







## Article

# Effects of Different Microbial Inocula on Tomato Tolerance to Water Deficit

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**Abstract:** Several recent reports have highlighted some of the mechanisms involved in the enhanced tolerance to abiotic stresses induced by root-associated microorganisms, although additional efforts are still required to exploit and optimize these strategies. Particularly, arbuscular mycorrhizal fungi (AMF) play an important role as “bio-fertilizing microorganisms”, establishing mutualistic symbioses with the roots of most crops. In this work, different microbial inocula (a single AMF species, a combination of three different AMF species, a combination of two plant growth-promoting bacteria (PGPB) strains and a more complex commercial inoculum) have been used to inoculate tomato plants (cv San Marzano nano), in order to verify their effects on the tolerance to a water deficit condition in pots, through the evaluation of biochemical stress markers and hormonal profiles (ABA and IAA). Results showed differences among tomato responses to water limitation depending on microbial inocula, confirming the importance to characterize the optimal plant/microorganism genotype combination(s) to maximize plant performance and tolerance. These findings open new perspectives for a better exploitation of these microorganisms.

**Keywords:** tomato; AM fungi; PGPB; water deficit; biostimulant

## 1. Introduction

Environmental stresses are becoming a serious threat and productivity is declining at an unprecedented level [1]. Water deficit, salinity, extreme temperatures, flooding, nutritional limitations, pest and pathogen attacks are key threats to plant growth and crop productivity and constitute major constraints to actual agriculture [2]. The extent of agricultural soil affected by water stress and exposed to a loss of fertility is predicted to progressively increase due to climate change [3]. Conventional agriculture’s dependence on chemical fertilizers and pesticides has encouraged the thriving of industries producing these products that are not only hazardous for human consumption but also exert negative effects on the environment [1]. Biofertilizers, and biostimulants, could help to solve the problem of feeding an increasing global population at a time where agriculture faces several environmental stresses [1]. A number of recent reports have highlighted some of

the mechanisms involved in the enhanced tolerance to abiotic stresses induced by root-associated microorganisms [4–6]; however, additional efforts are still required to exploit the useful aspects of the different root-associated microorganisms and to optimize these strategies, supporting their application to current agricultural practices [1]. Arbuscular mycorrhizal (AM) fungi play an important role as “bio-fertilizing microorganisms” as they establish mutualistic symbioses with the roots of most crops [7,8]. These symbiotic fungi colonize plant roots and enhance the uptake of water and nutrients by the host plants, while, they receive the carbon compounds. These fungi are considered essential elements for plant nutrition, mainly in low-nutrient conditions, as their hyphae can extend for many meters in the ground, helping the plants to acquire mineral soil nutrients. Since AM fungi play an instrumental role in protecting the plants against abiotic stresses such as nutrient deficiency, extreme temperatures [9] and drought [10–14], they can benefit their hosts in both wild and agricultural environments [15]. Consequently, AM fungi are thought to have a great impact in natural environments [16–18], as in managed conditions in agriculture, horticulture and forestry [8]. Although there is no hard symbiont specificity in AM interactions, the efficiency of these associations depends on the interacting partner genotypes and the environmental conditions [19,20]. Recent findings suggest a certain degree of functional specialization in AM interactions [8]. Some plant/fungus genotype combinations are more efficient than others in terms of nutrition or stress tolerance/resistance [13,14,20–25]. Despite the low host specificity of AM under controlled conditions, the presence of several symbionts might result in the most effective mutualistic combination [8]. Berruti and colleagues [26] demonstrated that the AM fungal communities originating from cells containing the arbuscules, which represent the functional structures in AM symbiosis, and the whole root samples of *Camellia* plants (grown in natural soil) differed remarkably. These results suggested that not all the AM fungal isolates present in soil could form a functional symbiosis. Symbiotic fungi, however, are only part of the soil and root-associated microbiota. Plant growth-promoting bacteria (PGPB), genera like *Bacillus*, *Azospirillum* or *Pseudomonas*, also exert beneficial effects on plant metabolism and primes tolerance mechanisms against biotic and abiotic stresses [6]. Interestingly, it was recently demonstrated that grapevine roots differently respond to a pure AM inoculum with respect to a mixed inoculum containing different microbial isolates/strains [27]. Here, we have used different microbial inocula on the commercial tomato cv San Marzano nano to verify the impact on the tolerance to a water deficit condition. One of the most important challenges in this research area is to dissect the actual mechanism of mode of action for different strains/isolates to evaluate their efficacy, alone or in combination, towards its use in sustainable agriculture.

## 2. Materials and Methods

### 2.1. Inoculation of Tomato Plants and Growth Conditions

Tomato (*Solanum lycopersicum* ‘San Marzano nano’) seeds were surface sterilized in sodium hypochlorite for 20 min, washed five times in sterile water, and germinated on wet filter paper. For this pot experiment, pots (10 cm × 10 cm × 12 cm) with a volume of 1 L containing substrate (sterilized quartz sand) were arranged on a growth chamber in a randomized block design including five treatments: (i) non-inoculated control (CTRL); (ii) AM fungi mono fungal inoculum (Myc\_Rhizo); (iii) AM fungi multi fungal inoculum (MULTISTRAIN); (iv) PGPB (LC3.5 + 5.2) and (v) a mixed commercial inoculum containing both bacteria and fungi (Commercial MIX). Each treatment was replicated ten times (10 pots), each pot contained one seedling.

Tomatoes were inoculated with AM fungi at planting time by placing the inoculum in the planting hole and in contact with the roots, as follows: 10 plants were inoculated with 15 g/pot of a mono fungal inoculum (Myc\_Rhizo) based on *Rhizoglossum irregulare* BEG140 and 10 plants with 15g/pot of multi fungal inoculum (MULTISTRAIN) constituted by *Claroideoglossum claroideum* BEG96, *Funneliformis caledonium* BEG97 and *F. geosporum* BEG199; both the pure AM fungi inocula were provided by Symbiom Ltd., (Lanškroun, Czech Republic). The commercial MIX Opera-Rizon (MsBiotech, Larino, Italy) was

used to inoculate other 10 tomato plants. This formulate, as reported in the product label, consists of AM fungal species (*Glomus* spp. 0.001%) and rhizospheric bacteria ( $1 \times 10^2$  Colony Forming Unit CFU). In addition, two PGPB (LC3.5 and LC5.2) were selected based on their high levels production of auxin: 46  $\mu\text{g/mL}$  and 24  $\mu\text{g/mL}$  for LC3.5 and LC5.2 respectively (Gritli and Bacem unpublished results). Both bacterial strains were isolated from roots of *Lathyrus cicera* in the northern of Tunisia. They were prepared as following: pure colonies of PGPB strains were multiplied separately in Luria Bertani (LB) broth by incubating them in a shaker for 72 and 24 h respectively at 27 °C. The optical density was adjusted to 1 (at 660 nm for PGPR. One mL of the bacterial suspension ( $10^9$  CFU/mL) of the two PGPRs (LC3.5 + LC5.2) was inoculated to 10 pots, where 15 g/pot of the carrier material (without AM fungi) was applied.

Ten plants were left as non-inoculated control plants: in these pots 15 g of carrier material (without AM fungi) was applied. Plants were grown in controlled conditions, with a temperature of 23 °C/21 °C day/night, 16/8-h light/dark photoperiod, and relative humidity 65%. From transplanting to the beginning of the water deficit experiment (after about 6–7 weeks), all the plants were watered twice per week with tap water and, once per week, with a modified Long-Ashton nutrient solution [28] containing 3.2  $\mu\text{M}$  inorganic phosphate.

