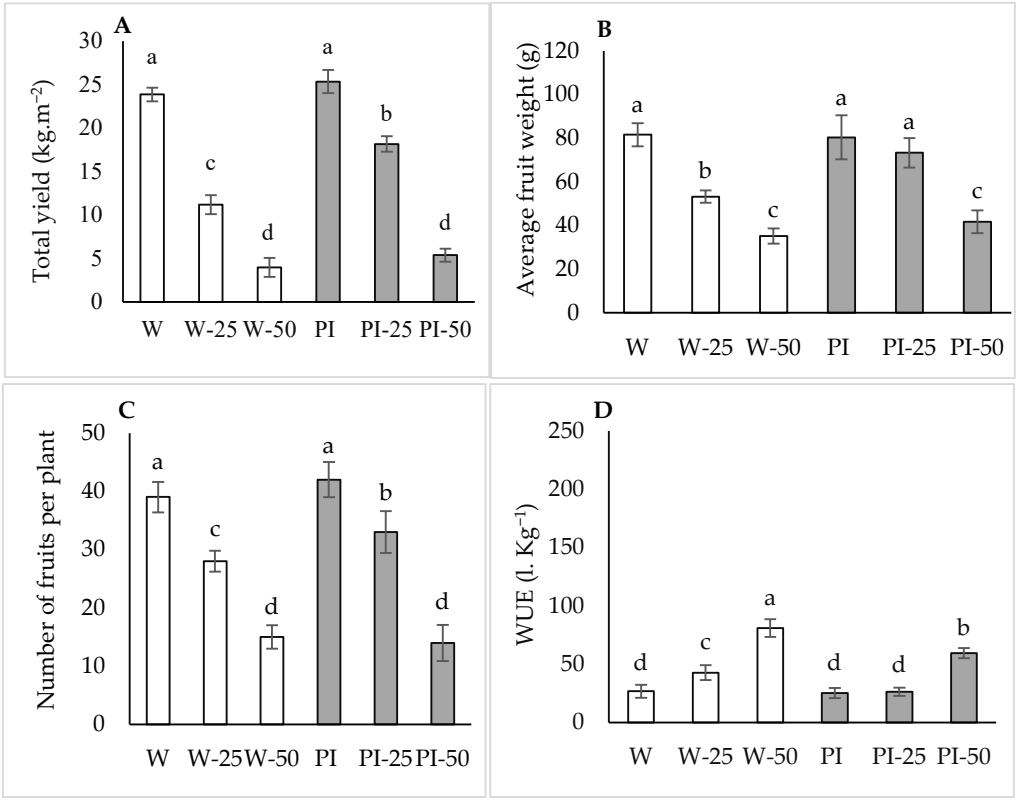

Figure 3. (**A**) Total yield, (**B**) average fruit weight, (**C**) number of fruits per plant, (**D**) water-use efficiency (WUE) of cucumber grown under well and limited water irrigation regimes. W (normal irrigation), W-25 (moderate water stress; equal 25% less than normal irrigation), W-50 (severe water stress; equal 50% less than normal irrigation), PI (inoculation with *P. indica* under normal irrigation), PI-25 (inoculation with *P. indica* under 25% less than normal irrigation), and PI-50 (inoculation with *P. indica* under 50% less than normal irrigation). Data are means of combined analysis of two growing seasons. Means marked by the same lower-case letter are not significantly different ($p < 0.05$). Error bars represent standard error of the means (SE), $n = 24$.

3.6. P. indica Colonization Reduces the Stomatal Closure of Water-Stressed Cucumber Plants

The leaf surfaces were scanned with SEM to observe the stomatal closure of the water-stressed cucumber plants. As shown in Figure 6A–F, water stress induced stomatal closure in the leaves of the stressed cucumber plants. Likely, stomatal closure was higher in severe stress treatment (10% PEG-6000) than in moderate stress (5% PEG-6000). Colonization with *P. indica* reduced stomatal closure under both water-stress levels (Figure 6E,F). This enhancement was more evident under severe water-stress treatment in colonized plants than in non-colonized ones (Figure 6C,F).

