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Leaf Gas Exchange and Growth Responses of Tomato Plants to External Flavonoids Application as Biostimulators under Normal and Salt-Stressed Conditions

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Abstract: The exogenous application of natural metabolites, such as phenolic compounds, is a useful strategy to stimulate growth and reduce the adverse effects of abiotic stress on crops, such as salinity. Salinity stress is one of the most damaging abiotic stresses to plants, causing reductions in growth by changes in the physiology, biochemistry, and gene expression. In this work, we investigated the effect of the foliar application of flavonoids (CropBioLife, CBL) on control and salt-stressed (NaCl 60 mM) tomato plants grown in controlled conditions. The results showed that CBL mainly influenced the stimulation of photosynthesis, increasing CO_2 fixation and promoting growth. Furthermore, a higher stomata number in an open state was found in CBL-treated plants in relation to the higher CO_2 fixation, which also resulted in a higher H_2O uptake due to increasing stomatal conductance and nutrient uptake that plants need for growth. The results were due to the increase of phenolic metabolism and the expression of most of the aquaporins, which could be the triggering signal for the rest to the changes observed. The effect of the biostimulation of CBL under salinity was related to higher levels of photosynthesis, the increase of some mineral nutrients, and the increase of some PIP aquaporins expression, although no effect on growth was observed. The results of this work showing the mechanism of action of flavonoids in tomato plants open a new line of investigation with great importance for the future of agronomy.

Keywords: aquaporins; phenolic compounds; biostimulants; salinity; tomato

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1. Introduction

Flavonoids are the largest family of chemical products in plants, belonging to secondary metabolism [1]. Their chemical formula basically contains three phenolic rings of six carbons bound to a central three-carbon ring (C6-C3-C6), which can produce several derivatives of the basic structure [2]. The flavonoid subgroups are mainly flavonols, flavones, flavanones, flavanonols, flavanols, anthocyanins, isoflavonoids, and chalcones [3]. The low molecular weight of these secondary metabolites allows them to be involved in the main plant physiological functions, often demonstrating protective effects against abiotic stresses such as salinity [4].

Although flavonols have been well-documented for their antioxidant capacity in vitro [5], their antioxidant capacity in plants has been a matter of controversy until the last few years, when they were reported to be not as efficient as other secondary metabolites in absorbing wavelengths in the 290–320 nm spectral region, although they showed a higher capacity to maintain homeostasis and to regulate growth under abiotic stress conditions [6]. In this way, some studies have provided direct evidence that demonstrates that improving flavonol accumulation regulates stomatal movement, revealing new insights into guard cell signaling and allowing a higher gas exchange for concomitant photosynthesis and water uptake and transport [7]. Furthermore, recently, stomata density was significantly related to the increase in phenolic compounds and flavonoid contents, as well as the

antioxidant activity in lettuce, revealing an increase in the concentration of chlorophylls and photosynthesis [8].

Soil and water salinization is an increasing problem worldwide [9]. The salinization of crop land has become one of the most important global factors, especially in arid and semi-arid zones, which have to study mechanisms [10] and agronomical practices [11] to overcome and tolerate salinity. The excess of soluble salts in the soil solution may limit plant growth, primarily through two mechanisms: osmotic stress and ion toxicity. First, a low solute/osmotic potential due to the increased ion concentration (NaCl) in the soil water reduces the total soil-water potential (Y), which, in turn, reduces the ability of plant roots to uptake water, eventually resulting in diminished plant growth [12]. Second, ion toxicity in plant tissues, more frequently due to sodium accumulation, causes cellular damage by membrane disruption, and disturbs the plant's physiological processes, including photosynthesis, respiration, transpiration, and osmoregulation, resulting in necrosis or chlorosis, and leading to reduced plant growth [13]. The increased accumulation of flavonoids by 40%, when plants face salinity stress, has been reported as a phytochemical strategy to combat salt stress and subsequent toxic reactions, contributing to cellular protection by detoxifying the accumulated salts [14].

Tomato (*Solanum lycopersicum* L.) is one of the most produced crops in the world, as tomato is not only consumed fresh, but is also in a large variety of processed products [15]. This crop is considered to be moderately sensitive to salinity, but several parameters that have an influence on the growth and development of the plants, such as water relations or photosynthesis, are affected at a medium-salinity stress [16]. In this way, due to the high economic importance of producing tomato in all areas of the world, the study of the response to salinity, and the technologies for improving this tolerance, has strongly increased in recent years [17].

The use of biostimulants on crops has been proposed as an agronomical practice that improves yield and could help to increase the sustainability of agriculture by reducing inputs such as fertilization and water [18]. The mechanisms of metabolism activation of the different biostimulants available need to be addressed, as it involves benefits in physiology responses and helps to overcome environmental stress conditions [19]. The already discovered effects of biostimulant compounds include the stimulation of the enzyme activities of glycolysis and the Krebs cycle [20], but further studies should be conducted to investigate the mechanism of action of each biostimulant when applied to plants. The external application of flavonoids has been little documented, but recent results obtained with the application of individual flavonoids such as coumarin [21], ferulic acid, salicylic acid [22], and vanillic acid [23] have lead us to believe that they could work as biostimulants. Furthermore, the investigation of the application under salinity conditions revealed some metabolic positive changes but no effect on growth [22]. Therefore, the need for deep physiological study was reported.

In our experiments, we explored the responses of leaf gas exchange and the growth of tomato plants to the external application of flavonoids as biostimulators under normal and salt-stressed conditions. The growth and physiological determinations related to water and nutrient uptake and transport, such as gas exchange, osmotic adjustment, photosynthesis, stomata, and mineral nutrient composition, were associated with the expression of aquaporins and the metabolism of phenolic compounds to determine if the mechanism of action was related to water uptake and transport.