Out of 50 plants, 25 (five plants for each treatment) were used as controls (irrigated or non-stressed, NS) and maintained in a well-watered state (at container capacity). The remaining 25 plants (five plants for each treatment) were subjected to a water stress (WS) treatment. To this aim, about 6 weeks after fungal inoculation irrigation was withheld and the experiment was stopped when the first plants reached a stress level (measured by infrared gas analyzer, IRGA, ADC-LCPro+ system; Analytical Development Company Ltd, Hoddesdon, UK).

## 2.2. Miniprep Bacterial Strains DNA Isolation and 16S SSU rRNA Amplification

The two PGPB strains were subjected to molecular characterization by means of amplification with conventional PCR using DNAs isolated from bacterial strains LC3.5 and LC5.2 as a template. An almost complete small subunit (SSU) bacterial ribosomal RNA gene (16S) was amplified with bacterial universal primers 27F-1492R that amplified a fragment of about 1465 bp [29]. The PCR were carried out in a final volume of 25  $\mu\text{L}$  containing 10  $\mu\text{L}$  of Platinum Hot Start PCR Master Mix (2X), 0.5  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ), template DNA (1  $\mu\text{L}$ ) and 13  $\mu\text{L}$  of PCR-grade water. Bacterial PCR amplification was performed using a T3000 thermal cycler (Biometra, Göttingen, Germany) with the following profile: initial denaturation for 5 min at 95 °C; 35 cycles of denaturation (60 s at 94 °C), annealing (60 s at 58 °C) and extension (60 s at 72 °C) and a further 7 min at 72 °C. All the PCR products were checked using 1.5% (*w/v*) agarose gel stained with ethidium bromide (Merck KGaA, Darmstadt, Germany). The two PCR products replicates for each strain were pooled and purified using Wizard SV Gel and a PCR Clean-Up System kit (Promega, Madison, WI, USA). Purified PCR products were sequenced, using either the universal primer 27F or 1492R, by LMU sequencing services (Munich, Germany). The two sequences were deposited at NCBI (accession # MN879506 and MN879507 for LC3.5 and LC5.2, respectively).

## 2.3. Eco-Physiological Parameters

Measurements of transpiration rate ( $E$ ), stomatal conductance ( $g_s$ ) and net photosynthetic rate ( $A_N$ ) were performed on adult, non-senescent leaves at the same physiological age (in the middle part of the plant, considering the third to fourth leaf from the shoot apex). Intrinsic water use efficiency ( $iWUE$ ) was calculated as the ratio between  $A_N$  and  $g_s$ . Measurements were taken with an IRGA instrument. During measurements, light intensity in the leaf chamber was set at 1200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ , temperature was 25 °C, and the concentration of  $\text{CO}_2$  was maintained between 450 and 470 ppm. Measurements were taken between 10:00. and 13:00. The chlorophyll content index (CCI) was determined at the end of the experiment (about 9 DAT) using the portable chlorophyll meter SPAD 502 (CCM-200; Opti-Sciences, Hudson, NH, USA).

#### 2.4. Assessment of Symbiosis Development

At the end of the experiments, plants were harvested, and plant height and fresh weight (not shown) were recorded. A part of the root apparatus of at least three plants (up to five depending on the treatment) was stained with 0.1% Cotton Blue in lactic acid. For each plant, sixty randomly chosen 1-cm-long root segments were mounted on slides and fungal colonization was quantified according with the Trouvelot system [30] using MYCOCALC software, while the remaining root systems were stored at  $-80^{\circ}\text{C}$  until further analyses.

#### 2.5. Preparation of Extracts and Biochemical Parameter Evaluation

After the measurement of plant morphological parameters, leaf and root samples were dried by lyophilization, then, grounded and homogenized using a mortar and pestle. About 30 mg of the obtained dried powders were extracted with 90% (*v/v*) methanol using a 1:50 (*w/v*) ratio. Samples were mixed by vortexing for 5 min, and sonicated for 15 min at  $8^{\circ}\text{C}$ . Following a centrifugation step (10 min at 8000g,  $4^{\circ}\text{C}$ ), the supernatants were filtered using a filter tips, and directly used for chemical determinations.

##### 2.5.1. Determination of Total Chlorophyll Content (TCC)

The leaf extracts were employed for the determination of the total chlorophyll content (TCC), according to Lichtenthaler and Buschmann [31]. Briefly, 1 mL of appropriate diluted sample was subjected to spectrophotometric measurements at 665.2 nm and 652.4 nm. TCC, expressed as  $\mu\text{g}$  per g of dry weight (d.wt), was calculated for each sample using the following equation:

$$\text{TCC} = \frac{[(16.82 \times \text{Abs}_{665.2} - 9.28 \times \text{Abs}_{652.4}) + (36.92 \times \text{Abs}_{652.4} - 16.54 \times \text{Abs}_{665.2})] \times V_{\text{extr}} \times \text{DF}}{\text{WH}} \quad (1)$$

$V_{\text{extr}}$  = volume, expressed as mL, used for the extraction process;  $\text{DF}$  = dilution factor and  $\text{WH}$  = weight of each sample expressed in grams.

##### 2.5.2. Determination of Proline Concentration (TpC)

The proline concentration (TpC) was determined according to Carillo and Gibon [32]. Briefly, 500  $\mu\text{L}$  of undiluted samples were incubated with 1 mL of the reaction mix containing 1% (*w/v*) ninhydrin solubilized in 60% (*v/v*) acetic acid and 20% (*v/v*) ethanol. The mixture was incubated at  $95^{\circ}\text{C}$  for 20 min in the dark, and then centrifuged at 10.000 rpm for 1 min at room temperature in a table microfuge. The absorbance was then measured at 520 nm. Quantification was performed using an external calibration curve prepared using a pure standard of proline, whose concentration ranged from 0.01 to 1.00 mmol.

##### 2.5.3. Determination of Total Polyphenol Content (TPC)

The total polyphenol content (TPC) was evaluated both in leaf and root extracts following the method of Ainsworth and Gillespie [33]. Briefly, each sample was appropriately diluted in 90% (*v/v*) methanol and then 930  $\mu\text{L}$  were incubated with 30  $\mu\text{L}$  of Folin–Ciocalteu reagent and 40  $\mu\text{L}$  of 20% (*w/v*) sodium carbonate ( $\text{Na}_2\text{CO}_3$ ). The samples were then incubated for 1 min at  $80^{\circ}\text{C}$  and for 20 min at room temperature in the dark. Then the absorbance was monitored at 725 nm. An external calibration curve using gallic acid (GA), ranged between 50 and 400 mmol, was employed to quantify TPC in the samples. The results were expressed as  $\mu\text{mol}$  of gallic acid equivalent (GAE) per g of dry weight (d.wt).

