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Achromobacter xylosoxidans and *Enteromorpha intestinalis* Extract Improve Tomato Growth under Salt Stress

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Abstract: The effect of seed coating salt-stressed tomato with the bacterium *Achromobacter xylosoxidans* BOA4 and/or irrigation with an extract of the marine algae *Enteromorpha intestinalis* (EI) is herein evaluated. The plant shoots and roots were harvested separately on day 50, following extensive saline stress. The addition of BOA4 and/or EI extract resulted in an average increase of 33% in plant shoot DW, but an averaged decrease of 44% in the root to shoot biomass ratio. Anthocyanin content increased by over 34% and 44% with EI and BOA4 plus EI treatments, respectively. Since enhanced protein tyrosine nitration (PTN) is a known plant response to salt stress, the PTN level was inspected through 3-nitrotyrosine content determination. This was drastically increased by salt stress; however, BOA4, EI or both caused an averaged PTN decrease of 30% in stressed roots or shoots. This PTN response could be associated with tomato phenotypic characteristics and is postulated to be inversely correlated to cytokinin contents in stressed plants, namely cis-zeatin-type-cis-zeatin (cZ) plus cis-zeatin riboside (cZR), and isopentenyladenine (iP). The latter showed a drastic average increase by 3.6-fold following BOA4 and/or EI treatments of salinized tomato. This increment could be related to cytokinin biosynthesis induced by the applied bio-stimulants; IP and derivatives are the main cytokinins in seaweeds, and *Achromobacter xylosoxidans* BOA4 was shown to produce up to 17.5 pmol mL^{-1} of isopentenyladenine. This work is the first report on the influence of bio-stimulants, used to improve salt stress tolerance, on plant PTN levels; BOA4 and/or EI treatments decreased PTN, while increasing cis-zeatin-type and iP cytokinins in tomato, the latter showed an enhanced tolerance to salt stress.

Keywords: cis-zeatin-type cytokinins; iP cytokinins; *Enteromorpha intestinalis*; plant growth promoting bacteria; protein tyrosine nitration; salt stress

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1. Introduction

More than fifty per cent of agricultural lands will be at risk of being affected by salinity by the year 2050 [1]. Soil salinization urges researchers to design new strategies for plants to cope with salt stress. Cultivating new salt-tolerant varieties, producing genetically modified crops, and applying exogenous osmoprotective compounds are regarded as critical strategies for improving agricultural performance under salt stress [2]. For example, the use of algae extracts as a source of nutrients and osmoprotectants has been envisaged for salt stress relief ([3] and ref therein). Plant Growth-Promoting Rhizobacteria (PGPR) have also been considered for improving crop growth under salt stress or under water deficit stress, which is often linked to soil salinization, e.g., [4–6]. PGPR mechanisms useful

for improving plant tolerance to salt stress include the production of osmoprotectants, non-enzymatic antioxidants and antioxidant enzymes. Additionally, PGPR produce phytohormones, for instance, the auxin indole-3-acetic acid (IAA), which is needed for cell division and elongation in salt-stressed plants, and cytokinins, which are important for cellular proliferation and differentiation [7]. Additionally, PGPR can improve nutrient acquisition by salt-stressed plants via their ability for phosphate solubilization or siderophore production for iron uptake by the roots [7].

Plant ionic and osmotic stresses are cell primary effects of water deficit, associated with salinity and drought, and oxidative stress occurs secondarily through the accumulation of harmful reactive oxygen species (ROS). These highly reactive species are associated with nitric oxide (NO) chemistry, as NO reacts with ROS, namely with superoxide (O_2^-), to produce highly reactive nitrogen species (RNS), e.g., peroxynitrite ($ONOO^-$). Although enhanced $ONOO^-$ formation causes nitrosative stress in various biological systems, peroxynitrite may lead to nitrosation (addition of NO) or nitration (addition of NO_2) with the formation of compounds that intervene in NO-dependent cell signaling pathways—e.g., S-nitrosoglutathione and nitrotyrosine (NO_2-Tyr) in proteins, the latter of which is known as protein tyrosine nitration (PTN) [8]. PTN is a post-translational modification, where the nitro (-NO_2) group is added to one of the two equivalent ortho carbons of the aromatic ring of tyrosine residues [9], forming 3-nitrotyrosine (3-NT).

While PTN may be involved in stress signaling designed to cope with initial low stress, a stress situation may escalate from a regular stress level to an elevated level and further to an excessive degree; severe stress could cause the accumulation of nitrated proteins and protein aggregation with irreversible cell damage [10]. In fact, plant cells have a basal endogenous nitration level, but when exposed to adverse conditions plants are usually in nitro-oxidative stress and show an increase in NO_2-Tyr content thanks to increased PTN [10]. Thus, it is important to distinguish between large-scale protein nitration due to a greater amount of RNS, which is able to mediate damage to biomolecules, and the nitration of key targets of cell signaling processes. PTN is a low-yield process: 1–5 NO_2-Tyr residues per 10,000 tyrosines have been reported for stressed tissues [11]. These lower levels of NO_2-Tyr compared with Tyr content are indeed suggestive of PTN as a physiological regulator of signaling pathways involving nitrated proteins. PTN is also selective, as not all proteins can become nitrated nor can all tyrosine residues within a protein undergo nitration [11]. Proteins subjected to PTN may dramatically change their structures and examples of either losses or gains of function have been published [12]. Under salt stress, an increase in NO_2-Tyr and $ONOO^-$ content has been registered for different plant species together with the identification of proteins involved in photosynthesis, disease/defense, energy, and storage [10]. It was suggested that salt stress promotes a NO release from peroxisomes to the cytosol for the generation of $ONOO^-$; the raised level leads to increased PTN and consequent nitrosative stress [13].

Evidence of an association between NO signaling, protein nitration and phytohormone regulation has been described by several authors [10,14]. Synergistic and antagonistic interactions between NO and cytokinins have been reported depending on the physiological process under study, plants species and experimental procedure [14]. Liu et al. [15] proposed that NO and cytokinins might modulate each other's homeostatic levels and activity, as they showed that the cytokinins trans-zeatin and isopentenyladenine can react with peroxynitrite, and form cytokinin derivates with near to no biological activity. Additionally, exogenous trans-zeatin alleviated *Arabidopsis* severe mutant phenotypes attributed to high levels of NO. Thus, NO chemistry via peroxynitrite formation and PTN level might be correlated to the cytokinin level, namely with trans-zeatin and isopentenyladenine levels. Studies on NO signaling associated with PTN and cis-zeatin-type cytokinins are missing. However, it has been suggested that cis-zeatin-type cytokinins, e.g., cis-zeatin cZ and cis-zeatin riboside cZR, herein named cZ(R), which have been largely overlooked when compared to trans-zeatin (tZ) isomers, have a main role in abiotic stress, replacing tZ(R), which has higher cell division promoter activity [16]. It is assumed that cZ(R) is used for the

maintenance of plant minimal cytokinin activity, more compatible with stress responses involving growth suppression associated with the formation of stress-protective compounds.