2. Materials and Methods

2.1. Experimental Design and Culture Conditions

Seeds of tomato (*Solanum lycopersicum* cv Marmande from Ramiro Arnedo, La Rioja, Spain) were pre-hydrated with de-ionised water and aerated continuously for 24 h. After this, the seeds were germinated in vermiculite in the dark at 28 °C for three days. Then, the seedlings were transferred to a controlled-environment chamber with a light–dark cycle of 16-8 h, a temperature of 25–20 °C, and a relative humidity of 60–80%. A photosynthetically

active radiation (PAR) of 400 μmol m^{-2} s^{-1} was provided by LEDs (Pacific LED, WT 470 C, LED8OS/840 PSD WB L1600 lights, Philips, Amsterdam, Netherlands). After 7 days, the seeds were transferred to hydroponic conditions in 16-L containers (6 plants each), each filled with Hoagland's solution, pH 5.5. The solution was continuously aerated and changed every week. The composition of the solution was: 6 KNO_3, 4 $Ca(NO_3)_2$, 1 KH_2PO_4, and 1 $MgSO_4$ (mM), and 25 H_3BO_3, 2 $MnSO_4$, 2 $ZnSO_4$, 0.5 $CuSO_4$, 0.5 $(NH_4)_6Mo_7O_{24}$, and 20 Fe-EDDHA (μM). Sixty mM NaCl was applied after five days of growing in a hydroponic culture to half of the containers.

After 10 days of growing in a hydroponic nutrient solution, a first foliar spray of CBL (CropBioLife, Aussan Laboratories Pty Ltd, Campbellfield, Victoria, Australia, composed by a botanical extract based on *Citrus* sp. containing 12% of flavonoids) was applied (diluted to 3 mL L^{-1}) to half of the control plants, and to half of the NaCl-treated plants. Applications of 25 mL per plant were performed. After 5 days of the first CBL spray, another application of CBL was performed. The measurements and collection of samples were carried out after 3 days from the second foliar treatment (scheme of the experimental design in Figure S1). Growth, determined by weight, gas exchange parameters, leaf water relations, and relative water content were determined.

2.2. Dry Weight

After these two-week treatments, four plants from each group were weighed to obtain the fresh weight, separating the root from the shoot, and were then kept in an oven (60 °C) for five days until they were completely dry. Then, they were weighed again to obtain the dry weight (DW).

2.3. Relative Water Content

Relative water content (RWC) was calculated using a 1 cm^2 piece from 4 fully developed middle leaves per plant, in which fresh weight, full-turgor weight, and dry weight were measured. For the turgor weight, the fragments were kept in darkness and humidity in a 4 °C chamber for 24 h. For the dry weight, the fragments were placed in a 60 °C oven for 2 days.

2.4. Chlorophylls and Fluorescence of Photosystem II

Chlorophyll content was determined with a Chlorophyll Meter SPAD-502Plus (Konica Minolta, Langenhagen, Germany), while the fluorescence of photosystem II was determined with a mini-PAM (miniaturized pulse amplitude–modulated photosynthesis yield analyser; Walz, GmbH, Germany). The measurements were performed in a total of 5 leaves per plant and per treatment. The measurements were performed during the middle part of the photoperiod.

2.5. Gas Exchange Parameters

The gas exchange parameters, such as transpiration, stomatal conductance, assimilation rate, and internal CO_2, were measured in fully developed leaves, using a TPS-2 Portable Photosynthesis System gas exchange meter (PP Systems, Inc., Amesbury, MA, USA) in a total of 5 leaves per plant and per treatment. The measurements were performed during the middle of the photoperiod.

2.6. Leaf Osmotic Adjustment

The leaf water relations, such as water potential (Ψ_w), were determined with a Scholander pressure chamber. For the osmotic potential (Ψ_π), one leaf from each plant was harvested, put into tubes, and frozen at −20 °C. They were subsequently thawed, pressed, and centrifuged (1000× *g*) to extract the cell sap. The osmotic potential of the leaf sap was calculated after measuring sap osmolarity using an osmometer (Digital Osmometer, Roebling, Berlin, Germany). Turgor pressure (Ψ_p) was calculated as the difference between water and osmotic potentials (Ψ_w-Ψ_π).

2.7. Number of Stomata

Imprints of leaf epidermis were made by pressing the leaf surface on the non-sticky side of adhesive tape, weakened with a small drop of acetone. The number of open and closed stomata per unit area was calculated with the Leica-Q-500 (Wetzlar, Germany) computer program. Imprints were done on a total of 5 leaves per plant and per treatment during the middle of the photoperiod.

2.8. Ion Analysis in Leaf Dry Matter

All the leaves from five plants from each treatment were collected. Samples were lyophilized and finely ground before digestion with HNO_3:$HClO_4$ (2:1). The elements were detected by inductively coupled plasma (ICP) analysis (Optima 3000, PerkinElmer, Waltham, MA, USA).

2.9. Phenolic Extraction and Analysis

Lyophilized-dried powder from leaves (0.1 g) was extracted with 1.5 mL of a mixture of methanol:water:formic acid (25:24:1) and sonicated for 1 h, followed by an overnight maceration at 4 °C. Samples were centrifuged for 15 min at 15,000× *g*, and the supernatants were collected and filtered through a 0.22 µm pore diameter PVDF membrane. The chromatographic analyses were performed in an Agilent HPLC 100 series (Agilent Technologies, Qaldbronn, Germany), with a Phenomenex reverse-phase column (250 × 4.6 mm, Luna lm C18 (2), 100A). The gradient conditions and phases used were as described in [24].

2.10. Aquaporins Expression

The gene expression of all known aquaporin isoforms of tomato was analyzed in leaf tissues. The isoforms analyzed corresponded to the following groups: SlPIP1, SlPIP2, SlTIP1, SlTIP2, SlTIP4, SlNIP1, SlNIP2, SlNIP4, SlNIP5, SlSIP1, SlSIP2, and SlXIP1.

2.10.1. RNA Extraction and Reverse Transcription

For RNA extraction, leaf samples deep-frozen at −80 °C were used. The extraction process was performed using the NZY Total RNA Isolation kit (NZYtech, Lisbon, Portugal), following the manufacturer's protocol, using 50 mg per sample. As an additional step, the ground samples were vortexed for 20 s after the addition of the extraction buffer. Possible traces of contaminating DNA were removed with DNase I included in the kit. The concentration and purity of the RNA was quantified with a UV/Vis NanoDrop 1000 microvolume spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and its integrity was verified by agarose gel electrophoresis. The extracted RNA was stored at −80 °C until analysis.

2.10.2. RNA-Seq Analysis and Differential Expression

RNA-Seq libraries were prepared using the QuantSeq 3′ mRNA-Seq Library Prep Kit FWD (Lexogen, Wien, Austria) and sequenced in an Illumina Hiseq XTen, San Diego, CA, USA (150 × 2) following vendor specifications. The bioinformatic analysis was performed with the Lexogen Bluebee pipeline, including adapter trimming (BBDuk software, part of BBTools v. 38.91; https://sourceforge.net/projects/bbmap/, California, USA), quality control, and mapping against the *Solanum lycopersicum* reference genome (STAR mapper). The workflow used HTSeq count and Deseq2 to calculate normalized gene counts and for differential expression analysis. Differential expression steps were performed by comparing treatments with the control. Genes with adjusted p-values below 0.1 were selected as being differentially expressed.