#### 2.6. Determination of Absciscic Acid (ABA) and Indole-3-Acetic Acid (IAA) Content

About 500 mg of homogenized leaf and root samples were freeze-dried and transferred with 1 mL of methanol:water (8:2 *v/v*) acidified with 0.1% (*v/v*) of acetic acid in an ultrasonic bath for 1 h. Samples were centrifuged for 10 min at  $4^{\circ}\text{C}$  and 15,000 rpm, and the supernatant was analyzed by

high-performance liquid chromatography (HPLC, Agilent, Waldbronn, Germany). Original standards of abscisic acid (ABA, purity  $\geq 98.5\%$ , Merck KGaA, Darmstadt, Germany) and indole acetic acid (IAA, purity  $\geq 99\%$ , Merck KGaA, Darmstadt, Germany) were used for the identification by comparing retention time and UV spectra. The quantification was made by external calibration method. The HPLC apparatus was an Agilent 1220 Infinity LC system (Agilent R, Waldbronn, Germany) model G4290B equipped with gradient pump, auto-sampler and column oven set at  $30\text{ }^{\circ}\text{C}$ . A 170 Diode Array Detector (Gilson, Middleton, WI, USA) set at 265 nm was used as detector. A Nucleodur C18 analytical column ( $250\text{ mm} \times 4.6\text{ mm i.d.}$ ,  $5\text{ }\mu\text{m}$ , Macherey Nagel) was used. The mobile phases consisted in water acidified with formic acid  $0.1\%$  (A) and acetonitrile (B), at a flow rate of  $0.500\text{ mL min}^{-1}$  in gradient mode, 0–6 min: 30% of B, 6–16 min: from 30% to 100% B and 16–21 min: 100% B;  $20\text{ }\mu\text{L}$  was injected for each sample.

### 2.7. Statistical Analysis

All measurements are the average of five different biological replicates. Each biological replicate was analyzed three times in each experiment. The content of chlorophylls (TCC), proline (TpC), polyphenols (TPC), ABA and IAA were reported both as relative content (Figures 1–6) and as absolute content (Supplementary Tables S1–S4). The relative content was calculated comparing the content observed in inoculated plants (treated with MULTISTRAIN, Myc\_Rhizo, LC3.5 + 5.2 or the Commercial MIX) with the content of unstressed and/or untreated plants (control plants). In both cases, data are expressed as mean values  $\pm$  standard deviation (SD). Significant differences were evaluated by performing one-way ANOVA followed by Tukey's HSD test ( $p \leq 0.05$ ) or  $t$ -test ( $p \leq 0.05$ ) using SPSS ver. 24 software.

## 3. Results and Discussion

The effect of several microbial inocula on tomato tolerance to a water deficit condition was verified. The beneficial effects of root-associated microbes (i.e., AM fungi and PGPB) on plant growth and performance under water limitation have already been reported for several plant species [5,34], including the tomato genotype considered in the present research [13,14,35]. These previous works already showed a different plant response to a water deficit condition depending on the AM fungal species associated to plant roots. An untargeted metabolomic analysis in tomato roots colonized by three AM fungi of different genera showed that some responses to drought and salt stress were common to all AM fungi tested, while others were specifically related to single isolates [25]. Here, several microbial inocula (a single AM fungal species, a combination of three different AM fungal species, a combination of two PGPB strains and a more complex commercial inoculum) were tested for the effect on tomato tolerance to water limitation. Both the bacterial strains used in this work (LC3.5 and LC5.2) showed sequence identity with *Bacillus* spp. In detail, a sequence identity with *Bacillus subtilis* (first hit: MN704441.1, query cover 100%,  $e$ -value 0.0, identity 99.78) and *B. megaterium* (first hit: MK791705.1, query cover 100%,  $e$ -value 0.0, identity 98.64%) was found for LC3.5 and LC5.2, respectively. The AM fungal colonization using several formulates was also evaluated, showing some relevant differences among the two AM fungal inocula (Myc\_Rhizo and MULTISTRAIN), while the presence of AM fungal structures were not observed in the roots inoculated with the mixed inoculum (Commercial MIX; Table 1). A very low colonization by AM fungi was already observed using a commercial mixed inoculum on grapevine rootstocks [27]. In detail, a different grapevine root transcriptome profile was observed after inoculation with a pure AM inoculum (*Funneliformis mosseae*) and the mixed one, although this last elicited an important transcriptional regulation probably due to the predominantly presence of PGPB.



**Table 1.** AM fungal colonization using three different inocula. F%, Frequency of mycorrhization in root system; M%, Intensity of mycorrhizal colonization in the root system; a%, arbuscule abundance in mycorrhizal parts of root fragments; A%, arbuscule abundance in the whole root system. Values are expressed as a mean  $\pm$  SD ( $n = 3$ ). Data were subjected to statistical analysis using SYSTAT 10 software, applying the nonparametric Kruskal-Wallis test adopting a probability level of  $p < 0.05$ . Data followed by different superscript letters indicate significant statistical differences among samples.

	F%	M%	a%	A%
Myc_Rhizo NS	33.33 $\pm$ 10.27 <sup>a</sup>	2.69 $\pm$ 1.16 <sup>a</sup>	68.09 $\pm$ 10.84 <sup>a</sup>	1.75 $\pm$ 0.46 <sup>a</sup>
Myc_Rhizo WS	23.67 $\pm$ 12.21 <sup>a</sup>	2.59 $\pm$ 2.38 <sup>a,b</sup>	68.53 $\pm$ 26.69 <sup>a</sup>	1.4 $\pm$ 0.88 <sup>a,b</sup>
MULTISTRAIN NS	28.02 $\pm$ 8.02 <sup>a</sup>	4.23 $\pm$ 2.62 <sup>b</sup>	41.13 $\pm$ 27.38 <sup>a</sup>	1.39 $\pm$ 1.33 <sup>b</sup>
MULTISTRAIN WS	35.96 $\pm$ 5.35 <sup>a</sup>	4.28 $\pm$ 2.74 <sup>b</sup>	52.3 $\pm$ 29.09 <sup>a</sup>	2.24 $\pm$ 2.11 <sup>b</sup>
Commercial MIX NS	0 $\pm$ 0 <sup>b</sup>	0 $\pm$ 0 <sup>c</sup>	0 $\pm$ 0 <sup>b</sup>	0 $\pm$ 0 <sup>c</sup>
Commercial MIX WS	0 $\pm$ 0 <sup>b</sup>	0 $\pm$ 0 <sup>c</sup>	0 $\pm$ 0 <sup>b</sup>	0 $\pm$ 0 <sup>c</sup>