Tomato is an economic relevant crop in the Mediterranean, and its production is threatened by climate change, which is increasing the area of salinized soil. *Achromobacter xylosoxidans* BOA4 has been previously identified as a diazotrophic-PGPB and has several important PGPR biochemical traits in addition to nitrogen fixation, such as siderophore and IAA production, and phosphate solubilization [3]. Marine macro-algae are known for their tolerance to water stress, such as salinity and drought. Such adaptations are linked to their chemical composition, which suggests that seaweeds might be used as efficient sources of a wide range of compatible solutes; Rai et al. [3] previously observed that *E. intestinalis* extracts significantly promoted BOA4 halotolerance under salt stress. Considering *A. xylosoxidans* BOA4 and *E. intestinalis* biochemical traits, we aimed to assess their potential to promote tomato growth under extensive saline stress, and thus their prospective use in the actual context of arable soils of increasing salinity. Plant biomass and leaf pigment vegetation indices designed to provide a measure of stress-related pigments were determined. Plants show an enhanced protein tyrosine nitration (PTN) in response to salt stress; bio-stimulants may improve plants' tolerance to salt stress, but it is not known if they act on PTN levels. Thus, we also performed an endpoint PTN analysis, through the determination of 3-nitrotyrosine content, to investigate PTN level as an indicator of the magnitude of plant stress under the different treatments. Moreover, in view of the above-mentioned relation between NO chemistry, protein nitration and cytokinins, we aimed to relate the observed plant characters for the different treatments with the phytohormonal content, namely with abiotic stress responsive phytohormones, e.g., cis-zeatin-type cytokinins.

2. Materials and Methods

2.1. Biological Materials

The strain BOA4 was previously isolated from an agricultural land in the province of Bouira (36°7′41.29″ N, 3°32′55.89″ E, northern Algeria) and molecularly identified as *Achromobacter xylosoxidans.* BOA4 was selected for further studies based on its biochemical PGP traits [3]. Herein, it was further characterized in terms of carbon source use with Biolog Ecoplate^{TM} (Biolog Inc., Hayward, CA, USA), which contains 31 of the most useful carbon sources for soil community analysis. Although Biolog Ecoplate ^{TM} was designed for the community-level metabolic profiling of environmental samples, it was recently used to evaluate the nutritional diversity of distinct *Bacillus subtilis* isolates [17]. For the characterization of the BOA4 isolate, this was grown in a rich medium, TYG, containing 5 g tryptone, 2.5 g yeast extract, 2 g glucose in 1 L of distilled water, at 28 °C, 120 rpm, until the early stationary phase and diluted to an OD of ca 0.45 (λ = 600 nm), measured with a CamSpec photometer M105. The culture was centrifuged at 4000 rpm for 15 min in an Eppendorf 5810R centrifuge, washed twice with sodium chloride 0.85% and the pellet was resuspended in Phosphate Buffered Saline (PBS) at the same OD. Next, 150 µL of the suspension was placed in each well of the microplate and incubated at 28 °C. The assay was performed following the manufacturer's instructions and the use of the different substrates over time was summarized through the integration of the area under the corresponding curves (AUC).

Enteromorpha intestinalis (EI) was collected from Boulimat beach in the province of Bejaïa, Northern Algeria (36°48′54.5″ N 4°59′11.1″ E). The collected material was cut into fine pieces, air dried in darkness and then powdered using an electric grinder.

Tomato seeds (*Solanum lycopersicum*, Marmande variety) were provided by the Centre for Ecology, Evolution and Environmental Changes (cE3c) of the Faculty of Sciences-University of Lisbon (Portugal). This variety is characterized by early maturation, facilitating short-term studies. They are marketed under the license "Soares & Rebelo, Lda".

2.2. Greenhouse Assay

Tomato seeds were softened by gentle agitation in distilled water for 1 h. Afterwards, they were surface sterilized for 3 min with 70% ethanol, washed three times with sterile distilled water and subsequently treated for 5 min with a hypochlorite-based bleach, 2% active chlorine. Seeds were finally washed ten times with sterile distilled water. They were then brought into contact with a suspension of strain BOA4 in sterile distilled water for 40 min under agitation at 450 rpm using a platform shaker, Titramax 100 (Heidolph Instruments, Schwabach, Germany); uninoculated seeds were left in contact with sterile distilled water. The BOA4 suspension was prepared from a bacterial culture in TYG (6.3×10^9 cfu/mL, OD = 2.2; λ = 600 nm), which was centrifuged at 4000 rpm for 20 min in an Eppendorf 5810R centrifuge. The pellet was collected, washed three times in sterile distilled water and standardized at an OD = 0.8; λ = 600 nm.

A soil with 1.94% organic matter, provided by the Center for Ecology, Evolution and Environmental Change (cE3c) of the Faculty of Sciences of the University of Lisbon, was mixed with vermiculite (50/50 v/v) before use. Other physicochemical properties of this soil (Supplementary Materials Table S1) were determined at "Laboratório de análises agrícolas e ambientais, Guimarães" following its passage through 4.75 mm and 2 mm sieves, assuring that no aggregates were retained before analysis. The above tomato seeds (uninoculated and BOA4 coated) were placed in 14 × 15 cm pots (five seeds/pot at a depth of about 1 cm in the soil–vermiculite mixture). The experimental setting was completely randomized with 8 treatments and five replicates, resulting in a total of 40 pots, as pots with seeds were watered with 30 mL of water, 150 mM NaCl (controls) or EI extract (prepared in either water or 150 mM NaCl). EI extracts were prepared by autoclaving EI-dried powder at 1% (w/v) in distilled water or in 150 mM NaCl.

Over the first 18 days, pots were watered similarly every 3 days with 15 or 30 mL/pot of the above solutions, and then with tap water or 150 mM NaCl every 3 days until day 35. At this point, germinated plants in each pot were collected, leaving only one plant per pot; the shoot fresh weight (FW) of the collected 35 days plants ($8 \leq n \leq 19$, depending on the treatment) was determined, as well as the dry weight (DW) after drying to constant weight at 65 °C. The experiment was conducted for 50 days with the five remaining plants per treatment (one plant/pot) and watering was carried out with tap water for the last 8 days. By day 50, 54 mmol NaCl had been added in total throughout the experiment to each pot that received the 150 mM watering. Before the final harvest after 50 days, assessing plants' shoots and roots to determine FW and DW, a portable handheld spectro-radiometer, Polypen RP410 (Photon Systems Instruments-PSI), was used for the measurement of the spectral reflectance of plants leaves and calculation of reflectance indices. PSI manual provides the list of the vegetation indices automatically calculated for the measured samples based on their reflectance spectra. The normalized difference vegetation index, previously found to be sensitive to salinity [18], was compared among treatments. The indices AR (ARI1 and ARI2) and CR (CRI1 and CRI2) were also calculated in order to measure stress-related leaf pigments, anthocyanins and carotenoids. ARI2 corrects for leaf density and thickness, as the near-infrared spectral band (760–800 nm), related to leaf scattering, is added to ARI1. CR indices negate the effect of chlorophyll on the reciprocal reflectance at 510 nm, the wavelength of maximal sensitivity to the total carotenoid content, using a reciprocal reflectance at either 550 nm (wavelength absorbed both by chlorophylls a and b) in CRI1 or 700 nm (absorbed mainly by chlorophyll a) in CRI2.

2.3. Tyrosine Nitration Quantification

To quantify tyrosine nitration, plant tissue homogenates of plants grown for 50 days were prepared: fragments of fresh shoots and roots were weighted, and sections of the plant tissues were minced to small pieces in liquid nitrogen, homogenized in PBS (100 µL PBS to 10 mg tissue) and distributed in aliquots, which were stored at −20 °C until the assay. After thawing, samples were mixed using a vortex, and particles were removed by centrifugation at 3000 rpm for 15 min in a Labnet Spectrafuge 16M centrifuge. Plant 3-Nitrotyrosine (3-NT)

ELISA Kit (MyBioSource, Vancouver, Canada) was used to quantify 3-NT in triplicate 50 µL aliquots of the collected supernatants following the manufacturer's instructions. Briefly, 100 µL of HRP-conjugate reagent was added to each well of a Microelisa Stripplate, where 50 µL of the sample, control or standard was previously added. The wells were covered with a closure plate membrane. After a 1 h incubation at 37 °C, the wells were washed four times with 350 µL of the reconstituted Wash solution provided with the kit. After the final wash, the plate was dried by hitting it onto an absorbent paper. Fifty microliters of Chromogen Solution A and 50 µL of Chromogen Solution B were added to each well successively. The plate was incubated, protected from light, for 15 min at 37 °C. Finally, 50 µL of stop solution was added to each well and the optical density at 450 nm was read using a Zenyth 3100 Microplate reader. The 3-NT values were reported to the total protein concentration of the above supernatants, which was determined by the Bradford assay [19] using BSA fraction V (Sigma, Burlington, MA, USA) as standard.