2.11. Statistical Analysis

Data analyses were performed using RStudio software (RStudio 16 PBC, Boston, MA, USA) with R version 4.1.0. All the parameters were analyzed using a one-way ANOVA, followed by Duncan's post hoc multiple comparison test, to determine significant

differences between the values for all the parameters at $p \leq 0.05$. The outliers were identified and excluded using Rosner's test ($p \leq 0.05$).

3. Results

3.1. Shoot and Root Dry Weight (DW)

The DW of shoots and roots (Figure 1) showed similar responses with respect to the treatments. A significant increase was observed with the CBL treatments. However, no significant differences were observed in any of the other treatments with respect to the control.

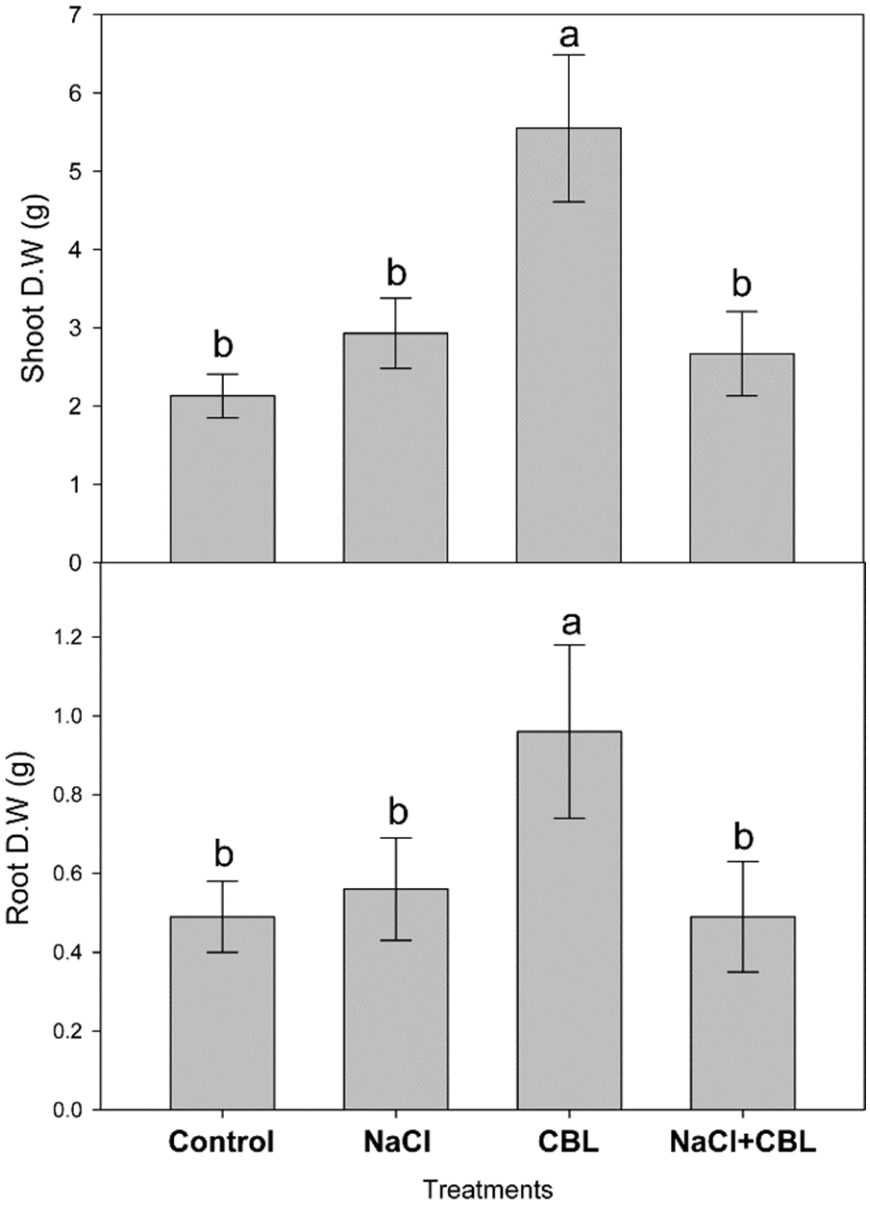

Figure 1. DW (g) of the tomato shoots and roots treated with NaCl, CBL, and NaCl + CBL. Values are means ± SE (n = 5). For each treatment, different letters show significant differences according to Duncan's test at $p < 0.05$.

3.2. Relative Water Content (RWC)

The RWC (Figure 2) of the leaves of tomato plants showed a significant decrease with all the treatments, except in those where the NaCl and CBL were combined (NaCl + CBL), which did not show differences with respect to the control.

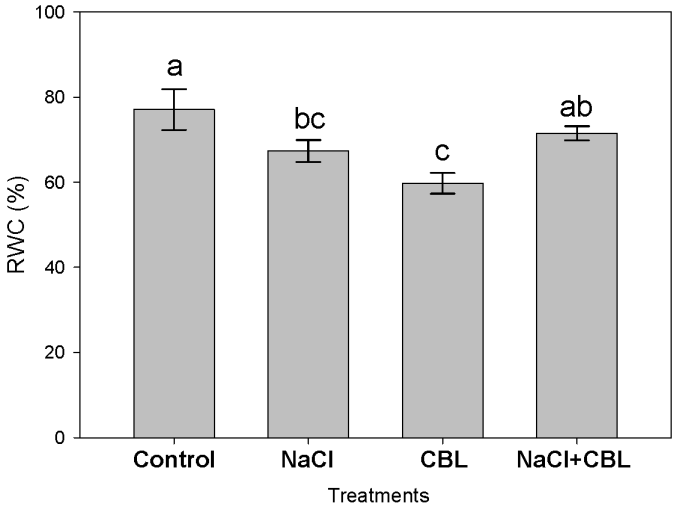

Figure 2. Relative water content (%) of the tomato leaves treated with NaCl, CBL, and NaCl + CBL. Values are means ± SE (n = 5). For each treatment, different letters show significant differences according to Duncan's test at $p < 0.05$.