### 3.1. Impact of Treatments and Water Stress on Eco-Physiological Parameters

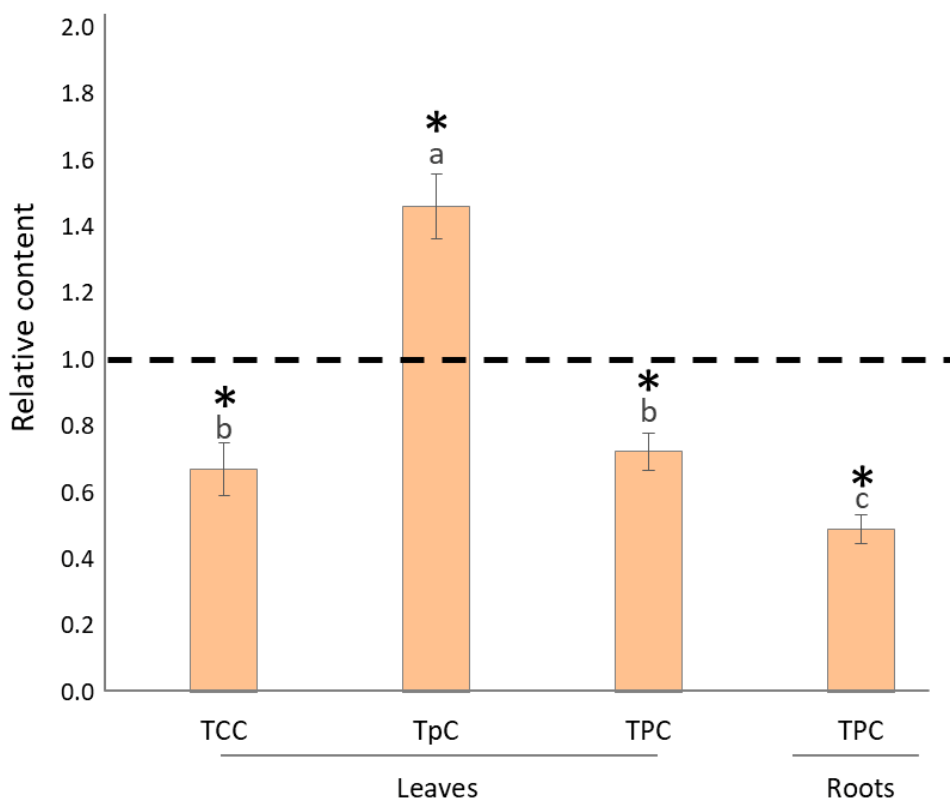
Eco-physiological parameters were recorded at the end of the experiment, considering both gas exchanges and CCI (Table 2 and Table S1). In general, assimilation rates (A) decreased under water deficit condition with the lowest values in the treatment with bacteria (LC3.5 + 5.2 and commercial mix), while no effect was observed on *e*-values. Brilli et al. [34] already found that the tomato inoculation with a PGPR (*Pseudomonas chlororaphis*) did not affect the physiological parameters. In addition, the highest “A” values under water stress for the AM-inoculated plants are in agreement with the data from Chitarra et al. [13] and Volpe et al. [14] on the same tomato genotype, although a difference between two AM fungal species was observed. As expected, stomatal conductance ( $g_s$ ) decreased under water limitation mainly in inoculated plants (Figure 2). This is not surprising since a different timing in reaching a stress level has been already reported from AM-colonized and non-colonized tomato plants [13]. Similarly, although not statistically significantly different, a decrease in  $g_s$  was observed in the presence of bacteria inoculation [34]. On the contrary, an increasing trend in iWUE was observed in WS inoculated plants, in agreement with previous works [13,14,34]. Regarding CCI, a general decrease was observed in WS plants. Interestingly, a different impact of the several inocula was observed (e.g., MULTISTRAIN vs. Myc\_Rhizo) both in NS and WS plants, confirming species-specificity in affecting physiological traits. Concerning plant height, no significant results have been obtained among treatments and stress conditions. Taken together our results highlighted that symbiotic fungi (i.e., AM fungi) differently affect plant traits important for the tolerance to stressful conditions with respect to root-associated bacteria.

**Table 2.** Eco-physiological and biometric parameters. Photosynthetic rate (A), transpiration rate (E), stomatal conductance (gs), intrinsic water use efficiency (iWUE), stem height and SPAD in non-stressed (NS) and water stress (WS) leaves no treated (control) or treated with the different inocula (MULTISTRAIN, Myc\_Rhizo, LC3.5 + 5.2 or the commercial mix). Values are represented as mean  $\pm$  SD. For each column, within the same series (NS or WS) different lowercase letters indicate significant differences at  $p \leq 0.05$  as measured by an ANOVA-one way followed by a Tukey's multiple range test. Letter "a" denotes the highest value. Among the WS series, the symbols "\*" ( $p \leq 0.05$ ), "\*\*\*" ( $p \leq 0.005$ ) and "\*\*\*\*" ( $p \leq 0.001$ ) indicate significant differences between NS and WS leaves (Student's *t*-test).

		A	E	gs	iWUE (A/gs)	Stem Height	CCI
		$\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$	$\text{mmol m}^{-2} \text{ s}^{-1}$	$\text{mol m}^{-2} \text{ s}^{-1}$	$\mu\text{mol CO}_2 \text{ mol}^{-1} \text{ H}_2\text{O}$	cm	
NS	Control	$2.73 \pm 0.27^a$	$0.86 \pm 0.16^a$	$0.14 \pm 0.04^a$	$22.49 \pm 3.83^a$	$23.56 \pm 2.03^a$	$31.83 \pm 2.29^b$
	Multistrain	$3.17 \pm 0.24^a$	$0.74 \pm 0.19^a$	$0.12 \pm 0.02^a$	$27.04 \pm 6.99^a$	$16.92 \pm 1.99^a$	$40.71 \pm 0.88^a$
	Myc_Rhizo	$2.79 \pm 0.15^a$	$0.65 \pm 0.06^a$	$0.11 \pm 0.01^a$	$27.91 \pm 1.55^a$	$16.92 \pm 4.19^a$	$31.47 \pm 1.59^b$
	LC3.5 + 5.2	$3.42 \pm 0.38^a$	$1.32 \pm 0.67^a$	$0.14 \pm 0.05^a$	$26.56 \pm 11.53^a$	$21.53 \pm 2.23^a$	$39.71 \pm 2.81^a$
	Commercial MIX	$3.43 \pm 1.24^a$	$0.76 \pm 0.24^a$	$0.11 \pm 0.04^a$	$32.03 \pm 11.34^a$	$24.03 \pm 3.43^a$	$39.91 \pm 3.41^a$
WS	Control	$1.28 \pm 0.11^{ab,***}$	$0.83 \pm 0.25^a$	$0.13 \pm 0.05^a$	$11.08 \pm 5.25^{a,*}$	$18.72 \pm 2.39^{a,***}$	$23.85 \pm 2.95^{b,***}$
	Multistrain	$1.96 \pm 0.59^{a,***}$	$0.66 \pm 0.32^a$	$0.09 \pm 0.06^a$	$24.09 \pm 11.82^a$	$18.02 \pm 2.27^a$	$36.22 \pm 2.28^{a,**}$
	Myc_Rhizo	$1.36 \pm 0.2^{a,b,***}$	$0.62 \pm 0.32^a$	$0.04 \pm 0.01^{a,***}$	$21.36 \pm 16.67^a$	$17.56 \pm 1.41^a$	$26.57 \pm 2.8^{b,**}$
	LC3.5 + 5.2	$0.49 \pm 0.35^{b,***}$	$1.15 \pm 0.51^a$	$0.05 \pm 0.02^{a,**}$	$13.62 \pm 10.23^a$	$17.46 \pm 2.24^{a,*}$	$33.34 \pm 2.48^{a,***}$
	Commercial MIX	$0.53 \pm 0.25^{b,**}$	$0.45 \pm 0.12^a$	$0.04 \pm 0.01^{a,*}$	$22.33 \pm 4.24^a$	$26.93 \pm 8.41^a$	$38.39 \pm 0.49^a$

### 3.2. Effect of Water Deficit on Total Chlorophyll Content (TCC), Total Proline Content (TpC) and Total Polyphenol Content (TPC)

Water stress implicates morphological, biochemical and molecular changes [36], and may affect plant growth during different developmental stages [37]. As a first point, we evaluated how a water stress (WS) condition could change some biochemical parameters of tomato plants grown in the absence of specific treatments. The biochemical profile of stressed plants was compared to that of unstressed ones grown in well-watered (WW) conditions (Figure 1; Tables S2 and S3). In our experimental conditions, the exposure of tomato plants to WS negatively influenced the TCC and TPC, while the TpC was positively affected. The major effect of drought in plants is correlated with the decrease of photosynthetic processes, leading not only to the reduction of leaf expansion, but also to fruit production [38]. The main reason for a decrease in photosynthesis is due to changes in photosynthetic pigment levels that are part of the photosynthetic apparatus [39]. The decrease in TCC ( $0.67 \pm 0.08$ ), observed here, was already reported in tomato plants subjected to WS [40,41]. Other abiotic stresses such as heat [42] and salt stress [43,44] also affected the amount of these molecules.