2.4. Cytokinin Quantification

The identification and quantification of cytokinins were performed for above shoot homogenates outcoming from stressed plants; aliquots of homogenates in PBS were centrifuged at 3000 rpm for 10 min and the supernatant was discarded. Sample pellets (min: 134 mg FW; max: 234 mg FW) were processed for cytokinin extraction, purification and quantification by high-performance liquid chromatography-electrospray-high-resolution accurate mass spectrometry (HPLC-ESI-HRMS). Cytokinins were extracted from frozen pellets with a methanol–water–formic acid (15:4:1, $v/v/v$) solution and deuterium-labeled internal standards were added to the extraction medium. An Oasis MCX column (ref. 186000254, Waters Co., Milford, MA, USA) was used in the purification procedure. The recovered eluate was evaporated to dryness and redissolved with 250 µL of methanol plus 250 µL of 0.04% formic acid and centrifuged at 20,000 RCF for 10 min before being injected into the HPLC-ESI-HRMS system for the detection and quantification of the cytokinins. The detailed protocol is described by Olaetxea et al. [20]; the studied cytokinins were trans- and cis-zeatin (tZ and cZ), dihydrozeatin (DHZ), trans- and cis-zeatin riboside (tZR and cZR), dihydrozeatin riboside (DHZR), isopentenyladenine (iP), isopentenyladenosine (iPR), benzyladenosine (BAR), meta-topolin (mT), metatopolin riboside (mTR), ortho-topolin (oT), and ortho-topolin riboside (oTR).

To assess the potential production of cytokinins by strain BOA4, inocula (ca 10^6 cfu/mL) were grown in TYG medium, at 28 °C, 120 rpm. Cultures were monitored and collected after 18 h, 22 h and 48 h. After centrifugation at 4000 rpm for 10 min, at 20 °C, in an Eppendorf 5810R centrifuge, the supernatants were frozen and processed as above. Non-inoculated TYG medium was used as the control.

2.5. Statistical Analysis

Statistical tests were performed using the IBM SPSS Statistics 27.0. software package. One-way ANOVA, followed by Tukey's post hoc test, was performed for multigroup comparisons. p-values ≤ 0.05 were considered statistically significant.

3. Results and Discussion

3.1. BOA4 Production of Cytokinin and Carbon Source Use

BOA4 is a diazotrophic strain isolated from soil with 0.7% of organic matter. BOA4 has several PGP traits and was identified by 16S rRNA gene sequencing as belonging to the *Achromobacter xylosoxidans* phylogenetic cluster [3]. The calculated AUC for the substrates used by the BOA4 strain is indicated in Table S2 (see Supplementary Materials). Among the tested substrates, xylose, not surprisingly, was a preferred carbohydrate. Recently, Santos et al. [17] demonstrated a correlation between the genetic and nutritional diversity (carbon consumption) of *B. subtilis* rhizobacterial isolates and their ability to promote plant growth. The isolate with the highest ability to promote plant growth was the one that consumed glycyl-L-glutamic acid. Glutamic acid, synthesized from plant-absorbed

nitrogen, is a key amino acid for plant protein synthesis and for plant stress tolerance due to its participation in the production of glutathione, which is involved in ROS scavenging [21]. Herein, the calculated AUC for Glycyl-L-Glutamic acid consumption by *A. xylosoxidans* BOA4 was among the highest values. BOA4 might use its diazotrophic ability, providing nitrogen for the plant synthesis of glutamate, which could be further exudated by the plant roots for BOA4 consumption, a scenario that benefits both the plant host and the bacterium.

BOA4 was able to synthesize isopentenyladenine (iP)—a precursor cytokinin in the zeatin biosynthesis pathway—and iP concentration in the supernatant increased during growth in TYG medium (see Section 2.4), from 1.1 pmol/mL in the exponential growth phase to 4.7 pmol/mL in the stationary growth phase. A longer incubation led to higher values of iP production in the stationary phase; a maximum of 17.5 pmol/mL was determined.

Isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) are precursors for the biosynthesis of isoprenoid molecules used in diverse processes such as hormone (e.g., iP, cZ and tZ) synthesis [22]. In bacteria, and in the chloroplasts of higher plants, the biosynthesis of these precursors occurs through the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway [23]. The MEP pathway initiates with the generation of 1-deoxy-D-xylulose 5-phosphate from pyruvate and glyceraldehyde 3-phosphate. Distinct carbon sources may influence fluxes for precursors in carbon metabolism, e.g., pyruvate and glyceraldehyde 3-phosphate, and phytohormone synthesis. However, very few studies have been conducted to inspect a potential differential in phytohormone production by soil bacteria, namely in iP production, with different carbon sources. Examples of such studies are a report by Shahzad et al. [24], who showed *Bacillus amyloliquefaciens* alteration in phytohormone production when using methanol as a carbon source, and the recent work by Alfonso et al. [25], where propionate was shown to be the preferred carbon source to produce 3-indoleacetic acid in *Bacillus subtilis*, as it increases the fluxes towards precursors. Nonetheless, the pattern for carbon source use registered for BOA4 (Supplementary Materials Table S2) with a high AUC value for pyruvate is compatible with the potential for iP formation and secretion. Future studies to assess the relation between tomato root exudates, carbon source use, and bacterial phytohormone production are envisaged; pyruvic and malic organic acids, glutamic acid, and xylose are major components of tomato root exudates [26,27], a composition compatible with the carbon use profile of BOA4. Moreover, the increased release of organic acids by plant roots can constitute an important mechanism of response to environmental stress [28] and of soil bacteria recruitment. Therefore, recruited root-associated bacteria may be potential cytokinin providers under stress.

3.2. Effect of BOA4 and EI Extract on Plant Growth

Without salt, the pre-treatment of seeds with the BOA4 strain increased the final germination by 33% (Table 1). BOA4 stimulation of tomato seeds' germination in the absence of salt stress was found in our precedent work, where BOA4 and/or the EI extract enhanced germination similarly (by more than 31%) in the absence of salt [29], which could be explained by the nutrients and phytochemicals present in the EI extract and by the PGP traits of BOA4, respectively [3]. Herein, however, such stimulation from EI or the joint treatment is not registered, and the different seed and soil provenience could have been the cause for these results. As compared with the "no salt" control percentage, the germination percentage of tomato seeds under salt stress decreased only by 11%. This small decrease suggests a relative "insensitive-salt" behavior from the seeds in initial salt-watering procedures. However, BOA4 treatment caused a larger decrease of 28% (Table 1). Previous experiments with a different soil resulted, the same as above, in a similar improvement in germination (by more than 30%) for EI and BOA4 plus EI treatments under salt stress, except for BOA4, which caused also a 25% decrease in germination compared with the control without salt [29]. This BOA4 seed-coating effect on germination is unexpected considering the previous report on the BOA4 promotion of wheat seed germination on sterile filter paper under saline conditions [3]. This outcome exemplifies

the frequent differences obtained when using different plant substrates/soils. Even if the halotolerance of BOA4 has been previously demonstrated, with the growth of the bacterium up to 1 M NaCl when in a rich medium [3], the scavenging of water and other seed nutrients by the BOA4 strain might have occurred in the soil in order to cope with salt addition.

Table 1. Final germination percentage of tomato seeds in the absence and presence of salt stress †.