3.3. Chlorophylls and Fluorescence of Photosystem II

Figure 3 shows the chlorophyll concentration and the fluorescence of the photosystem II. In our results, the chlorophyll concentration was significantly higher in plants treated with CBL, either in standard conditions or grown under salinity stress conditions (60 mM NaCl), when compared with the controls. However, the fluorescence of the photosystem II only showed significantly higher values in the plants treated only with CBL in comparison with the control.

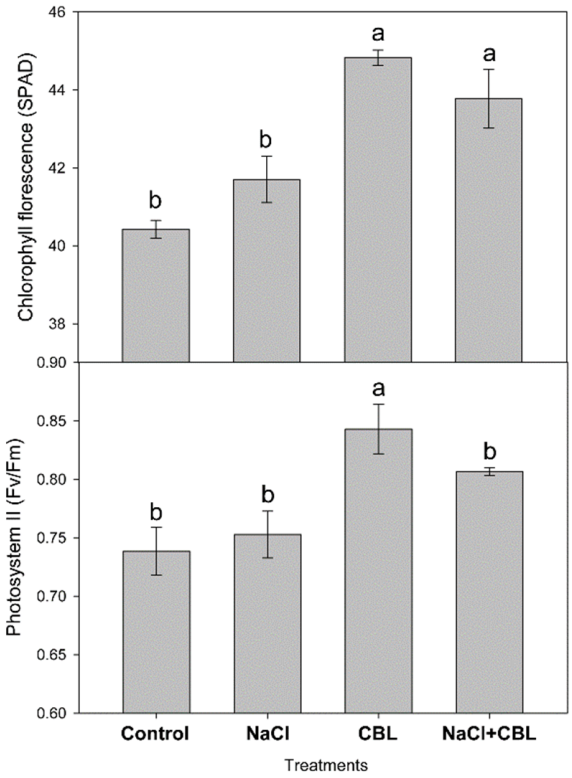

Figure 3. Chlorophyll concentration and the fluorescence of the photosystem II of the leaves of tomato plants treated with NaCl, CBL, and NaCl + CBL. Values are means ± SE (n = 5). For each treatment, different letters show significant differences according to Duncan's test at $p < 0.05$.

3.4. Gas Exchange

Figure 4 shows the results of the internal concentration of CO_2 (Ci), stomatal conductance (Gs), and CO_2 assimilation (Pn). During photosynthesis, the concentration of carbon dioxide in the intercellular spaces of a leaf (Ci), determines the flux of carbon dioxide into the leaf if stomatal apertures and external concentration remain constant. Our results showed higher Ci in plants treated only with CBL. A significant reduction was observed when plants were treated with NaCl. However, when the CBL was applied to plants grown under salinity, there was no significant variation in comparison with the control results. Stomatal conductance (Gs) and CO_2 assimilation (Pn) significantly increased in the CBL-treated plants in comparison with the control. Nevertheless, a significant decrease was observed in the plants treated with NaCl, either alone or with CBL.

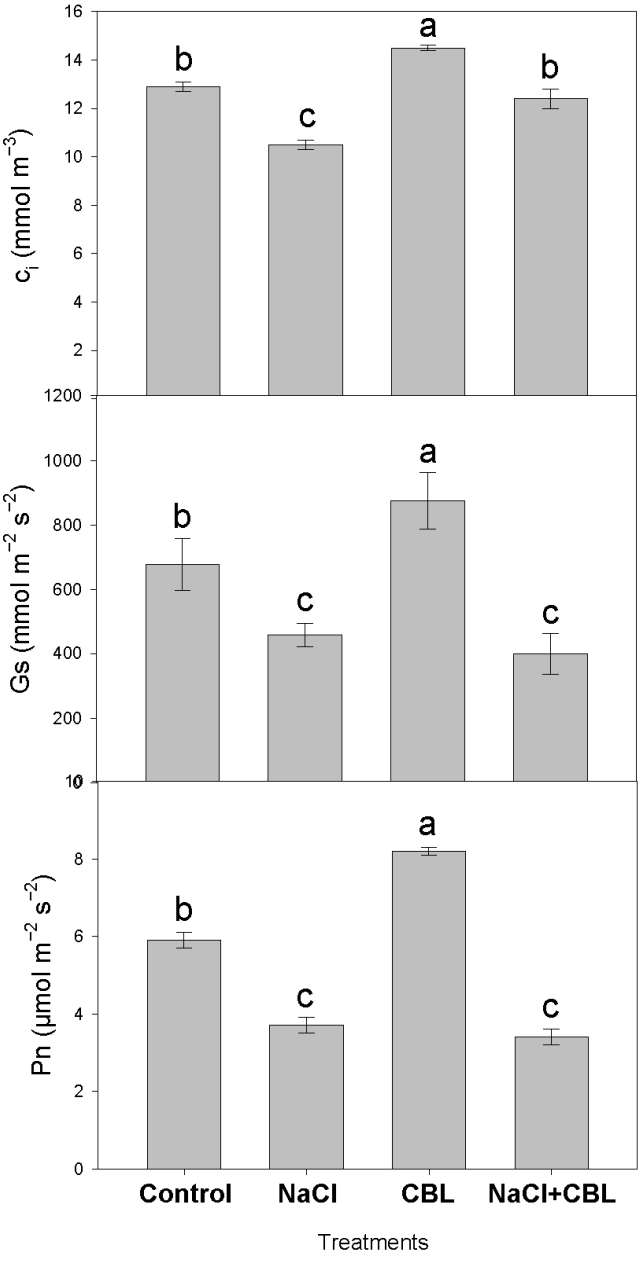

Figure 4. Gas exchange parameters, internal CO_2 (mmol m^{-3}) (Ci), stomatal conductance (mmol m^{-2} s^{-1}) (Gs), and CO_2 assimilation (µmol m^{-2} s^{-1}) (Pn) of the leaves of tomato plants treated with NaCl, CBL, and NaCl + CBL. Values are means ± SE (n = 5). For each treatment, different letters show significant differences according to Duncan's test at $p < 0.05$.