**Figure 1.** Effects of water-stress (WS) on the tomato total content of chlorophylls (TCC), proline (TpC) and polyphenols (TPC). Data for each quantification are expressed as relative content, comparing the measurements obtained for water-stressed plants with those of unstressed plants. The dotted line indicates the basal expression of NS. Absolute quantification of each parameter is also reported in Supplementary Table S2. Bars with different lowercase letters indicate significantly different values at  $p \leq 0.05$  as measured by a one way-ANOVA followed by a Tukey's HSD post hoc test (see Supplementary Table S4 for additional information). The symbol "\*" indicates significant differences ( $p \leq 0.05$ ) between untreated-water stressed and untreated-non stressed plants, as measured by a *t*-test.

On the other hand, the role of polyphenols, which represent the soluble antioxidant defenses of the plant, in stressed samples has been widely discussed and contrasting. Although several studies already reported the increase of polyphenols to contrast the oxidative damage generated after the exposure to different abiotic stresses [45], in other cases a substantial decrease of these molecules was



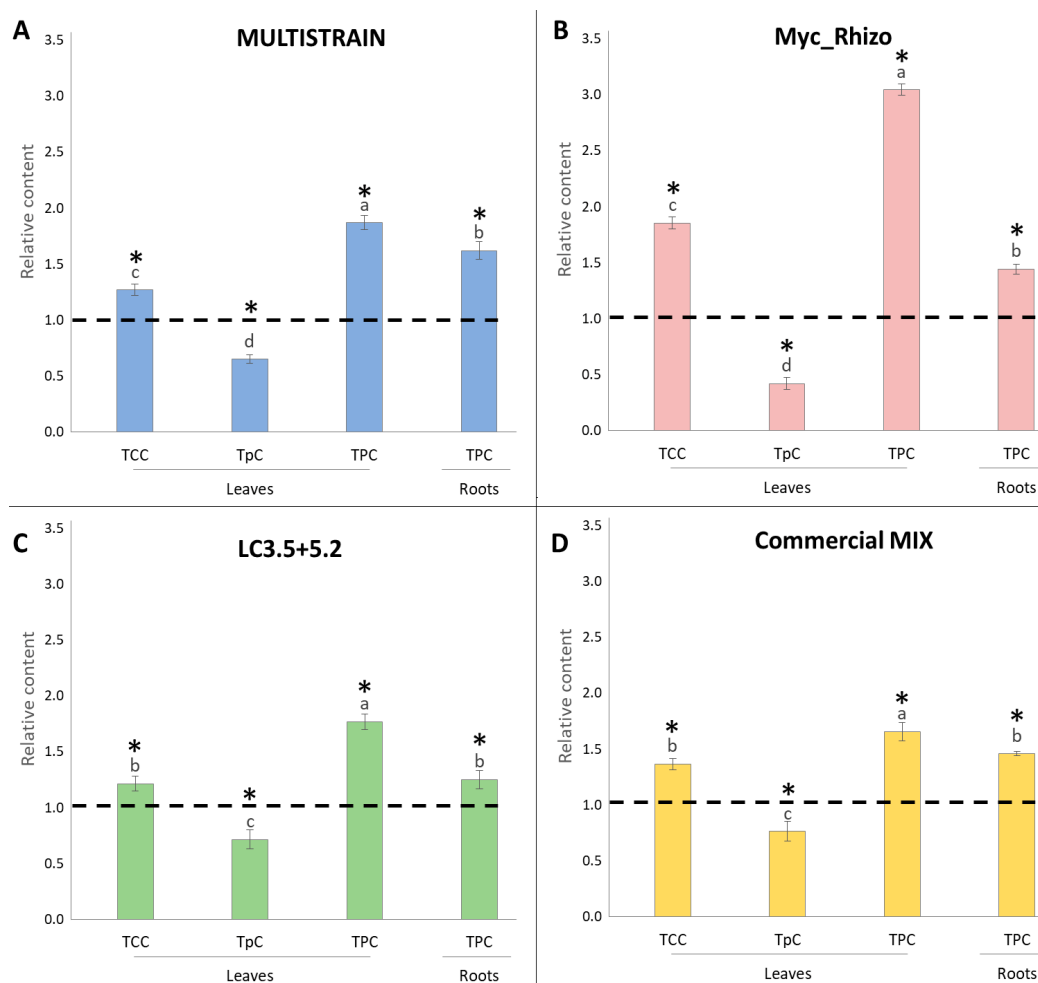
observed [46,47]. This response is probably due to the loss of the plant capability to synthesize *ex novo* the soluble antioxidant defenses. In our experiment, the TPC, evaluated on both leaves and roots, decreased after the exposure to WS. Moreover, a stronger effect was observed in leaves compared to roots ( $0.72 \pm 0.06$  and  $0.49 \pm 0.04$ , respectively).

Finally, in order to respond to unbalanced water repartition, plants generally accumulate compatible solutes with the aim to raise osmotic pressure and thereby to maintain both turgor and driving gradient for water uptake [32,48]. Among these solutes, proline plays a key role in these processes. The accumulation of proline in leaves can be considered as a strong indicator of abiotic stresses such as drought, salt and heat stresses. In accordance with our results ( $1.45 \pm 0.09$ ), an increase of proline in leaves of stressed plants was previously reported, not only in tomato but also in other plants [32,48–51].

### 3.3. MULTISTRAIN, *Myc\_Rhizo*, LC3.5 + 5.2 and the Commercial MIX are Able to Recover the Biochemical Parameters in Water Stressed Plants

In order to check if the treatments with the different microbial inocula were able to restore the correct plant homeostasis, tomato plants were inoculated with four different inocula. To allow the successfully establishing of the relationship between roots and employed microorganisms, plants were grown in well-watered conditions for a period of about six weeks before to start with water limitation. Figure 2 shows the change in TCC, TpC and TPC values of treated-WS plants compared to untreated-WS plants (dotted-line). All the treatments promoted a recovery of the TCC and TPC amount in WS plants, suggesting beneficial properties of the formulations, and a decrease of water stress in treated plants. However, significant differences ( $p \leq 0.05$ ) among the four treatments were found. In particular, *Myc\_Rhizo* (Figure 2B) was the most effective in increasing TCC in leaves ( $1.86 \pm 0.06$ ), followed by the commercial mix ( $1.37 \pm 0.05$ ; Figure 2D). The highest recovery in term of TPC in the leaves was recorded in WS plants treated with *Myc\_Rhizo* ( $3.04 \pm 0.05$ ; Figure 2B), while the best recovery of TPC in roots was observed with MULTISTRAIN ( $1.62 \pm 0.08$ ; Figure 2A) followed by *Myc\_Rhizo* ( $1.44 \pm 0.04$ ; Figure 2B).

Finally, TpC was also affected by the different treatments, with *Myc\_Rhizo* and MULTISTRAIN (Figure 2A,B) that showed again the highest decrease ( $0.42 \pm 0.05$  and  $0.65 \pm 0.03$ , respectively). Moreover, a very strong and negative correlation was found between TCC/TpC ( $\rho = -0.89$ ) and leaf-TPC/TpC ( $\rho = -0.96$ ), as revealed by Pearson analysis (Table S4). On the other hand, no correlation was found between root-TPC and TpC ( $\rho = -0.15$ ).