3.7. Gene Expression of the Chlorophyll Metabolism-Related Genes in Cucumber

The transcript levels of the Glu-TR gene involved in chlorophyll biosynthesis were increased in cucumber leaves grown in the presence of *P. indica* colonization under severe water stress compared to the non-colonized plants (Figure 7A). However, *P. indica* colonization strongly upregulated POR expression up to six-fold and four-fold under moderate (PEG5%, equivalent to W-25) and severe (PEG10%, equivalent to W-50) water stress, respectively, in comparison to normal irrigation (Figure 7B). Moreover, water shortage gradually increased CAO expression in non-colonized plants by 20-fold and 40-fold under stress conditions (Figure 7C), while colonized plants presented 3-fold and 11-fold increases over control. Regarding the effect of *P. indica* on chlorophyll degradation genes, data in Figure 7D–F show that colonizing cucumber roots with the fungus showed reduced levels of PPH and RCCR genes when compared to non-colonized plants, particularly under

severe water-stress conditions (PEG10%). At the same time, no significant differences were observed among colonized and non-colonized plants under normal or moderate water stress.

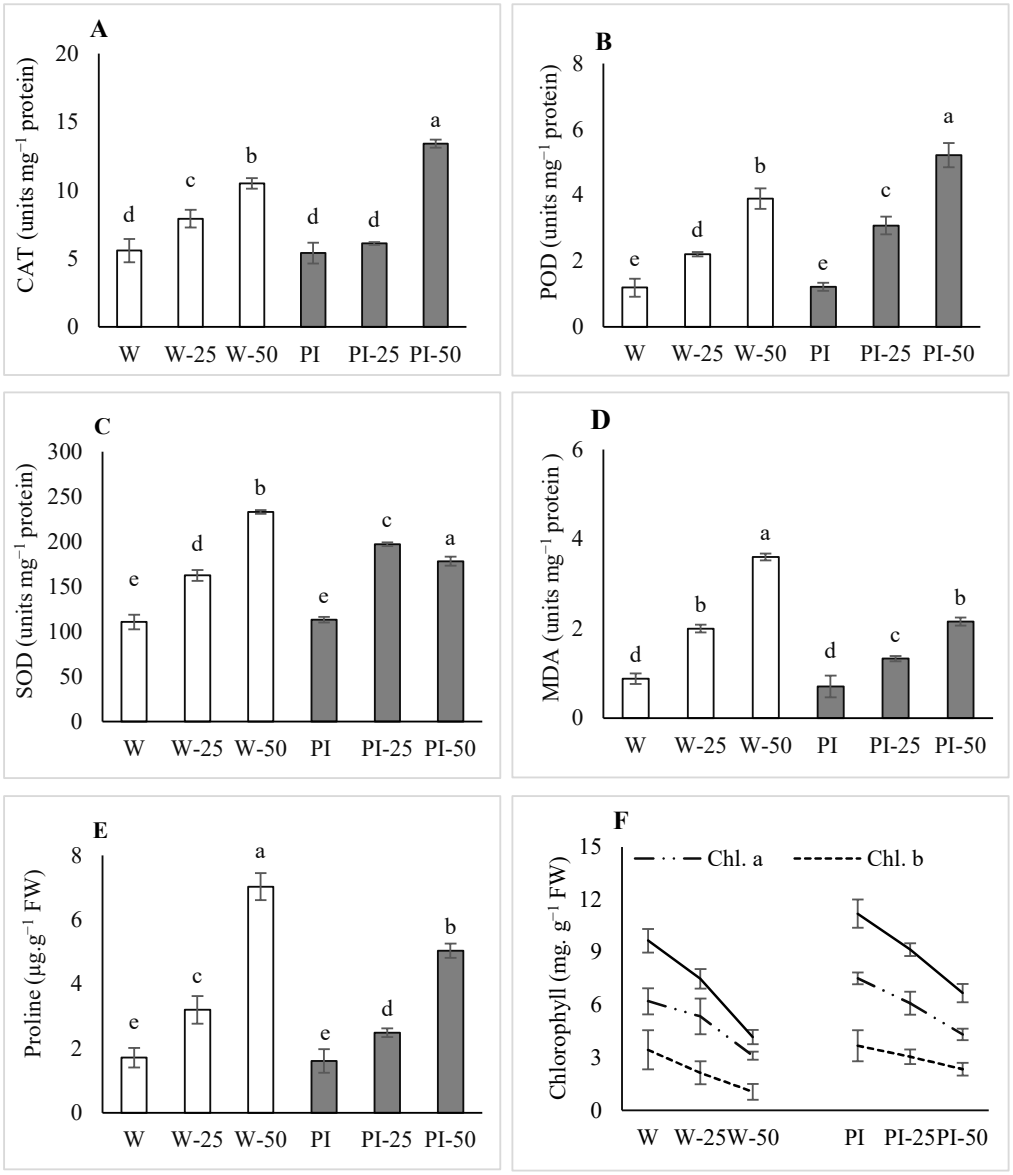

Figure 4. (**A**) Catalase (CAT), (**B**) peroxidase (POD), (**C**) superoxide dismutase (SOD), (**D**) malondialdehyde (MDA), (**E**) proline, (**F**) chlorophyll content in leaves of cucumber plants at 60 days post *P. indica* inoculation, under well and limited water irrigation regimes. W (normal irrigation), W-25 (moderate water stress; equal 25% less than normal irrigation), W-50 (severe water stress; equal 50% less than normal irrigation), PI (inoculation with *P. indica* under normal irrigation), PI-25 (inoculation with *P. indica* under 25% less than normal irrigation), and PI-50 (inoculation with *P. indica* under 50% less than normal irrigation). Data are means of combined analysis of two growing seasons. Means marked by the same lower-case letter are not significantly different ($p < 0.05$). Error bars represent standard error of the means (SE), $n = 6$.