3.5. Leaf Osmotic Adjustment

The osmotic adjustment results (Table 1) showed that the water potential (Ψ_w) and osmotic potential (Ψ_π) were not altered in the NaCl and CBL independent treatment. The turgor potential (Ψ_p) was significantly higher in the leaves of the plants treated with CBL but not in the NaCl plants, in which no significant differences with respect to the control were observed. Only the NaCl + CBL treatment produced decreases in the water potential (Ψ_w) and osmotic potential (Ψ_π) parameters. When the turgor potential (Ψ_p) was calculated in leaves from NaCl + CBL-treated plants, a significant decrease was observed.

Table 1. Measurements of the leaf water potential (Ψ_w), osmotic potential (Ψ_π) and turgor potential (Ψ_p) in tomato. Each value represents the mean of four biological replicates ± SE. Rows with different letters differ significantly according to Duncan's test ($p \leq 0.05$).

Treatment	Ψ_w (MPa)	Ψ_π (MPa)	Ψ_p (MPa)
Control	−0.22 ± 0.10 b	−1.01 ± 0.05 b	0.81 ± 0.01 b
NaCl	−0.27 ± 0.07 b	−1.09 ± 0.04 b	0.82 ± 0.01 b
CBL	−0.23 ± 0.02 b	−1.22 ± 0.18 b	1.02 ± 0.00 a
NaCl + CBL	−1.36 ± 0.25 a	−2.02 ± 0.18 a	0.64 ± 0.05 c

3.6. Number of Stomata

As observed in Table 2, the number of stomata per surface area of the leaves increased significantly and highly in plants treated with CBL. Thus, the number of open stomata was also much higher in CBL plants as compared with the control and in the NaCl + CBL-treated plants, as compared with the NaCl treatment alone.

Table 2. Number of stomata, number of open and closed stomata per area in leaf tissue of tomato plants treated with NaCl, CBL, and NaCl + CBL. Values are means ± SE (n = 5). For each treatment, different letters show significant differences according to Duncan's test at $p < 0.05$.

	Mean Stomata in 0.048 mm^2		
Treatments	**Total Stomata**	**Stomata Open**	**Stomata Closed**
Control	13.8 ± 0.60 b	5.5 ± 1.12 bc	8.3 ± 1.23 a
NaCl	11.3 ± 0.49 b	3.8 ± 0.54 c	7.5 ± 0.96 a
CBL	20.5 ± 1.65 a	12.3 ± 1.82 a	8.2 ± 1.19 a
NaCl + CBL	12.2 ± 0.79 b	6.3 ± 0.49 b	5.8 ± 1.01 a

3.7. Mineral Content

The concentration of all mineral nutrients was determined in dried leaf samples. Table 3 only shows those with significant differences. The table shows that Ca was only significantly decreased in the treatments with NaCl. Furthermore, K decreased only in the treatment with NaCl, but no significant differences were found between NaCl-treated and CBL+NaCl-treated plants. The concentration of Mg in leaves decreased with the NaCl and CBL+NaCl treatments, but there were no significant differences with CBL-treated plants. Na strongly increased in the salinity treatments (NaCl and CBL + NaCl) at a similar rate. A decrease in the Cu concentration was observed in the treatments that contained CBL (CBL and CBL + NaCl). However, Fe significantly increased in these latter treatments. An increase in the Mo concentration was observed only in the plants treated with CBL + NaCl, while no significant differences were observed in CBL plants. Zn decreased only in CBL plants.

Table 3. Concentration of macronutrients (mmol kg^{-1} D.W.) and micronutrients (µmol kg^{-1} D.W.) in tomato shoots. Results are expressed as the mean of four biological replicates ± standard error. Columns with different letters differ significantly according to Duncan's test ($p \leq 0.05$).

	Control			NaCl			CBL			NaCl + CBL		
Macronutrients (mmol kg^{-1} D.W.)												
Ca	716.21	±	7.18 a	504.31	±	24.21 b	713.67	±	49.56 a	782.37	±	54.75 a
K	998.13	±	19.43 a	618.93	±	14.27 b	966.92	±	9.78 a	798.44	±	18.25 ab
Mg	94.27	±	5.18 a	70.53	±	2.46 b	97.27	±	9.64 a	63.06	±	7.25 b
Na	8.01	±	1.36 b	580.08	±	9.60 a	4.92	±	0.87 b	800.48	±	93.52 a
Micronutrients (µmol kg^{-1} D.W.)												
Cu	162.60	±	19.09 a	195.96	±	19.53 a	166.25	±	6.66 b	91.33	±	2.10 b
Fe	1574.36	±	66.69 b	1757.73	±	161.54 ab	1881.67	±	61.24 a	1817.24	±	112.41 a
Mo	146.39	±	2.01 b	165.39	±	2.93 ab	149.38	±	3.98 b	174.32	±	5.41 a
Zn	502.53	±	48.68 a	508.02	±	32.87 a	303.14	±	42.03 b	616.33	±	88.56 a

3.8. Phenolic Compounds Analysis

The phenolic compounds (Table 4) showed that CGA-I only increased significantly in the treatments with NaCl (NaCl and CBL + NaCl), while CGA-II and CGA-III increased in all treatments. However, the increase in CGA-III was higher in plants treated with both CBL and CBL + NaCl. A similar response was observed for caffeic acid and rutin, which increased in all treatments, but to a higher extent in the CBL treatments. For the total phenolics concentration, the increase was again, high in all treatments, although this increase was significantly stronger in the CBL treatments.

Table 4. Concentration of phenolic compounds in tomato (mg g^{-1} D.W.). Each value represents the mean of four biological replicates ± SE. Rows with different letters differ significantly according to Duncan's test ($p \leq 0.05$).

Treatment	Cafeoyl Glucaric Acid (CGA)			Caffeic Acid	Rutin	Total
	I	II	III			
Control	0.93 ± 0.008 b	1.61 ± 0.02 b	4 ± 0.03 c	0.62 ± 0.04 c	0.52 ± 0.03 c	7.69 ± 0.12 c
NaCl	1.17 ± 0.05 a	2 ± 0.06 a	5.5 ± 0.15 b	0.8 ± 0.19 b	0.78 ± 0.03 b	10.3 ± 0.35 b
CBL	0.83 ± 0.24 b	2.43 ± 0.13 a	6.1 ± 0.2 ab	1.1 ± 0.03 a	0.85 ± 0.02 a	11.24 ± 0.5 ab
NaCl + CBL	1.15 ± 0.01 a	2.72 ± 0.2 a	6.31 ± 0.15 a	1.05 ± 0.07 a	0.82 ± 0.1 a	12.06 ± 0.26 a