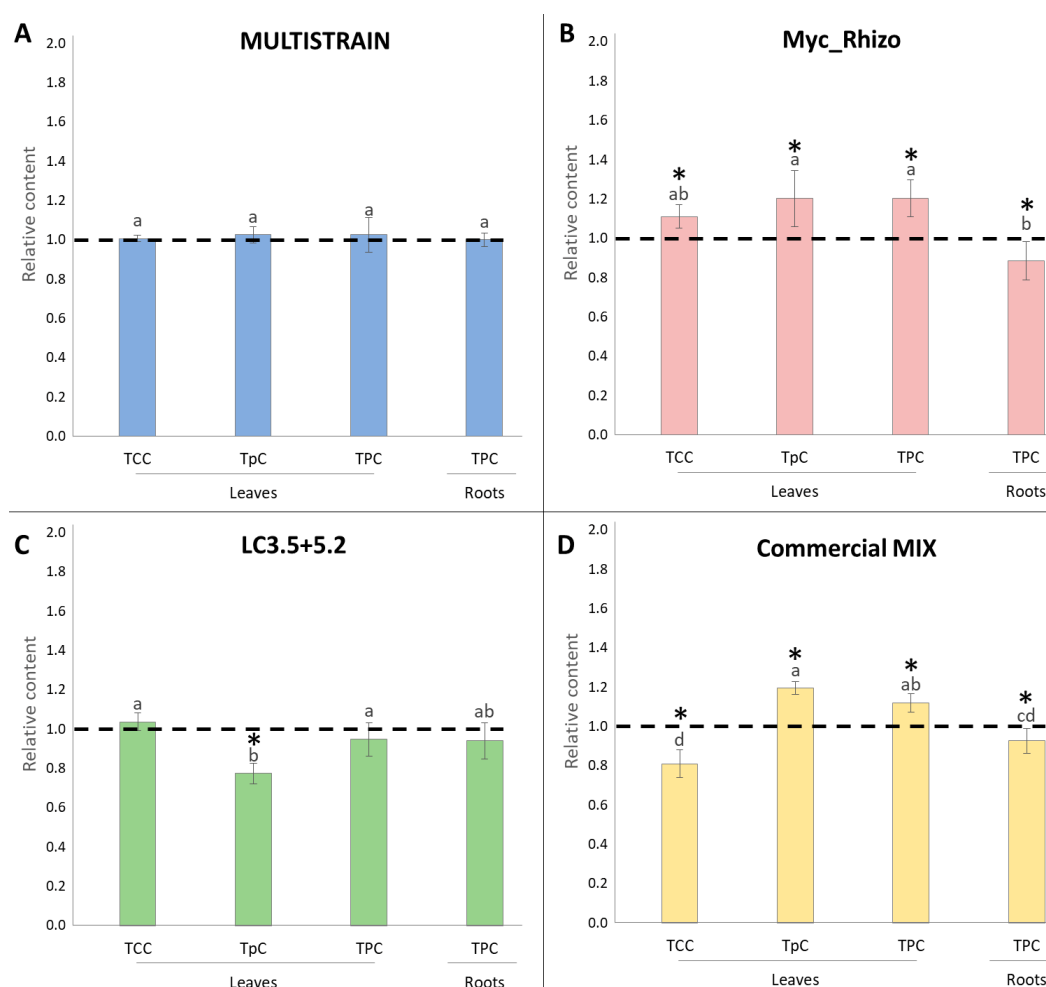


**Figure 2.** Effect of the treatment with MULTISTRIN (A), Myc\_Rhizo (B), LC3.5 + 5.2 (C) and the commercial mix (D) on the total content of chlorophylls (TCC), proline (TpC) and polyphenols (TPC) evaluated on water-stressed plants. Data for each quantification are expressed as relative content, comparing the measures obtained by treated-and untreated-water stress plants. The dotted line indicates the basal level of untreated water stressed plants. Absolute quantification of each parameter is also reported in Supplementary Table S2. Bars with different lowercase letters indicate significantly different values at  $p \leq 0.05$  as measured by a one way-ANOVA followed by a Tukey's HSD post hoc test (see Supplementary Table S4 for additional information). The symbol “\*” indicates significant differences ( $p \leq 0.05$ ) between treated-WS and untreated-WS plants as measured by a  $t$ -test.

### 3.4. MULTISTRIN, Myc\_Rhizo, LC3.5 + 5.2 and the Commercial MIX Affect Total Chlorophyll, Polyphenol and Proline Content in Absence of Stress

In order to evaluate the performance of different formulations without a water stress condition, NS plants treated with MULTISTRIN, Myc\_Rhizo, LC3.5 + 5.2 or of the commercial mix were analyzed. Figure 3 shows the relative content of treated-non stressed plants in comparison to untreated-non stressed plants (dotted-line). As a general trend, the treatments with MULTISTRIN (Figure 3A) and LC3.5 + 5.2 (Figure 3C) did not change the content of the analyzed biochemical parameters, with the exception of TpC in non-stressed plants treated with LC3.5 + 5.2. A more evident effect was instead observed in non-stressed plants inoculated with Myc\_Rhizo or with the commercial mix (Figure 3B,D). In these cases, TpC and TPC in the leaves statistically ( $p < 0.05$ ) increased with respect to untreated non-stressed plants ( $1.20 \pm 0.18$  and  $1.20 \pm 0.09$  for Myc\_Rhizo and commercial mix, respectively). On the other hand, TPC decreased in roots (Figure 3B,D). The slightly significant changes in the biochemical parameters could be associated to the functional traits of the considered

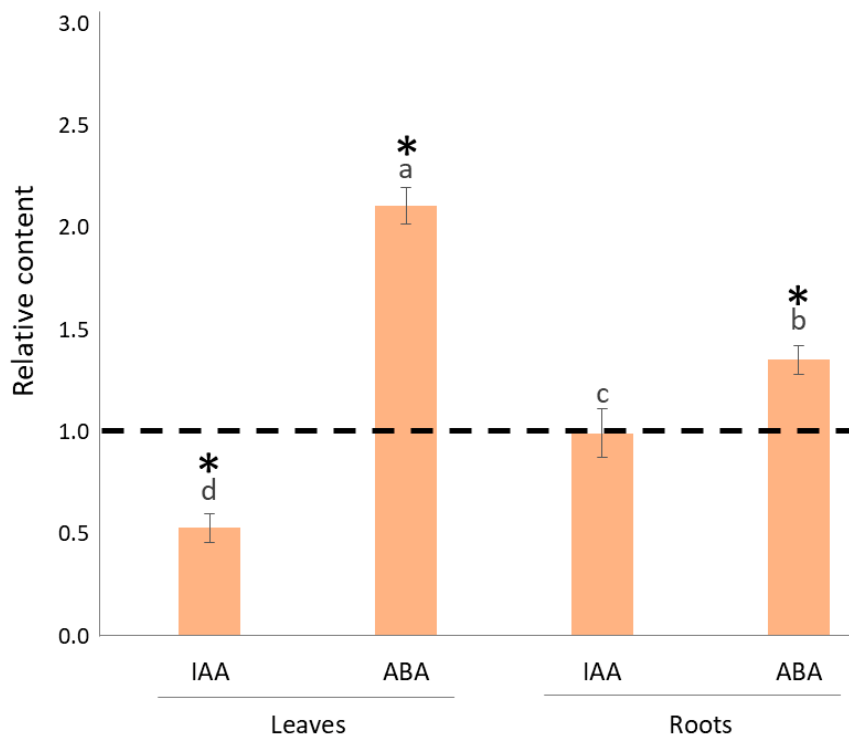
microorganisms that led to a priming status, also in the absence of stress, as previously reported ([6] and reference therein). Physiological, transcriptional and metabolic changes stimulated by the colonization of soil root-associated microorganisms can prime plants for enhanced defense ahead of abiotic and biotic stress occurrence [52]. Evidence of a possible priming of the plant defensive system induced by AM-inoculation was recently suggested in *Arundo donax* [53], where a significant increase in proline accumulation in AM-colonized roots was reported. Brilli et al. [34] suggested that *Pseudomonas chlororaphis* acted as a ‘priming stimulus’ triggering in inoculated tomatoes enhanced tolerance to water stress. Interestingly, a simultaneous increase in the activity of superoxide dismutase (SOD) and catalase (CAT), and in proline accumulation was observed in tomato leaves from inoculated plants, independently by the stress level (well-watered or water stressed plants). However, the contribution of the root-associated microorganisms in plant adaptation to environmental stress factors needs to be still extensively evaluated, particularly in natural conditions, where a complex soil microbiota is present, and upon multiple stresses.