Treatment	No Salt Stress (%)	Salt Stress (%)
Control	72 ± 4.9 ^{a}	64 ± 9.8 ^{a}
BOA4	96 ± 4.0 ^{b}	52 ± 8.0 ^{a}
EI	76 ± 9.8 ^{ab}	60 ± 6.3 ^{a}
BOA4 + EI	68 ± 10.2 ^{a}	64 ± 7.5 ^{a}

BOA4—*Achromobacter xylosoxidans* BOA4 strain; EI—*Enteromorpha intestinalis* extract †. Values shown are means ± SEM values, which were calculated for the percentage of plants germinated in each pot. Different letters indicate means that differ significantly ($p = 0.022$).

The shoot fresh weight (FW) and dry weight (DW) of plants collected at 35 days are represented in Figure 1. Without salt addition, the EI extract led to plant growth improvement in the poor N soil. Tomato shoot FW increased by 52% and 32% following the addition of EI and EI plus BOA4 treatment, respectively (Figure 1A), while the DW increased by 61% and 32% (Figure 1B). The effect of EI extract addition persisted until day 50 (Figure 2); e.g., the addition of EI led to shoot FW and DW increases of 34% and 40%, respectively, compared with the control. The tendency for FW and DW increments when the EI extract was added to the BOA4 treated non-stressed tomato (Figures 1 and 2) could be due to benefits given by the extract to both plants and bacteria. *E. intestinalis is* rich in minerals, sugars, polyols, amino acids, and other N- and S-organic molecules, being an important source of nutrients for both plants and bacteria in soil, and participating in plant growth enhancement [3,30,31].

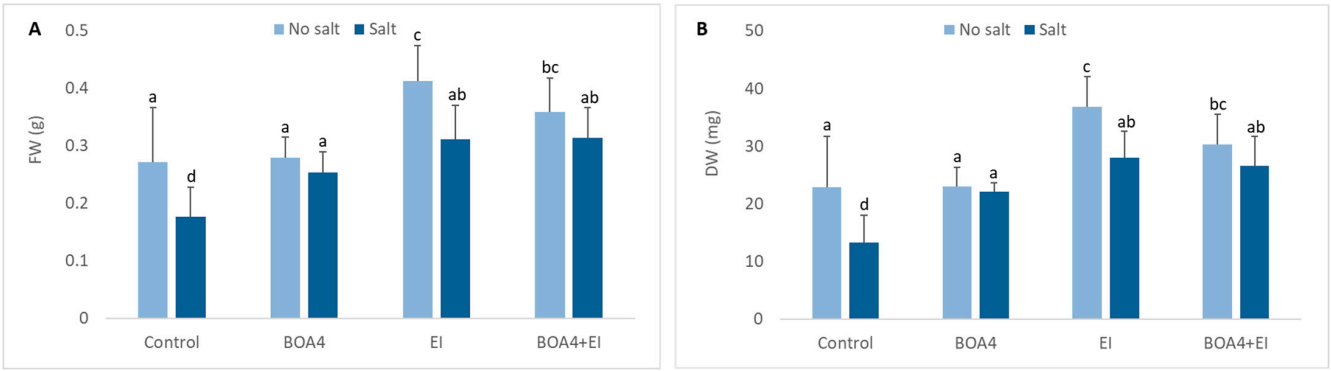

Figure 1. Shoot biomass of plants collected at day 35. Fresh weight (FW) (panel (**A**)) and dry weight (DW) (panel (**B**)) are depicted. No-salt and salt stress conditions are represented by light-colored and dark-colored columns, respectively. BOA4: *Achromobacter xylosoxidans* strain BOA4; EI: *Enteromorpha intestinalis* extract; BOA4 + EI: *Achromobacter xylosoxidans* strain BOA4 plus *Enteromorpha intestinalis* extract. Values shown are means and error bars represent standard deviation. Different letters above the bars indicate means that differ significantly ($p < 0.001$).

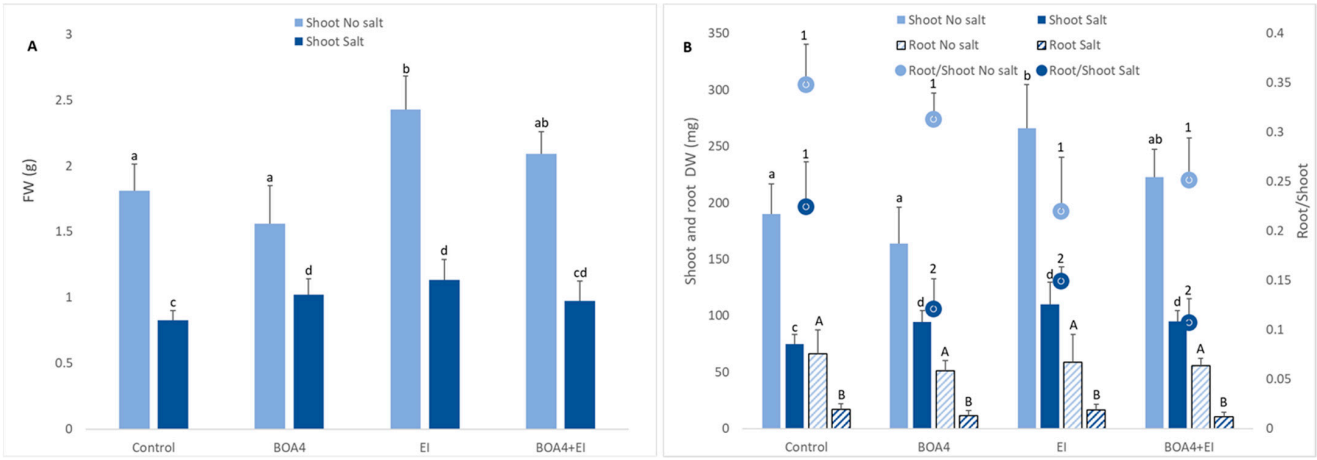

Figure 2. Shoot and root biomass and root/shoot ratio of plants collected at day 50. In panel (**A**), shoot fresh weight (FW) is depicted. No-salt and salt stress conditions are represented by light-colored and dark-colored columns, respectively. In (**B**), shoot and root dry weights are represented by filled and pattern columns, respectively. No-salt and salt stress conditions are represented by light-colored and dark-colored columns, respectively. Filled circles in (**B**) represent root/shoot ratio, light-colored and dark-colored circles correspond to no-salt and salt stress conditions, respectively. BOA4: *Achromobacter xylosoxidans* strain BOA4; EI: *Enteromorpha intestinalis* extract; BOA4 + EI: *Achromobacter xylosoxidans* strain BOA4 plus *Enteromorpha intestinalis* extract. Bars represent standard deviation. Different lower-case letters, upper-case letters or numbers above the bars indicate means that differ significantly ($p < 0.001$).

Salt stress reduced shoot FW and DW, after 35 days, by 35% and 42%, respectively (Figure 1A,B), but the bacterial inoculation and/or EI treatment restored it. EI extract, alone or with BOA4, improved tomato FW and DW (a ca twofold augmentation relative to the salt-control). As it happened for non-salinized plants, increments in FW and DW, of 24% and 20%, respectively, were observed with the addition of the EI extract to BOA4 inoculated plants. *Enteromorpha* has been previously used for bacterial growth promotion under salt stress as a source of osmoprotectants and other nutrients [32], which could explain the partial increment observed for the BOA4 plus EI treatment. Rai et al. [3] also described an increase in *A. xylosoxidans* BOA4 halotolerance given by EI extract, and this could account for the salt stress relief in the plants when subjected to bacterial inoculation and the addition of EI.

At the end of the assay, at day 50, salt stress caused an over twofold decrease in shoot FW and DW, i.e., reductions of 54% and 61%, respectively (Figure 2A,B). Even with further salt addition by additional watering with 150 mM NaCl (corresponding to an accrual of 13.5 mmol of sodium chloride/pot after day 35) treatments with BOA4, the EI extract or both still improved plant biomass, resulting in final shoot DW increments of 26%, 47% and 27%, respectively. Thus, after this longer period, FW and DW were only partially restored; for example, with the EI treatment, tomato shoot FW and DW reached 63% and 58% of the average weight of the control plants without salt, respectively.