3.9. Gene Expression of Aquaporins

According to the expression of aquaporins (Figure 5), the treatment with NaCl increased the expression of most PIP aquaporins, such as *SlPIP1;3*, *SlPIP1;5*, *SlPIP1;7*, *SlPIP2;4*, *SlPIP2;6*, *SlPIP2;9*, and decreased the expression of the TIPs, *SlTIP1;1*, *SlTIP1;3*, *SlTIP2;1*, and *SlTIP2;2*, as well as all NIPs, *SlSIP1;2*, and both XIPs. The treatment with CBL increased the expression of all 9 PIPs, except for *SlPIP1;7*, and *SlPIP2;4*. The expression of all the TIPs increased except for *SlTIP2;2*, *SlTIP2:3*, and *SlTIP4;1*, which remained unchanged. The expression of all the NIPs increased, except for *SlNIP4;2*; the SIPs remained unchanged with the exception of the decrease in expression of *SlSIP1;2*, and all measured XIPs increased without exception. However, the NaCl + CBL treatment increased the expression of only *SlPIP1;2*, *SlPIP1;3*, *SlPIP2;4*, *SlPIP2;9*, *SlTIP1;2*, *SlTIP2;1*, *SlTIP5.1*, and *SlNIP2;1*. All the others remained unchanged. When comparing NaCl treatments, CBL increased the PIPs: *SlPIP1;2*, *SlPIP2;8*, and *SlPIP2;12*. From the rest of the groups, *SlTIP1;1*, *SlTIP2;1*, *SlTIP2;2*, and *SlNIP2;1* were upregulated.

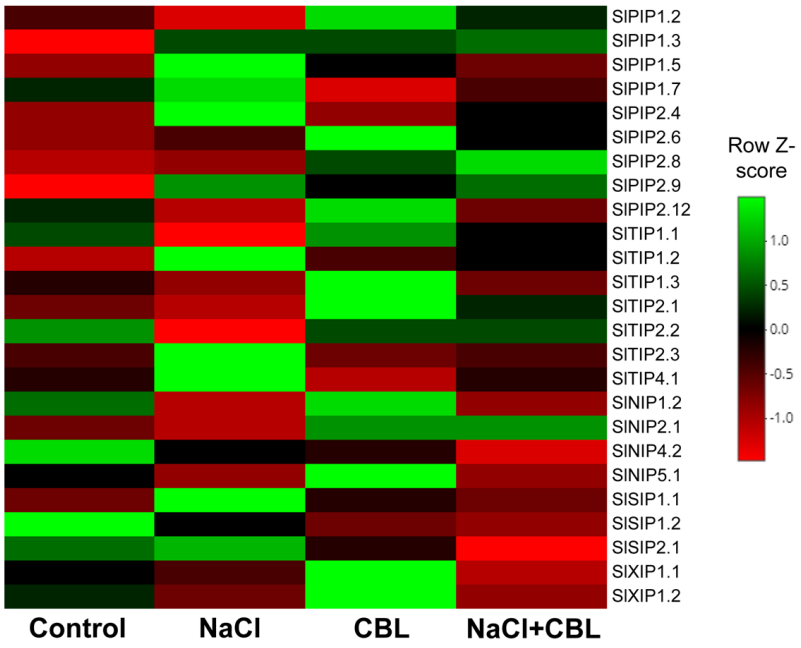

Figure 5. Z-score-based heatmap showing the differential gene expressions of all the aquaporin isoforms analyzed in tomato for each treatment. Each cell represents the mean of three middle leaves mixed from five different plants. The color spectrum indicates the intensity associated with the normalized expression values for each row independently, from a lower expression (red) to a higher expression (green). Individual expression in supplemental material (Figures S2–S7).

4. Discussion

The synthesis of phenolics has been proven to be related to plant growth, with a positive correlation observed between the synthesis of phenolics and the seed vigor response [25]. In addition, exogenously applied phenolics were able to stimulate the synthesis of endogenous phenolics in plants, inducing germination and growth [21,26]. In our results, we observed a significant increase in plant growth with the application of CBL. However, under the salinity treatment, no effects were observed, although it has been shown that under abiotic stress, the application of external individual phenolics increased growth under nutritional stress [21]. The lack of an effect in our plants could be related to the level of salinity, the time of application, or the stage of development, which are very relevant for the specific response of tomato plants [27].

The decrease in the RWC in salinity-treated plants has been associated with the accumulation of toxic ions, such as Na^+ and Cl^-, and/or to the accumulation of the synthesized organic compounds necessary to achieve a proper osmotic adjustment for maintaining water uptake and transport [28]. In this way, the reduction in the RWC in CBL plants could be a consequence of secondary metabolism activation. However, in this case of salinity, the decrease in the growth rate did not occur along with the putative metabolic changes reported as a response to salinity [29]. Moreover, the RWC in tolerant cultivars has been reported to be less influenced as compared with sensitive ones [30], as observed in the NaCl + CBL-treated tomato plants as compared to NaCl-treated ones, pointing to an enhanced tolerance with CBL application. In this way, the associated parameters, such as potentials (Ψ_w, Ψ_π, Ψ_p), were maintained under salinity stress, indicating a well-adapted system in this long-term stressful condition, which was able to maintain the water flux. The potentials showed only significant changes in the NaCl + CBL treatment, with a strong decrease in Ψ_w and Ψ_π, and a higher Ψ_p inside cells, which point to better water movement via the symplastic pathway in these leaves. In a similar manner, although the CBL-treated plants also showed lower values of Ψ_π than the control, the differences were not significant. However, the turgor was higher in the plants treated with CBL, indicating that, not only

were the synthesis or accumulation of solutes involved, but also that the water transport through cells increased by CBL.