**Figure 3.** Effect of the treatment with MULTISTRRAIN (A), Myc\_Rhizo (B), LC3.5 + 5.2 (C) and the commercial mix (D) on the total content of chlorophylls (TCC), proline (TpC) and polyphenols (TPC) evaluated on unstressed plants. Data for each quantification are expressed as relative content, comparing the measurements obtained by treated- and un-treated-non stressed plants. The dotted line indicates the basal level of untreated-non stressed plants. Absolute quantification of each parameter is also reported in Supplementary Table S2. Bars with different lowercase letters indicate significantly different values at  $p \leq 0.05$  as measured by a one way-ANOVA followed by a Tukey’s HSD post hoc test (see Supplementary Table S4 for additional information). The symbol “\*” indicates significant differences ( $p \leq 0.05$ ) between treated-NS and untreated-NS plants, as measured by a  $t$ -test.

### 3.5. Effects of the Different Formulations Applied on ABA and IAA Content

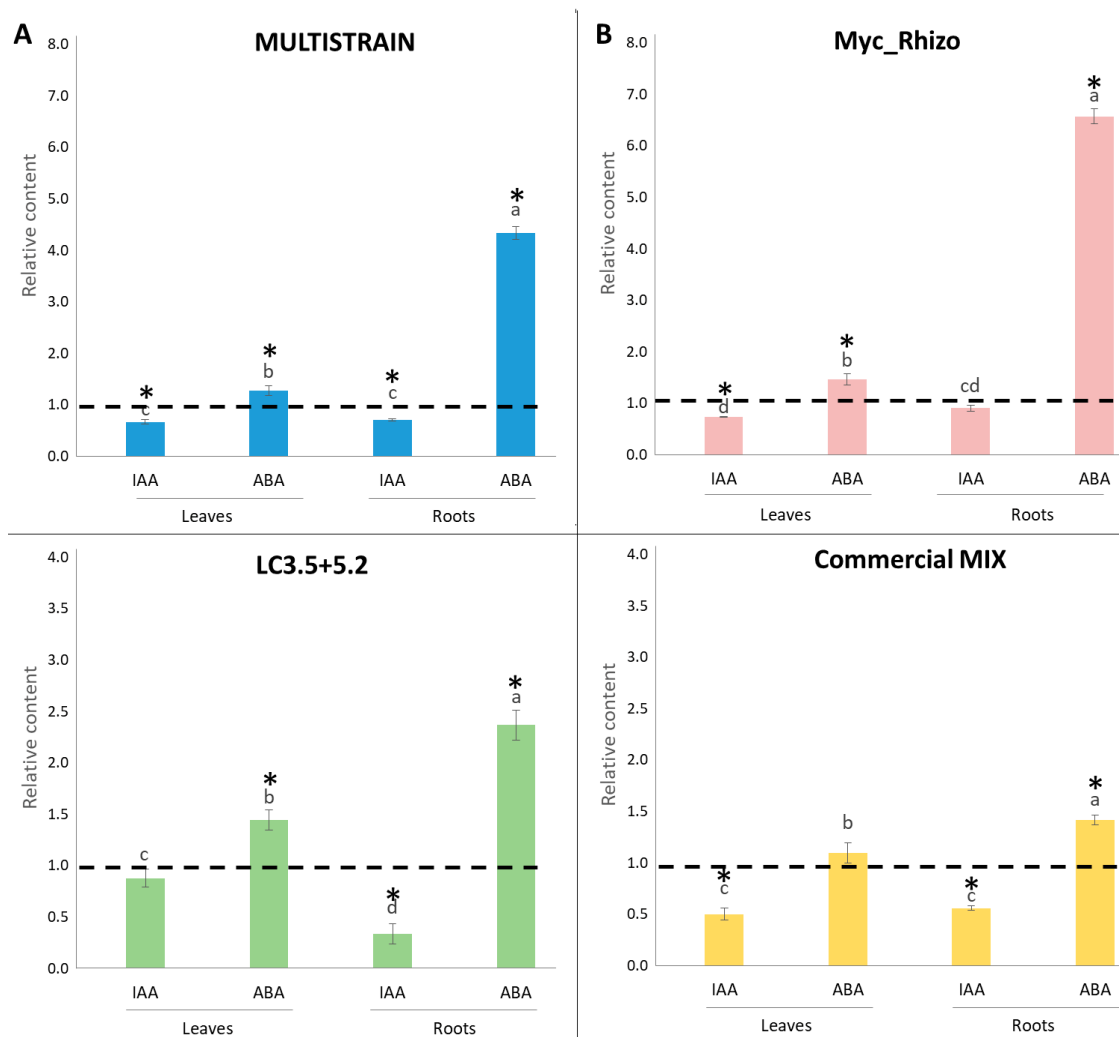
Regardless of the water regime conditions the pattern of ABA and IAA content were strongly affected by the applied consortia. The plant hormone ABA is a chemical signal produced in leaves and roots, largely studied because of its pivotal roles in stomata movement and molecular-mediated responses under water stress [54]. In general, ABA content was less affected in roots of treated plants with respect to the controls in both WS and NS conditions (Figure 4).