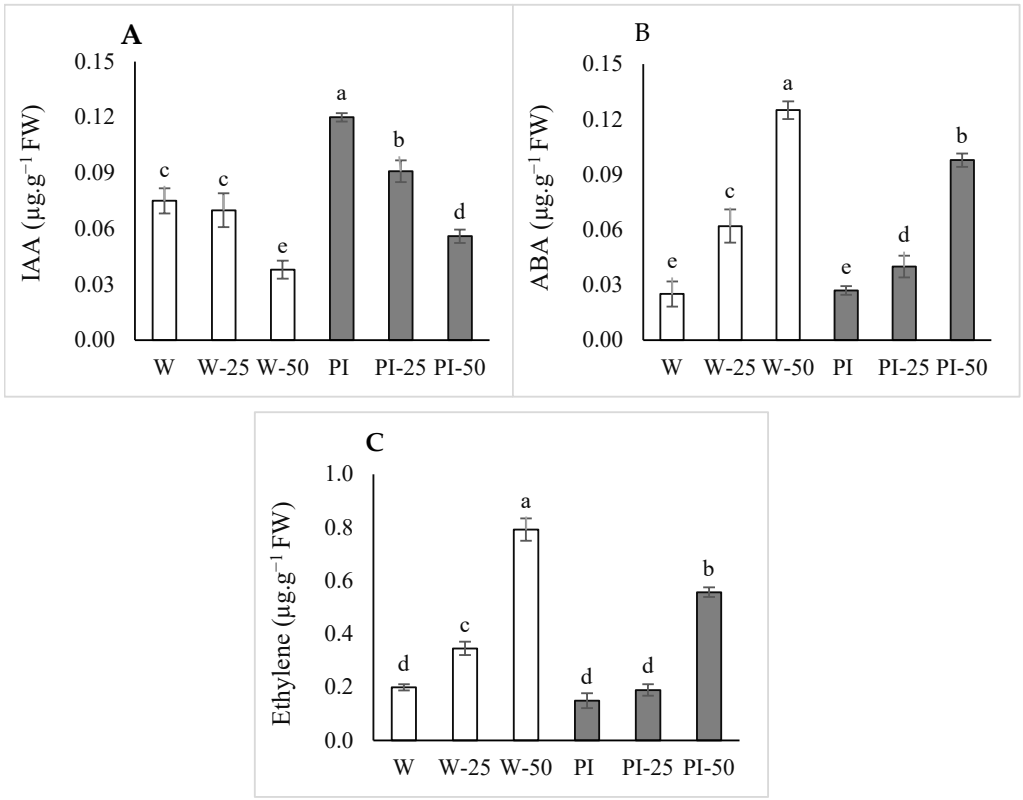

Figure 5. Hormone quantification (**A**) IAA, (**B**) ABA, (**C**) ethylene in leaves of cucumber plants grown under well and limited water irrigation regimes. W (normal irrigation), W-25 (moderate water stress; equal 25% less than normal irrigation), W-50 (severe water stress; equal 50% less than normal irrigation), PI (inoculation with *P. indica* under normal irrigation), PI-25 (inoculation with *P. indica* under 25% less than normal irrigation), and PI-50 (inoculation with *P. indica* under 50% less than normal irrigation). Data are means of combined analysis of two growing seasons. Means marked by the same lower-case letter are not significantly different ($p < 0.05$). Error bars represent standard error of the means (SE), $n = 6$.

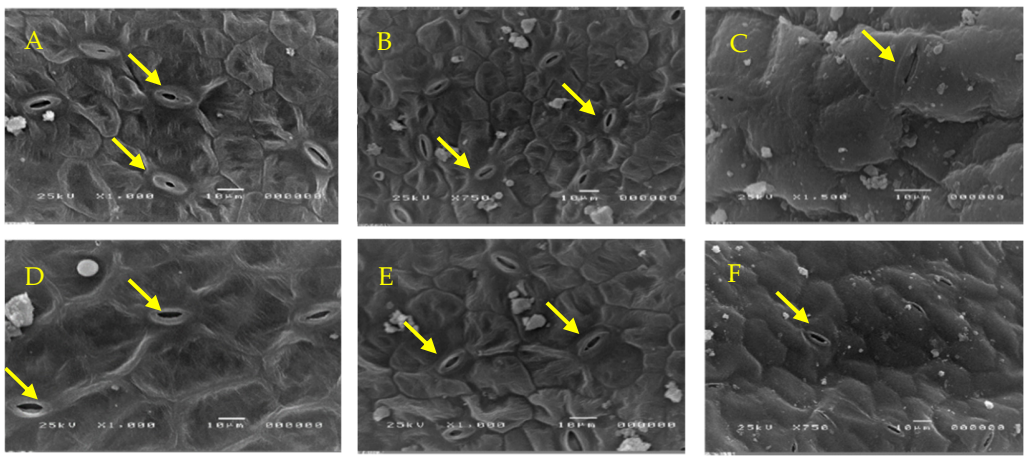

Figure 6. Scanning electron microscopy for stomatal closure of cucumber leaves grown under 2 PEG-6000 levels (5 and 10%) 15 days post *P. indica* co-cultivation. (**A**) No-stress, (**B**) 5% PEG-6000, (**C**) 10% PEG-6000, (**D**) *P. indica*-colonized plants, (**E**) *P. indica*+ 5% PEG-6000, (**F**) *P. indica* + 10% PEG-6000. Scale bar = 10 µm.