The promoting effect BOA4 has on plant growth observed under salt stress conditions could be due to mechanisms already described for other PGP. For instance, BOA4 might improve nutrient acquisition of salt-stressed plants via its ability for nitrogen fixation and phosphate solubilization, which are important in salinized soils (see also Section 3.3), and siderophore production for root iron uptake; iron bioavailability to plants is reduced in saline soils and siderophore-expressing rhizobacteria may tackle Fe limitation in saline soils. Additionally, a positive effect of BOA4 on osmoprotectants accumulation by the plant, as already described for PGPR [5,33,34], may be envisaged. In our previous work, using a different soil and less extensive salt-stress conditions, we have indeed observed that BOA4 inoculation leads to an increase in proline content in tomato [29]. Proline is

known to accumulate in both plants and bacteria, contributing to salt stress resistance as an osmolyte and a ROS-detoxifying molecule [35,36]. Other BOA4 mechanisms associated with plant growth promotion under salt stress could be related to an effect of the bacterial strain on the plant hormonal balance (Section 3.3).

3.3. Effect of BOA4 and EI Extract on Root/Shoot Ratios

Increased salinity drastically reduced tomato root biomass (ca 4-fold), which did not increase with EI treatment and/or the bacterial inoculation (Figure 2B). A reduction of 35% in the average root/shoot ratio was observed under salt stress. This ratio decreased further with the addition of EI and/or BOA4 inoculation (*a* 46%, 34% and 52% reductions for BOA4, EI, and joint treatments, respectively, compared with the salt control). These reductions differ from the results yielded in many reports regarding the effect of salt stress on shoot–root partitioning and the influence of PGPR on the development of roots of salinized plants ([7] and ref therein). However, salinity conditions, crops and cultivars, and growth substrates are factors that will affect root-shoot partition. For example, the shoot and root biomass accumulation by *Sulla carnosa* under non-saline and saline conditions, as well as the effect of saline-tolerant bacterial inoculants on those parameters, was dependent on the plant provenance [37].

Phytohormone production by rhizobacteria may interfere with the plant hormonal balance, namely with main hormonal pathways involved in regulating root development [38,39]. BOA4 is an IAA producer [3] and we have shown that BOA4 is also an iP producer. The BOA4 effect on the root/shoot ratio was similar to that reported for inoculated lettuce seedlings with cytokinin-producing *Bacillus*, which lowered the root/shoot ratios by stimulating shoot growth under conditions of water sufficiency and deficit [40]. Cytokinins may lower carbon allocation to the roots, an inhibition relieved under stress when cytokinin content decreases [41], with a consequent activation of root growth [42] for optimal water extraction from the soil [43]. Additionally, reductions in the cytokinin content reduce stomatal density, and thus leaf transpiration, thereby lowering the chance of foliage dehydration [44]. On the other hand, cytokinins are necessary for shoot growth and photosynthesis [42]; a decrease in their contents under drought may in fact be deleterious for droughted plants. Arkhipova et al. [40] concluded that compensating for the loss of cytokinins by bacterial soil inoculation may have a beneficial result in a set-up where the depth of rooting is not a critical factor in accessing water.

The iP secreted by the BOA4 strain might have caused tomato cytokinin biosynthesis, namely trans and cis-zeatin-type cytokinins, through distinct metabolic flows [16]. Both bacterial and plant cis-zeatin-type cytokinin synthesis involve the formation of isoprenylated tRNA by the activity of tRNA-isopentenyltransferases (tRNA-IPTs), and further modification of the isopentenylated tRNA to contain cytokinins, which are released upon tRNA turnover [16,45]. As an example, isopentenylated tRNA was shown to be the source of extracellular isopentenyladenine in a Ti-plasmidless strain of *Agrobacterium tumefaciens* [46]. Data suggest that the degradation of tRNAs, rather than isoprenylation by IPTs, is the rate-limiting step of cZ biosynthesis [16]. As increased tRNA turnover has been found under various stress conditions [16], these may lead to increased cZ levels. In this context, we performed an assessment of the cytokinin content in salt-stressed tomato plants. Trans-zeatin (tZ), cis-zeatin (cZ), cis-zeatin riboside (cZR), isopentenyladenine (iP), and isopentenyladenosine (iPR) were the cytokinins detected in shoot extracts from these plants (Figure 3). BOA4 in fact caused an alteration of cytokinin proportions in tomato shoots, as it drastically increased iP (3.5-fold), and increased cZ and cZR contents by 35% and by 19%, respectively, over the control values.