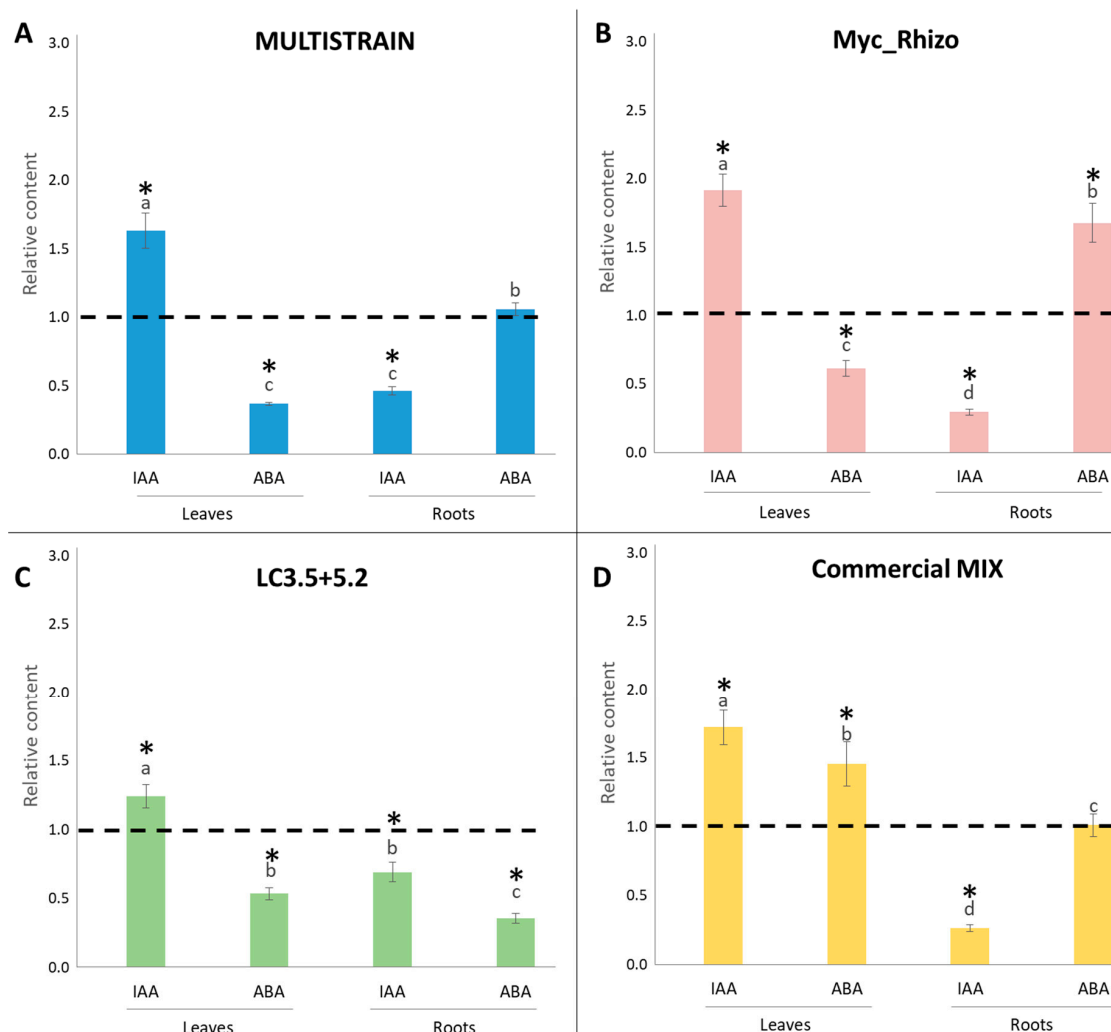
**Figure 4.** Effects of water-stress (WS) on the tomato content of indole acetic acid (IAA) and abscisic acid (ABA) evaluated both on leaves and roots. Data for each quantification are expressed as relative content, comparing the measurements obtained for water-stressed plants with those of unstressed plants. The dotted line indicates the basal expression of non-stressed plants. Absolute quantification of each parameter is also reported in Supplementary Table S2. Bars with different lowercase letters indicate significantly different values at  $p \leq 0.05$  as measured by a one way-ANOVA followed by a Tukey's HSD post hoc test (see Supplementary Table S4 for additional information). The symbol "\*" indicates significant differences ( $p \leq 0.05$ ) between untreated-water stress and untreated-non stressed plants, as measured by a  $t$ -test.

Under the NS condition, the ABA content in roots was significantly higher ( $p \leq 0.05$ ) in the treated plants when compared to their controls, suggesting an ABA-primed status induced by the microorganisms added in the substrates (Figure 5). In NS leaves, MULTISTRAIN, Myc\_Rhizo and LC3.5 + 5.2 showed significantly higher levels of ABA with respect to the controls. As expected, under WS conditions, ABA content was generally higher with respect to NS and only in roots of Myc\_Rhizo and leaves of the commercial mix was significantly higher with respect to their controls ( $p \leq 0.05$ ), pointing out a microbial-mediated role in WS sensing and in turn ABA synthesis on inoculated plants (Figure 6) [4,34].

In almost all conditions tested, under NS conditions, IAA content showed an opposite trend for ABA) confirming their negative correlation as previously reported by Saeedipour and Moradi [55], with the exception of LC3.5 + 5.2. Interestingly, under WS conditions, all the treatments showed significantly higher IAA levels in leaves whilst lower levels were observed in roots with respect to their controls ( $p \leq 0.05$ ; Figure 6).



**Figure 5.** Effect of the treatment with MULTISTRIN (A), Myc\_Rhizo (B), LC3.5 + 5.2 (C) and the commercial mix (D) on the content of indole acetic acid (IAA) and abscisic acid (ABA) evaluated both on leaves and roots of unstressed plants. Data for each quantification are expressed as relative content, comparing the measurements obtained by treated- and untreated-non stressed plants. The dotted line indicates the basal expression of untreated-non stressed plants. Absolute quantification of each parameter is also reported in Supplementary Table S2. Bars with different lowercase letters indicate significantly different values at  $p \leq 0.05$  as measured by a one way-ANOVA followed by a Tukey's HSD post hoc test (see Supplementary Table S4 for additional information). The symbol “\*” indicates significant differences ( $p \leq 0.05$ ) between treated- and untreated-non stressed plants, as measured by a *t*-test.



**Figure 6.** Effect of the treatment with MULTISTRRAIN (A), Myc\_Rhizo (B), LC3.5 + 5.2 (C) and the commercial mix (D) on the content of indole acetic acid (IAA) and abscisic acid (ABA) evaluated both on leaves and roots of water-stressed plants. Data for each quantification are expressed as relative content, comparing the measurements obtained by treated- and untreated-water stressed plants. The dotted line indicates the basal expression of untreated water stressed plants. Absolute quantification of each parameter is also reported in Supplementary Table S2. Bars with different lowercase letters indicate significantly different values at  $p \leq 0.05$  as measured by a one way-ANOVA followed by a Tukey's HSD post hoc test (see Supplementary Table S4 for additional information). The symbol "\*" indicates significant differences ( $p \leq 0.05$ ) between treated- and untreated-non stressed plants, as measured by a *t*-test.

#### 4. Conclusions

In conclusion, our results confirmed the fact that several microbial inocula have different impacts on the tomato's response under a water stress condition. Although aspects related to the persistence of the inocula at the end of the experiment were not considered, our results showed that the biochemical response of tomato to a stressful factor changed depending on the applied consortia of root-associated microorganisms. The latter were also able to induce a different effect on physiological traits. Moreover, the importance of symbiotic fungi, i.e., the AM fungi, in inducing a primed status and, in turn, a tolerance to water deficit was highlighted, reinforcing the consolidated evidence of the positive role played by these "biostimulants". However, many factors can affect the success of inoculation and persistence of inoculated microorganisms in soil, including compatibility with the target environment, the degree



of spatial competition with other soil organisms in the target niche and the timing of inoculation. For this reason, further efforts should be done, mainly for bacteria species, to implement methods for monitoring and characterizing the degree of root/rhizosphere colonization of the microbial inoculants.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2073-4395/10/2/170/s1>. Supplementary Table S1. Statistical analysis of absolute values of photosynthetic rate (A), transpiration rate (E), stomatal conductance ( $g_s$ ), intrinsic water use efficiency (iWUE), stem eight and CCI. Supplementary Table S2. Absolute determination of Total Content of Chlorophylls (TCC), Proline (TpC), Polyphenols (TPC), Indole Acetic Acid (IAA) and Absciscic Acid (ABA). Supplementary Table S3. Statistical analysis of absolute determination of Total Content of Chlorophylls (TCC), Proline (TpC), Polyphenols (TPC), Indole Acetic Acid (IAA) and Absciscic Acid (ABA). Table S4. Statistical analysis of the relative content of Total Content of Chlorophylls (TCC), Proline (TpC), Polyphenols (TPC), Indole Acetic Acid (IAA) and Absciscic Acid (ABA).

**Author Contributions:** All authors have read and agree to the published version of the manuscript. Conceptualization, R.B., E.L. and W.C.; investigation, G.M., L.N., T.G., C.M.B., E.L., M.N., W.C. and R.B.; formal analysis, G.M., L.N., W.C.; resources, T.G., M.B.; writing—original draft preparation, G.M., R.B., E.L., W.C.; writing—review and editing, G.M., L.N., C.M.B., M.N., V.F., W.C. and R.B.; supervision, R.B. All authors have read and agreed to the published version of the manuscript.

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