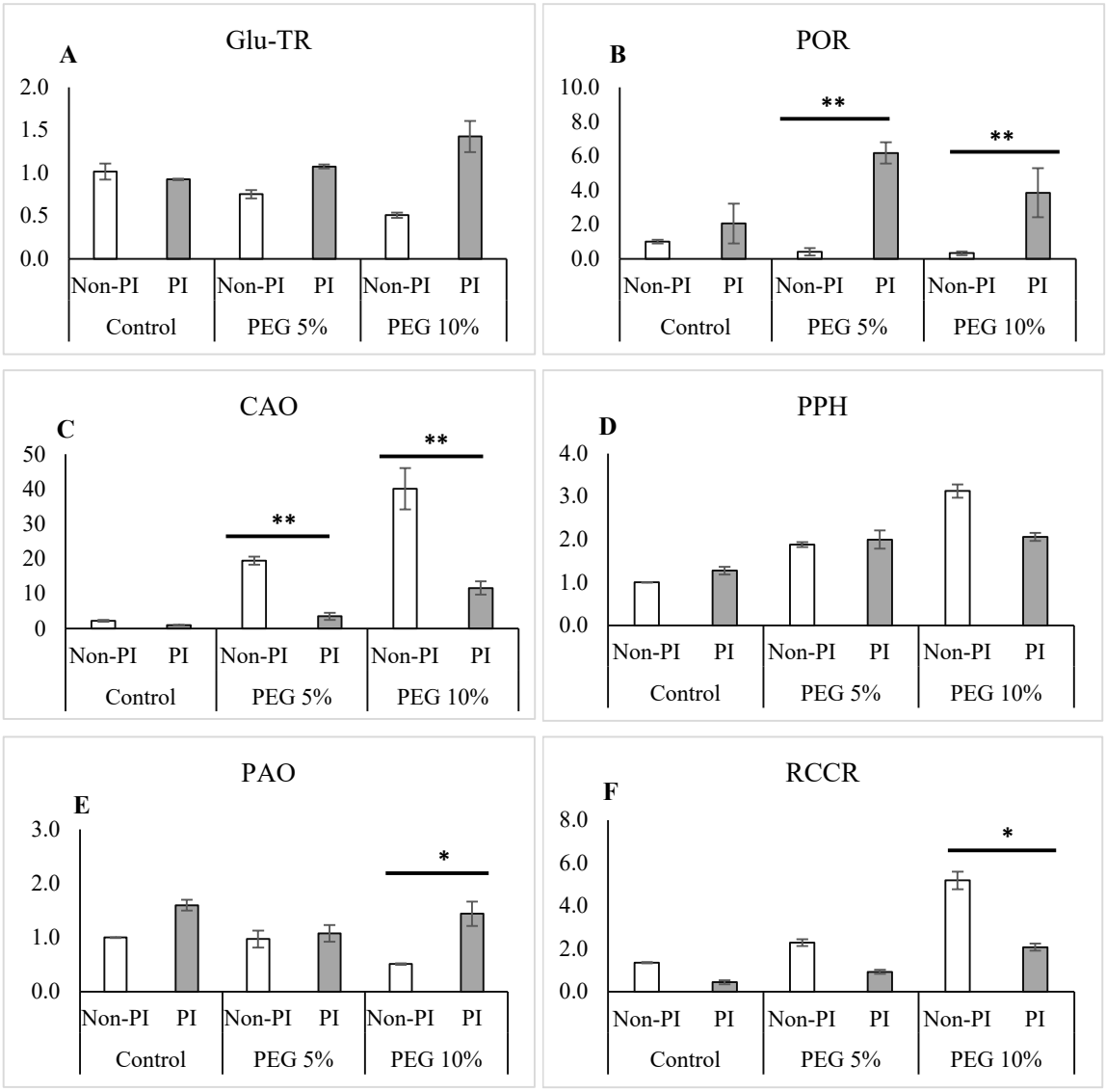

Figure 7. qPCR expression profile of chlorophyll biosynthesis and degradation genes in the third leaf of cucumber grown under 2 PEG levels (5 and 10%) 15 days post *P. indica* co-cultivation. (**A**) Glu-TR, glutamyl-tRNA reductase (**B**) POR, protochlorophyllide oxidoreductase (**C**) CAO, chlorophyll a oxygenase (**D**) PPH, pheophytinase (**E**) PAO, pheophorbide a oxygenase, (**F**) RCCR, red chlorophyll catabolite reductase for colonized and non-colonized *P. indica* (PI) plants. Actin gene was used for normalization. Data are mean fold changes of three independent experiments each containing three repeats. Asterisks indicate significance at ** $p < 0.01$ and * $p < 0.05$ by student *t*-test.

4. Discussion

Renewable freshwater resources for the agriculture industry are decreasing, meaning that one-third of irrigated crop production challenges are related to high-water stress [65,66]. The harmful effects of water deficits on plant growth performance and productivity have been intensively explained, with challenging efforts to enhance water-stress tolerance, particularly for crops with economic impact [67]. Therefore, the association between endophytic fungi and cost-effective crops can effectively overcome the adverse effects of water stress [68]. In this respect, the potential role of *P. indica* in sustainable agriculture has become promising in the past decade [69].