The measurement of the chlorophyll fluorescence provides detailed information on the state of the photosystem II (PSII) by determining the quantum efficiency of photochemistry and heat dissipation. This is related to CO_2 assimilation (Pn), as it provides energy for CO_2 assimilation. In the plants treated with CBL, this correlation was direct, as both the chlorophyll fluorescence and the Pn were higher than the control. However, in the plants treated with CBL + NaCl, higher values of chlorophyll fluorescence were found without a significant decrease in the Pn. This result can only be explained by the number of stomata (total and open), as a higher number of stomata were found in the CBL-treated plants. Thus, given that the environmental CO_2 in the crop chamber is constant, the higher CO_2 content in the intercellular spaces (c_i) could only be the result of a higher stomata opening or an increased stomata number. It has been reported that polyphenolic compounds extracted from spruce bark increased the photosynthetic rate and biosynthesis of assimilation pigments (chlorophyll a/b) [31], and in addition, phenolics have been reported to change the composition of thylakoids and mitochondrial membranes, resulting in a decrease in the energy needed for ion transfer [32]. Nevertheless, when a stress is present, such as nutritional stress, the production of phenolics such as *p*-coumarin increases, improving photosynthesis by reducing oxidative damage [33]. Thus, the effects of CBL under the NaCl + CBL treatment could be observed as an increase in the Pn, but it could also have an indirect effect due to the protection conferred by the reduced ROS, membrane integrity, better ion transfer, enhanced stomata number and aperture, and the observed increase in chlorophyll content.

Stomatal conductance (Gs) is a function of stomatal density, aperture, and size. However, in recent years, Gs has been associated with the water flow through leaf cell membranes, involving aquaporins. In our results, the stomatal conductance was lower in plants treated with salinity (independently of CBL application). In previous works, no significant differences were observed in the Pn or Gs in tomato and watermelon plants [34]. Furthermore, in other studies, a non-stomatal inhibition of photosynthesis was observed for several species grown under salinity, related to the high salt tolerance [35,36]. However, the time of salinity application is relevant for tomato [27]. Thus, although 60 mM could be outside of the tolerance range, the plants did not show a reduction in growth as the application was done from the start of the experiments (5-day-old plants). However, at the end (17-day-old plants), they started to show reductions in the Pn and Gs, but not in the chlorophyll fluorescence or Fv/Fn. Accordingly, this must be associated with the water and CO_2 transport through membranes, and not to the stomata or photosystems. Nevertheless, there was a significant increase in all the parameters related with gas exchange and photosynthesis in the plants treated with CBL. As it can be observed in Table 1, the number of stomata per surface area of leaves increased significantly in plants treated with CBL. Thus, the number of open stomata was also much higher in CBL plants as compared with the control, and in NaCl + CBL compared with NaCl. This indicates that CBL provided a higher number of stomata in standard conditions and a higher number of open stomata in salinity conditions. These results provide an explanation of why the concentration of carbon dioxide in the intercellular spaces of a leaf (Ci) increased, resulting in a higher CO_2 assimilation for photosynthesis leading to enhanced growth, and ultimately, a greater CO_2 fixation by the plant. In other works, in barley Yan66, a metal-tolerant genotype, the flavonoid biosynthesis was much more strongly enhanced and, accordingly, more free flavonoid biomolecules (naringin, narirutin, and neohesperidin) were found than in the sensitive species. This was associated with a higher photosynthesis rate. Moreover, in *Arabidopsis*, it was demonstrated that flavonoids accumulate mainly in the vacuoles of epidermal cells [37]. Later, [38] it was demonstrated that molecules belonging to the flavonols sub-group accumulated specifically in guard cells and acted as ROS scavengers in these cells. In this way, flavonol accumulation in guard cells was described to be involved in the inhibitory effect of ABA-induced H_2O_2 accumulation and stomatal closure [7]. Increased atmospheric CO_2 and gamma irradiation

have a significant impact on the plant's photosynthetic apparatus and organic compound production. In a study carried out by Moghaddam et al. [39], they evaluated the effect of elevated CO_2 on the photosynthetic efficiency and production of defensive secondary metabolites (flavonoids) induced by gamma irradiation in *Centella asiatica*. They concluded that naringin, among others, increased carbon availability for the photosynthetic pathway.

The concentration of mineral nutrients suggested that CBL does not alter the uptake or transport of nutrients to leaves. The application of salinity decreased the concentration of some nutrients, due to the competitive effect of Na and Cl with the other nutrients. However, the CBL applied after the salinity application did not show any significant effect. It has been reported that phenolic compounds applied to soil could increase nutrient absorption, as they were able to chelate metallic ions, thus improving their mobility and uptake [40]. Similarly, phenolics in wheat root exudates improve Ca, N, and Zn nutrition, by increasing both mobilization and absorption [41]. However, the mechanism of the effect of the application of phenolics to the plant leaves on plant nutrition has not been investigated. In our results, no significant differences were observed in the concentration of macronutrients in the CBL-treated plants. In salinity conditions, the reductions in Ca, K, and Mg, were observed as a typical response to salinity, as compared to the control. However, plants treated with CBL + NaCl only showed significant reductions in Mg, while Ca and K were unchanged, although Na also increased, which is not a normal effect given the competition between ions in their uptake by the plants. In fact, Ca, K, and Mg have been reported to decrease in plants under salinity due to competition with Na [42]. Interestingly, the Ca content increased in NaCl-treated plants when CBL was applied. Given its essential role in preserving the structural and functional integrity of the plant's plasma membrane, it is important to determine the concentration of Ca under salinity conditions. NaCl stress has been reported to reduce Ca availability and mobility within the plant [43], resulting in reductions in Ca concentration in tissues [44]. Thus, tomato plants decreased the concentrations of Ca in leaves by modifying the concentrations of ions in the apoplast [45]. The application of CBL to NaCl-treated plants restored Ca to normal values in leaves, pointing to a better mobilization. Potassium has been pointed as a major factor of resistance to salinity stress [46], since a correlation between the ability to retain K and overall plant salt tolerance has been demonstrated. However, in our experiments, K concentration decreased in NaCl-treated tomato plants that showed no reductions in growth. Therefore, in such a multigenic response under salinity, parameters other than K should be considered. According to the correlation between salinity and Mg concentrations in tomato plants, it was reported that plant water relations were a key aspect that affected Ca-Mg and K-Na interactions [42]. In the NaCl + CBL treatment, the observed increase in Ca concentration could explain the decrease in Mg concentration. According to the micronutrients that changed in concentration with the treatments, we found some alterations with respect to control, with an increased Fe concentration in the CBL treatments. Fe is closely associated with photosynthesis and chlorophyll synthesis, as it is directly related to the enhanced chlorophyll concentration and the fluorescence of the photosystem II of the leaves. In addition to Fe, Mo concentration also increased in NaCl + CBL plants, what could be related to the physiological response in terms of the enhanced mobility of nutrients related to better membrane integrity and transport, even if a higher growth rate was not detected.