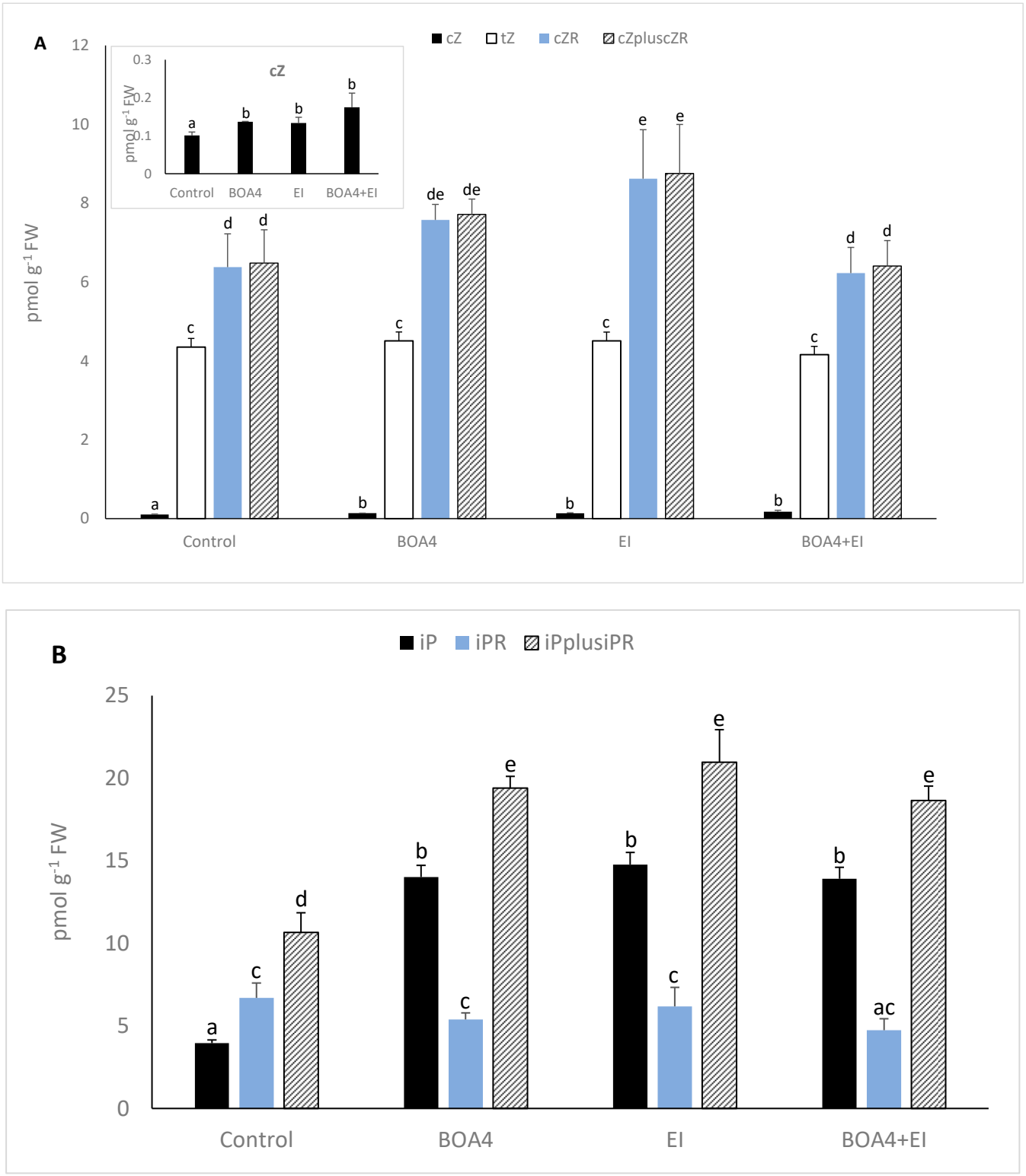

Figure 3. *Cont.*

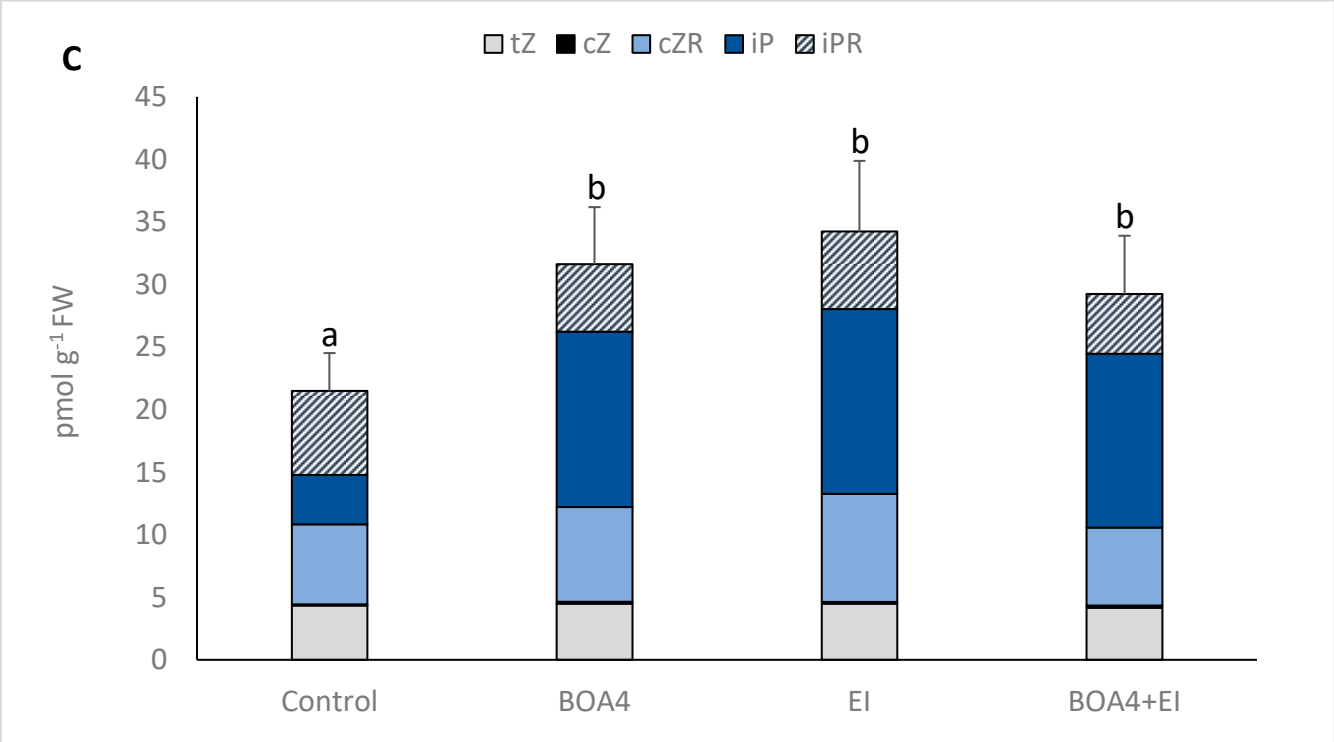

Figure 3. Shoot hormone content in salinized tomato. The hormones were quantified in shoots of plants grown in a greenhouse for 50 days. Identified bioactive cytokinins trans-zeatin (tZ), cis-zeatin (cZ), cis-zeatin-riboside (cZR), and cZ plus cZR are represented in panel (**A**). The smaller chart within panel (A) has a different Y-scale for better visualization of cZ content. Isopentenyladenine (iP) and the corresponding riboside, isopentenyladenosine (iPR), and iP plus iPR are represented in (**B**). Total and relative cytokinin contents for each treatment are represented in (**C**). BOA4: *Achromobacter xylosoxidans* strain BOA4; EI: *Enteromorpha intestinalis* extract; BOA4 + EI: *Achromobacter xylosoxidans* strain BOA4 plus *Enteromorpha intestinalis* extract. Different letters above the bars indicate means that differ significantly ($p < 0.05$).

Stress responses involve growth suppression associated with the formation of stress-protective compounds; less active cZ(R) may then replace tZ(R), which has high cell division promoter activity, assuring minimal cytokinin activity [16]. Silva-Navas et al. [47] showed that phosphate (Pi) starvation in *Arabidopsis* increases the cZ(R): tZ(R) ratio and Pi allocation to the roots. Herein, although the soil P_2O_5 content was high, 128 mg kg^{-1}, the addition of sodium chloride might have increased the requirement for phosphorus in tomato plants [48]. The substantial increment of cZ content but not of tZ supports the involvement of cZ in tomato response (and improved tolerance) to salt stress (Figure 3A). On the other hand, iP(R) [49] and the derived tZ(R) [50] accumulation have been implicated in faster shoot formation and reduced sodium ion accumulation in salinized plants. A timeline analysis of cytokinins would have been important to determine if the tZ(R) content was incremented by the bacterial (and/or EI) extract during the greenhouse assay at an earlier stress, since the shoot increment was observed earlier, as illustrated for the plants grown for 35 days (Figure 1).

EI treatment under salt stress resulted in 32% and 35% increments of cZ and cZR contents, respectively (Figure 3A); analogously to BOA4 treatment, the higher cZ(R) content may indicate a higher potential for the plant to cope with the imposed stress. It is known that seaweeds contain phytohormones [51]; an HPLC-MS analysis of several seaweed species from various taxa revealed that zeatin, IP and IP derivatives are mainly cytokinins and cis-zeatin forms are more common than trans-forms [52]. Moreover, adenine-based cytokinins show stability after autoclaving [53], thus hinting at their presence in auto-

claved dried EI extracts. However, EI extracts might have donated other compounds that contribute to tomato shoot growth and improved tolerance to salinity. Mathur et al. [54] identified benzoic compounds, reported as plant growth regulators, in *E. intestinalis* extract prepared from dried seaweeds by autoclaving.

The drastic iP accumulation for BOA4-treated salt-stressed plants, which might have been a direct consequence of BOA4 iP production, as well as for the plants treated with EI and BOA4 plus EI, respectively, of 3.7 and 3.5-fold compared with the stressed control plants (Figure 3B), could have also contributed to the observed tomato phenotype and increased salt tolerance. Isopentenyladenine was among the cytokinins with an enhanced accumulation in tomato transformants overexpressing SlIPT3 isopentenyltransferase, showing improved tolerance to salinity [49].