Here, we demonstrate that reducing irrigation water decreases cucumber growth, yield, and physiological traits. At the same time, colonization with *P. indica* could be utilized to improve cucumber water-stress tolerance mechanisms under moderate and severe irrigation stress at the commercial level. Data presented in this work show that

cucumbers exposed to a limited quantity of irrigation water, either 25% (W-25) or 50% (W-50) lower than normal irrigation water levels (W), resulted in a significant decrease in plant height, plant fresh weight, leaf area, and root biomass (Figure 1). Similar outcomes were obtained by Pokluda et al. [69], Abdelaziz and Taha [70], and Tsai et al. [71]. This negative effect of water stress on plant growth characteristics refers to the impact of water stress on cellular dehydration, reduction of cell volume and turgor [72], alongside inhibiting cell elongation by interrupting water flow from the xylem to the cells [73]. Particularly, reduction in the levels of irrigation water increased cucumber root length compared to non-stressed plants (Figure 2D). This consequence could be explained by the fact that plants subjected to water stress can increase root length as a defense technique to absorb more water under lower moisture conditions [74].

Furthermore, reducing irrigation water slightly (W-25) did not change the root biomass as much as severe water stress (W-50) (Figure 2E,F). A comparable conclusion was reported by Navarro et al. [75], who mentioned that moderate water stress caused non-significant changes in root development, while a severe water deficit clearly reduced the root dry weight of *C. albidus* by 48% [76]. Water stress caused a gradual decrease in cucumber yield traits under the W-25 and W-50 treatments. To mitigate the harmful impact of water stress, the mutualistic fungus *P. indica* was utilized to inoculate cucumber plants during the two cultivation seasons. In this experiment, successfully colonized cucumber roots 30 days post inoculation under normal and water-stress conditions were monitored by a light microscope (Figure S1). However, colonization with *P. indica* markedly improved shoot and root growth under water stress (W-25 and W-50) or normal water conditions (W). Nemours reports emphasize the potential of *P. indica* to mitigate abiotic stress in different host plants [77]. Besides, the positive effect of *P. indica* colonization on the growth of cucumbers under water stress was combined with a significant increase in fruit number per plant (17.8%), fruit weight (37.5%), and total yield (7.3%), mainly under moderate water stress (W-25). Simultaneously, no differences were recorded between colonized and non-colonized plants under severe water reduction (W-50) treatment, as shown in Figure 3A–C.

A similar trend was stated by Murphy et al. [78] and Ahmadvand and Hajinia [79], who validated the ability of *P. indica* to stimulate the yield of colonized plants under stress conditions. Earlier, Fakharo et al. [80] found that early harvest tomato colonized with *P. indica* produced twice as much fruit, while Abdelaziz et al. [43] and Atia et al. [44] reported 22% and 34% yield increases in tomato and cucumber, respectively. Therefore, our results clearly reflect the effect of *P. indica* inoculation in enhancing the growth performance of colonized plants compared to non-colonized under stress. In this respect, it could be suggested that the correlation between *P. indica* colonization and improvement in root systems might be a direct cause of elevated nutrient uptake efficiency. This suggestion is confirmed in Table 1, which shows that leaf photosynthesis rates and macronutrient content of colonized plants were significantly increased compared to non-colonized plants, either in normal or stress conditions [35,38,79,80].

Several reports have mentioned that *P. indica* promotes plant growth by facilitating nutrient acquisition [81–83] or by upregulating mineral transportation [82]. Moreover, from a sustainable agricultural perspective (Figure 3D), these improvements in plant growth and yield positively reflected WUE. WUE is considered an indicator of the actual irrigation water consumed to produce 1 kg of fruit [47] and shows whether the water has been used effectively [83–85].

According to our results, it could be concluded that *P. indica* improves WUE by supporting colonized plants to produce a higher yield per unit volume of water consumed by the crop in comparison to non-colonized plants under moderate stress or severe water stress. However, it is evident in our experiment that the fitness of colonized plants under water stress was better than non-colonized ones. Accordingly, many reports have explained the capability of *P. indica* to improve water-stress tolerance either by altering antioxidant enzyme activity, production of secondary metabolites, or hormonal modification [35,86,87].