The interest in biostimulation with algae-based compounds has greatly increased due to their ability to increase the phenolic content in plants [47,48]. However, little is known about the performance of other source materials, such as citrus flavonols and phenolic compounds, in the accumulation of biomolecules. In our work, the analysis of biostimulated tomato leaves reported an increase in the total phenolic content by 46% when CBL was applied, and an increase by 57% when combined with salinity stress. Nevertheless, the mechanisms underlying this effect remain unknown. One possible explanation is that perhaps certain phenolic acids, such as p-coumaric and cinnamic acids, acted as biostimulant components, as they are precursors of salicylic acid [49], with their

conversion occurring inside the plant. Thus, this increase in salicylic acid may have also increased the production of the tomato plants polyphenols. Furthermore, both flavonoid (rutin) and phenolic acids increased in concentration, revealing that the action of the CBL affected diverse biosynthesis pathways. In the work by Abd-Elkader et al. [50], zucchini plants were biostimulated with diverse plant-derived extracts, showing an increase in total phenolic acid and flavonoids, suggesting that a positive regulation feedback in the production of these biomolecules may have occurred.

In our results, the regulation of water flux and stomatal function was highly related with the expression of aquaporin genes. In the salinity treatments, even if a reduction in different gas exchange parameters were observed, photosynthesis and growth were not affected. Salinity by itself enhanced the expression of many PIP aquaporin isoforms, which are involved in cellular homeostasis by maintaining water potential and thus allowing plants growth. Furthermore, a general downregulation of TIP and NIP aquaporins was observed, which could be related to the reduction in RWC and the effect of increased toxic ions accumulation, which affect the transport of nutrients. According to salinity stress studies, it has been previously reported that the exogenous application of phenolic compounds, such as vanillic acid, reduced osmotic and ionic toxicity in salinity stressed-seedlings, by enhancing the RWC and reducing membrane damage, as the Na^+/K^+ ratio was reduced [23]. However, in our plants, the lack of an effect on the RWC and the growth in salinity-treated plants, made it difficult finding an effect of these phenolic compounds. Nonetheless, the results were correlated with aquaporin gene expression, when CBL was applied in addition to salinity. Many of the PIP aquaporins that were upregulated under salinity returned to normal values when CBL was applied. It is well known that salinity stress generates stress signals such as ABA, which lead to the production of ROS and specifically H_2O_2 as a secondary signal, with an effect on the regulation of AQP expression and activity [51]. However, flavonoids reduced stress signals such as ABA, promoting stomatal opening and reducing ROS concentrations, thus protecting membranes from damage [7]. Thus, it could be understood that the regulation of aquaporins expression under salinity could be counteracted by the effects of flavonoids. In addition, new, non-affected isoforms under salinity stress showed an increased expression with CBL application, such as *SlPIP1;2*; *SlPIP2;8*, and *SlPIP2;12*. This enhancement could be related to Ca increase, as Ca has been described to upregulate aquaporin functions [52]. As described above, one of the main effects of CBL in salinity-stressed plants could be related to increased water movements via the symplastic pathway, and thus, the expression of these aquaporins was perhaps related to the decrease in water potential but a high turgor pressure.

The CBL experiment results showed that the regulation of stomatal function was highly related with aquaporins expression, as most of the PIPs, except for *SlPIP1;7* and *SlPIP2;4*, increased in expression. This increase in expression has been previously reported to aid the movement of water and solutes [53]. In this way, the increase in growth of CBL-treated plants must be coupled with the higher stomata number and opening, and the higher water and CO_2 permeability. Thus, the involvement of PIP aquaporins in CO_2 transport has been directly associated with higher CO_2 diffusion and assimilation, increased stomatal conductance, and even higher stomatal density and sensitivity [54,55], ultimately promoting photosynthetic efficiency and growth. In addition, a correlation between TIPs such as SlTIP2;1 and Gs has been reported in *Vitis vinifera*, although the PIPs were more relevant to plasma membrane water flow [56]. However, as the tonoplast is very important to cell water flow, the authors reported a close interrelation between the regulation of the expression of this AQP, and stomatal control. It has also been reported that the exogenous application of the phenolic compound salicylic acid modulated the water transport in maize tissues via the fine regulation of aquaporins ZmSlPIP2:4 or ZmSlTIP1;1 [57]. In our results with CBL, all the either TIPs increased or maintained their expression levels. Therefore, along with determining the individual function of each aquaporin, these effects should be connected with gas and water exchange, as a reduction was also observed in aquaporins expression in NaCl-treated plants.

Finally, it should be noted that treatments with CBL increased the expression of one specific NIP isoform (*SlNIP2;1*) that has been directly related with metalloids transport and specifically to silicon uptake. In that sense, silicon has a double effect on plant physiology, as it has been associated with stomatal opening, and it also collaborates with ROS scavenging [58]. Both responses were promoted by the CBL treatments, thereby opening the door to future studies in this field.

5. Conclusions

Due to climate change, there is currently a pressing need to develop sustainable crop practices for agriculture. Therefore, studies on bio-based products such as biostimulants are of great interest. The phenolic compounds present in CBL have been shown to stimulate plant growth by improving CO_2 and water exchange in tomato leaves (photosynthesis and transpiration). This water and CO_2 exchange in cells was correlated with aquaporins that could trigger morphological changes such as the increase in the number of stomata on the surface of leaves. They also showed a sensitive response to phenolic application, which stimulated the tomato plants' own phenolic synthesis pathways. In addition, our plants showed a positive effect of CBL under salinity conditions in parameters such as chlorophyll concentration, fluorescence of photosystem II, Ci, nutrient uptake, and some PIP aquaporin expression. However, the fact that no effect on growth was observed deserves deep investigation. In this way, the timing for CBL and stress application, together with the specific levels of salinity and concentration of CBL, needs to be elucidated. In this way, the external application of phenolics and their effect as biostimulants opens a new line of research that deserves attention due to their modulation of the expression of aquaporin genes, which increase water and CO_2 transport, thereby improving photosynthesis and growth.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12123230/s1, Figure S1: Experimental design and growth stages of the tomato plant; Figure S2: Comparison of the growth of whole tomato plants under the different growth conditions; Figure S3: Relative expression of PIPs isoforms in tomato plants; Figure S4: Relative expression of TIPs isoforms in tomato plants; Figure S5: Relative expression of NIPs isoforms in tomato plants; Figure S6: Relative expression of SIPs isoforms in tomato plants; Figure S7: Relative expression of XIPs isoforms in tomato plants.

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