3.4. Leaf Content in Anthocyanins and Carotenoids

The spectral reflectance of leaves was measured to calculate the reflectance indices (Figure 4). Leaf pigment vegetation indices designed to provide a measure of stress-related pigments, carotenoids and anthocyanins, were significantly different among treatments. Anthocyanin reflectance indices ARI1 and ARI2 were higher under salt stress, and they increased by 35% and by 30% at 150 mM NaCl compared with the control without salt, respectively, indicating higher concentrations of anthocyanins in plants. These indices increased further with the addition of EI and with the BOA4 plus EI treatment; ARI1 and ARI2 increased by 34% and 37%, respectively, for EI addition and by 48% and 44% for the joint treatment, as compared with the 150 mM NaCl control (Figure 4B). Anthocyanins have a photoprotective function, having the ability to reduce chlorophyll photoinhibition, which is related to ROS production during stress, by modifying both the quantity and quality of light incident on chloroplasts [55,56]. It is known that anthocyanin accretion may be induced by abiotic stresses including drought and salinity [57]. Exogenous cytokinins have been shown to induce anthocyanin synthesis in plants [58,59]. Hence, BOA4 and EI extract, which led to higher tomato cytokinin content under salt stress (Figure 3C), might have contributed in this manner to anthocyanin accumulation. On the other hand, the addition of BOA4 alone did not result in an enhanced accumulation over the salt control's anthocyanin level, suggesting that the balance between different hormones, including cZ(R) and iP(R), might control anthocyanin synthesis under stress. The accumulation of anthocyanin in black carrot root following supplemental UV-B exposure was linked to a distinct hormonal profile and correlated with increased levels of cZ and tZR [60]. Here, the joint treatment, with the highest ARI values, did not increase the cZR level more than the salt control, but showed a 1.7-fold increase in cZ. Ours and other authors' data evidence the importance of further study of the potential role of cis-zeatin-type cytokinins, and their balance with other phytohormones, in anthocyanin-induced biosynthesis.

NDVI, which translates broadband greenness, decreased by 11% under salinity conditions and remained similar among treatments; salt had no significant effect on the NDVI spectral index, except for the BOA4 coating, which caused a 29% decrease in NDVI compared with the control value without salt (Figure 4A). It has been shown that increased anthocyanin levels seem to impair the use of NDVI to course greenness and chlorophyll content adequately [61], an effect that might account for the overall similarity of the NDVI values.

Under salt stress, carotenoid reflectance indices were reduced by salt addition in the control and BOA4 treatments (Figure 4C), but the addition of EI increased CR indices to non-stress control levels; EI and BOA4 plus EI treatments showed CR indices with average values higher and similar, respectively, than the corresponding values without salt; e.g., CRI2 increased by 55% with the addition of EI, as compared with the value for EI treatment of non-stressed plants. When compared with the BOA4 treatment under salinity, CRI1 and CRI2 increased by 92% and by 83%, respectively, with EI (by 39% when compared with the 150 mM control); these higher values indicate a greater concentration of carotenoid relative to chlorophyll. Carotenoids are powerful antioxidants and free radical

scavengers [62], having a role in stress conditions. Water deficit or salinity have been reported to depress the content of carotenoids in plant tissue [63,64], whose destruction is a consequence of the continuous formation of reactive oxygen species in the thylakoids [65]. Carotenoids are derived from IPP and DMAPP (Section 3.1); thus, as it happens in the synthesis of cytokinins, carotenoids synthesis is based on the MEP pathway in the plastids of plants. Consequently, a metabolic trade-off between the synthesis of carotenoids and cytokinins can be envisaged, as both syntheses require common precursors. Both BOA4 and EI increased cZ(R) and iP contents; while in BOA4, this could result in a decreased flux to carotenoid accumulation, the potential richness of the EI extract, e.g., in phenols and carotenoids [54,66], might have circumvented that process.

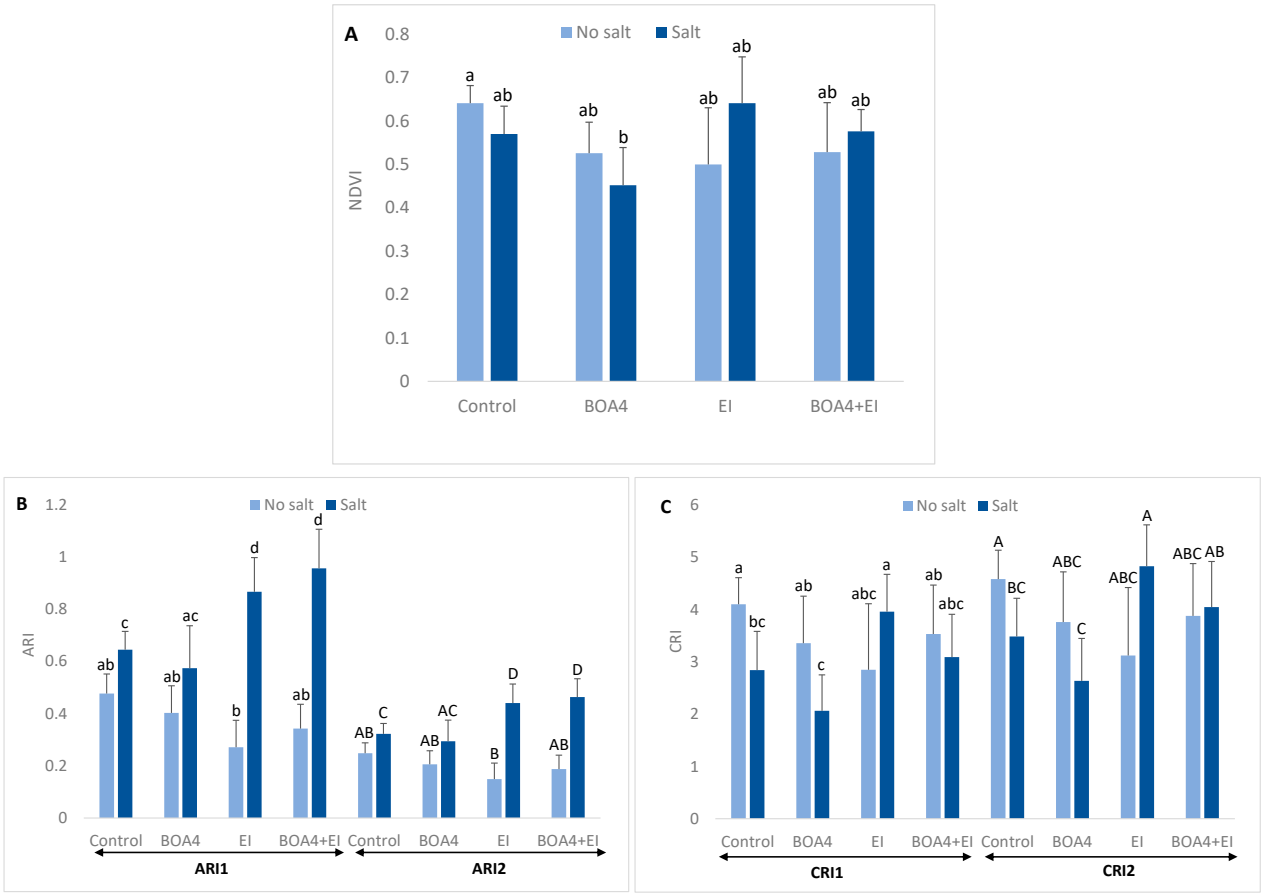

Figure 4. Reflectance indices determined for 50-day-old plants subjected to the different treatments. NDVI (panel (**A**)); ARI indices (panel (**B**)); CRI indices (panel (**C**)). No-salt and salt stress conditions are represented by light-colored and dark-colored columns, respectively. BOA4: *Achromobacter xylosoxidans* strain BOA4; EI: *Enteromorpha intestinalis* extract; BOA4 + EI: *Achromobacter xylosoxidans* strain BOA4 plus *Enteromorpha intestinalis* extract. Bars represent standard deviation. Different lower-case or upper-case letters above the bars indicate means that differ significantly ($p < 0.02$, $p < 0.001$ and $p = 0.01$, for panels (**A**), (**B**) and (**C**), respectively).