Generally, our findings revealed that *P. indica* colonization mediated water-stress tolerance by introducing CAT and POD enzymes (Figure 4A,B) and IAA content (Figure 5A). For indole-3-acetic acid (IAA), the *P. indica* promotion of IAA has been described as the primary mechanism that enhances plant growth under both normal and stress conditions by promoting root elongation and lateral root formation, stimulating adventitious roots [35,88]. The enhancement in IAA levels in the leaf tissue of colonized cucumber plants could be attributed to the interference of *P. indica* with auxin production and signaling cucumber plants to increase plant development. Several studies have reported that endophyte *P. indica* interferes with auxin metabolism and signaling in host plants and is accountable for, or at least contributes to, plant growth [88–94]. Nirenberg et al. [88] found that *P. indica* produced IAA separately in liquid culture and that fungal auxin production affects plant growth. The auxin content varies significantly in different plant species inoculated by endophyte *P. indica*, which has been determined directly or deduced from the expression of auxin-responsive genes. Growth promotion of colonized Chinese cabbage and barley seedlings has been associated with improved auxin content in plant roots [88–90]. Interestingly, Lee and his colleagues [88] found that auxin levels were raised in Chinese cabbage by *P. indica* colonization, indicating the vital role of the fungus in plant auxin production [88].

Regarding antioxidant enzyme activity, many studies have reported that drought stress increases the ROS levels (e.g., hydrogen peroxide, superoxide and hydroxyl radicals) as well as MDA level [95]. Levels of radical scavengers and antioxidant enzymes represent a crucial factor for alleviating oxidative stress in plants. Therefore, any imbalance in the ratio of antioxidants to ROS can cause severe cellular damage [96]. Recent studies have suggested that water stress increases oxidative pressure on plants, resulting in tissue damage [71]. Several studies have shown that *P. indica* improves the plant's drought tolerance by increasing the activity levels of antioxidant enzymes and coping with other water deficiency's harmful effects [95,96].

In contrast, contents of MDA, proline (Figure 4D,E), ABA, and ethylene (Figure 4B,C) were significantly decreased with the elevated water stress of colonized plants and with a significant induction in non-colonized plants. Abscisic acid mediates the early response to water stress by limiting the stomatal aperture to prevent water loss [97], while MDA is a well-known critical marker of oxidative damage to cells [98]. In addition, high proline content in the leaves of stressed plants has been reported to improve plant tolerance against osmotic stress [99]. Clearly, reducing the above three key water-stress tolerance markers confirms that *P. indica* colonization enhances the water-stress tolerance of colonized plants [99,100].

Another way to explore the impact of *P. indica* on WUE under water stress is to begin at the leaf level. It is well-known that water stress alters stomata closure to minimize water loss due to transpiration [100]. Simulation of water stress by polyethylene glycol (PEG-6000) increased osmotic stress on the treated plants and presented a significant deviation compared to the control [101].

The data in Figure 6 show that *P. indica*-colonized plants presented lower stomatal closure under stress than noncolonized. This result indicates that association with *P. indica* supports plants' coping with water stress, which is reflected in improving stomatal opening. In addition, colonized cucumber plants with *P. indica* have higher photosynthesis and transpiration rates (Table 1) combined with a significant increase in leaf chlorophyll *a* and *b* content (Figure 4F). These results agree with several reports showing a notable increase in chlorophyll and photosynthetic pigments due to *P. indica* colonization. In this respect, Atia et al. [44] stated that *P. indica* inoculation improved the chlorophyll content, photosynthesis rate, and water-use efficiency of cucumber plants compared to non-colonized plants. In tomatoes, *P. indica* colonization increased levels of chlorophyll *b* under salt stress [43]. Likewise, Shahabad et al. [102] indicated that the presence of *P. indica* significantly enhanced the chlorophyll *a* and *b* of sunflower plants under Cd stress. Chlorophyll plays a crucial role in harvesting and transducing light energy in plants [103].

Chlorophyll is indispensable for plant metabolic pathways and dynamically changes during stress responses [104]. Our findings revealed that the relative expression of Glu-TR, which handles 5-aminolevulinic acid (ALA) synthesis, decreased under drought conditions. Furthermore, this expression was accompanied by a decrease in the expression level of the POR gene (chlorophyll *a* synthesis), indicating that water stress reduced the expression of chlorophyll biosynthesis genes. However, the potential *P. indica* application can reverse the adverse effects caused by either moderate or severe water-stress conditions in cucumber leaves by boosting the expression levels of Glu-TR and POR genes [71]. Subsequently, overexpression of CAO plays a pivotal role in the chlorophyll cycle to convert chlorophyll *a* to chlorophyll *b*, resulting in increased chlorophyll *b*, which was obvious in the presence of *P. indica* under drought conditions [105]. Moreover, the fittest ratio of chlorophyll *a* to chlorophyll *b* may indicate that CAO tends to rebalance the photosynthesis rate, either in the presence of *P. indica* or drought conditions [103]. Finally, the PPH, PAO and RCCR are the central reactions of chlorophyll degradation genes. Previous studies have suggested that the significant reduction in these genes may result in accelerated cell death due to the high accumulation of the substrates of these enzymes [104]. The current results agree with the previous findings of Mo et al., (2016), who demonstrated that arbuscular mycorrhiza, a *P. indica*-like fungus mycorrhiza, in combination with drought stress, slightly increased the expression level of PPH and PAO in watermelon. Although both PI and non-PI treatments revealed an increased expression pattern either in normal or stress conditions, the expression of the RCCR gene was found to alleviate the chlorophyll degradation ratio in colonized stressed cucumber plants (PI at PEG 10%). Thus, this might explain why the expression of PAO and RCCR was fine-tuned by *P. indica* treatment under water-stress conditions.

Our results show that *P. indica* supports chlorophyll metabolism by inducing biosynthesis genes and downregulating selected chlorophyll degradation genes. These enhancements in chlorophyll content and leaf greening conclude that *P. indica* colonization indirectly delays leaf senescence under water stress. In this regard, ethylene has been quantified in the leaves of colonized and non-colonized plants under stress conditions to confirm this suggestion. Here, *P. indica* colonization reduced ethylene content under moderate and severe water stress compared to normal conditions. Ethylene is one of the most important hormones regulating leaf senescence under stress [106] and involves activating and regulating plant innate immunity [107]. Meanwhile, chlorophyll degradation and leaf abscission are the most common visual symptoms of leaf senescence [108,109].

5. Conclusions

The mutualistic fungus *P. indica*, like mycorrhiza, shows clear potential to support the growth performance and yield production of several plants under water stress. However, the *P. indica* mechanism used to support plants against different abiotic stresses is still not fully explained. Here, we present that *P. indica* enhanced growth, nutrient content, and stress tolerance traits and affected chlorophyll biosynthesis and degradation genes in the leaves of cucumber plants grown under two water-stress irrigations, 25% and 50% less than normal irrigation. This improvement in plant fitness, either under normal or water-stress conditions, was found to save irrigation water under greenhouse conditions by enhancing the efficiency of colonized plants. This work implies that *P. indica* plays an essential novel role in mitigating the harmful consequences of water stress.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae7100399/s1, Figure S1: Spores and hypha of *P. indica* in roots of cucumber at 30 days post inoculation, Table S1: list of selected chlorophyll synthesis and degradation genes, according to [4].

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E.A.A., T.A.A.I., M.A.M.A., M.A., and M.E.A.; resources, E.A.A., M.E.A., S.M.A., M.A., and M.A.M.A.; data curation, E.A.A., M.A., and M.E.A.; writing—original draft preparation, E.A.A., M.E.A., M.A.M.A., S.M.A., and M.A.; writing—review and editing, E.A.A., M.E.A., M.A., S.M.A., and M.A.M.A.; visualization, E.A.A., M.E.A., M.A.M.A., S.M.A and M.A.; supervision, E.A.A., M.A.M.A.; M.A. and M.E.A.; project administration, E.A.A., M.A.M.A. and M.E.A. All authors have read and agreed to the published version of the manuscript.

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