3.5. Effect of BOA4 and EI Extract on Tyrosine Nitration

Peroxynitrite, an RNS, is a major contributor to PTN. Its increased synthesis is linked to ROS generation during stress conditions, where both ROS and RNS lead to nitro-oxidative stress [67]. Thus, tyrosine nitration could be simply the consequence of stress and a biomarker of nitro-oxidative stress. However, in healthy, unstressed plants, a certain level of nitration is present, i.e., plants have a physiological nitroproteome [68], a fact that points to a regulatory role for PTN, which has been associated with the processes of cell growth and division, and development [68]. Herein, we registered an increase of ca 36% in 3-NT

content for the roots of non-stressed tomato treated with BOA4 or EI extract, and of 29% with the joint addition (Figure 5B). These results indicate that an increase in PTN is not always a consequence of stress onset, suggesting that the knowledge of which proteins are nitrated together with the extent of nitration would be important to distinguish plant physiological status under non-stress conditions, as well as stress levels.

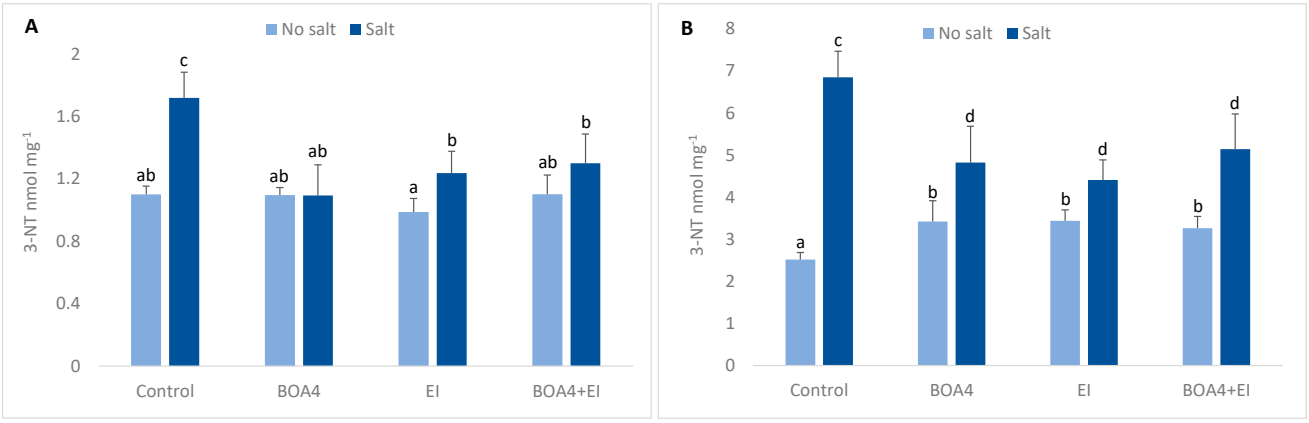

Figure 5. Nitrotyrosine (3-NT) content of tomato plants is represented for the different treatments. Panel (**A**) shows shoots 3-NT content and panel (**B**) roots 3-NT content. No-salt and salt stress conditions are represented by light-colored and dark-colored columns, respectively. BOA4: *Achromobacter xylosoxidans* strain BOA4; EI: *Enteromorpha intestinalis* extract; BOA4 + EI: *Achromobacter xylosoxidans* strain BOA4 plus *Enteromorpha intestinalis* extract. Bars represent standard deviation. Different letters above the bars indicate means that differ significantly ($p < 0.001$).

Tyrosine nitration values were increased under salt stress; 3-NT level increased 2.7-fold and 1.6-fold in roots and shoots, respectively, compared with the control level without salt (Figure 5A,B). However, the bacterial inoculation and/or the EI extract caused a significant 3-NT reduction either in the roots or shoots of salinized tomato. Decreases of 30%, 36% and 25% were registered for tomato roots treated with BOA4, EI or both, respectively. In the case of shoots, the reductions of 36%, 28% and 24% were observed, respectively.

PTN may directly regulate plant cell growth because 3-NT may impair microtubular functions, causing a dose-dependent inhibition of cell division [69]. In accordance with this, the decrease in tyrosine nitration registered for the BOA4 and EI treatments under salt stress might have been associated with a biomass increase thanks to "microtubular stress-relief" and consequent cell growth. The lower root/shoot biomass ratio, mentioned in Section 3.3, was suggested to be the result of phytohormone-intervened processes resulting from increased cytokinin levels. Phytohormone-dependent processes, such as those involved in cell division and differentiation, are known to be linked to NO (reviewed by [14]), a pivotal molecule in PTN. For example, NO mediates the auxin-regulated processes the of inhibition of primary root growth and the promotion of lateral root formation [70]. NO repress cytokinin signaling by inhibiting the phosphorelay activity central to the signaling transduction of this hormone [71]. A distinct hormonal balance and a NO–cytokinin antagonistic interaction fits our finding of a lower root/shoot ratio and a lower PTN (lower NO-derived RNS) for BOA4 and/or EI treatments of stressed tomato, but a higher cytokinin content.

PTN has been described as an oxidative post-translational modification that disrupts NO signaling and curves metabolism to pro-oxidant processes [72]. In this context, processes antagonistic to high NO levels and consequent extensive PTN are worth mentioning. Trans-zeatin can rescue mutant phenotypes in *Arabidopsis* resulting from high levels of endogenous NO, and peroxynitrite reacts with cytokinins in vitro (namely with isopentenyladenine, which we found to be produced by BOA4 and increased in bacterial and/or EI-treated salinized tomato) [15]; thus, cytokinins can act as NO suppressors. Anthocyanins, herein further accumulated by EI addition in salinized plants, constitute potent inhibitors in

the formation of nitrated tyrosine in vitro, by scavenging peroxynitrite [73,74]. By causing a reduction in PTN levels, the bacterial and/or EI treatments under salt stress might have the potential to shift plant cells from tyrosine nitration stress levels towards NO signaling involved in stress tolerance and growth, which is compatible with the cytokinin-mediated promotion of shoot growth. Indeed, under salt stress, PTN levels were decreased (Figure 5) while cytokinin cZ(R) and iP levels were increased by the addition of BOA4 or EI extract (Figure 3A,B), suggesting an inverse correlation between these cytokinins and PTN.

4. Conclusions

We have shown that the seed coating with *A. xylosoxidans* BOA4, the addition of EI extract, or both treatments improved tomato growth under a high-salt regimen, resulting in an averaged increase of 33% in plant shoot DW. These results indicate the potential for the utilization of the bacterium and/or EI extracts in crops in high-salinity conditions. We identified isopentenyladenine and cis-zeatin-type cytokinins as the responsive plant cytokinins to the applied treatments, confirming previous data on the role of cis-zeatin-type phytohormones in abiotic stress relief. The improved plant salt tolerance was herein correlated with a PTN decrease and associated with observed phenotypic traits, such as the anthocyanin accumulation in EI-treated salinized tomato. Additionally, a potential antagonistic relationship between cis-zeatin-type and iP cytokinins, increased by BOA4 and EI treatments, and PTN can be postulated, which requires further research. BOA4 itself was shown to be a cytokinin producer, and the potential differential in this phytohormone production with different carbon source use patterns in the rhizosphere should be studied. Overall, this report presents new directions in the research of plant tolerance to stress mediated by cytokinin-producing bio-stimulants.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12040934/s1, Supplementary Table S1: Soil physicochemical properties, Supplementary Table S2: Biolog substrates used by *Achromobacter xylosoxidans* BOA4.

Author Contributions: Conceptualization and investigation, M.M.S., A.R. and C.C.; methodology, A.P.R. and A.M.Z.; writing—original draft preparation, M.M.S.; writing—review and editing, J.M.G.-M. and C.C. All authors have read and agreed to the published version of the manuscript.

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