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Improved production and quality of peppers irrigated with regenerated water by the application of 24-epibrassinolide

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ABSTRACT

Water shortage for crop irrigation is reducing agricultural production worldwide and the use of sewage treatment plant (STP) water to irrigate horticultural fields is a solution to avoid the use of drinkable water in agriculture. In this study, two different genotypes of pepper (Red Cherry Small and Italian green) were irrigated with STP water, as an alternative to potable water. Moreover, the foliar application of a molecule with biostimulant properties (24-epibrassinolide; EBR) was tested as a strategy to ameliorate the production and quality of fruits. Both genotypes differed on their tolerance to the suffered oxidative stress due to their different salinity tolerance, but fruit commercial weight was reduced by 49% on the salt sensitive and by 37% on the salt tolerant. Moreover, ascorbic acid was also decreased by 37% after STP water irrigation in the Red Cherry Small peppers. However, EBR applications alleviated STP watering stress effects improving pepper plants fruit production and quality parameters, such as ascorbic acid and capsaicinoids. These results have important economic and environmental relevance to overcome present and future water deficiencies in the agricultural sector derived from climate change, guaranteeing the maintenance of production in peppers irrigated with STP water for a more sustainable agriculture following relevant circular economy actions.

1. Introduction

Plants are continuously exposed to numerous stresses that limit their development and net production wherever they are cultivated. Climate change is causing serious problems of water scarcity, affecting global crop irrigation (Lu et al., 2019), which is one of the major challenges in order to make up for the global food demand. To solve the negative impact of this lack of water on the agricultural sector, the European Parliament and Council Directive 2000/60EC establishing a framework Community action in the field of water policy proposes using water resources more efficiently and using non-traditional resources such as regenerated water from sewage treatment plant (STP). In this way, many countries have decided to convert sewage into a principal resource for irrigation of their crops in order to fulfil the requirement and address the water shortage problem (Hashem and Qi, 2021).

Globally, the use of these waters is a benefit to meet current demands, by taking advantage of the water resources at our disposal, while improving crop profitability. However, at local level, regenerated water is not free of inconveniences as, due to their nature and composition, they present problems associated with the high contents of solutes, which can cause osmotic stress in crops (Hashem and Qi, 2021). This increase in salts available at root level has a detrimental effect on crops, since most plants used in the agricultural sector are highly sensitive to saline environments. Under these conditions, a reduction in osmotic potential in roots ends up causing stomatal closure, resulting in a reduction on CO_2 assimilation rate, making the absorption of light exceed the demand of the Calvin-Benson cycle. In this way, the electron transport chain is affected, and several photoinhibition processes can occur by formation of reactive oxygen species (ROS; Pintó-Marijuan and Munné-Bosch, 2014; Toscano et al., 2023). If plant cells are unable to effectively reduce ROS with the various antioxidant systems they possess, it causes intracellular imbalance and ROS can cause damage to multiple cell structures (proteins, lipids, DNA, and RNA). Moreover, the high accumulation of ROS gives rise to oxidative stress with a reduction

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of photosystems efficiency, which has negative effects on plant growth and performance, leading to significant losses of productivity (Shahid et al., 2020). Antioxidant systems developed by plants to reduce ROS levels and maintain a suitable redox balance are very diverse (Kohli et al., 2019; Mansoor et al., 2022). First, strategies given to ensure photoprotection and minimize photoinhibition of photosystem II (PSII) are based on decreasing the excessive absorption of light and, hence, reduce ROS production. But, if ROS are overproduced, plants have mechanisms aimed at detoxification, classified into enzyme antioxidant systems and non-enzymatic antioxidants, which include ascorbic acid, glutathione, carotenoids and tocopherols (Foyer and Noctor, 2005; Munné-Bosch and Alegre, 2002; Takahashi and Badger, 2011).

Pepper is a high-quality crop whose fruit is enriched in anthocyanins, ascorbic acid, phenolic compounds, minerals, flavonoids, carotenoids, capsaicin and many vitamins, among others (Hernández-Pérez et al., 2020), meaning that it presents a wide variety of antioxidants and compounds important to human health. Because there has been a high interest in developing several nutritional and culinary qualities of the various genotypes of *Capsicum annuum*, peppers have been subjected to breeding for centuries and, nowadays, they can be classified in different groups depending on the morphology and purpose of their consumption. There are peppers that are often noted for their pungent character, due to their high content in capsaicinoids (Srinivasan, 2016), while other peppers are highly appreciated for their sweetness (Antonio et al., 2018), altogether making peppers one of the most cultivated horticultural crops worldwide. Particularly, in Europe, where *Capsicum annuum* was one of the crops with the largest annual production, with 3583,286 tons in 2020 (FAOSTAT, 2022). Nevertheless, the production of this horticultural crop requires a considerable amount of water which could be intended for human or animal drinking. The lack of water might cause severe consequences on crops reducing their production drastically (Steduto et al., 2012). The use of STP water at specific times during the plant growth is emerging in some areas to save potable water for drinking purposes. Therefore, it is of great interest to assess pepper crop responses to irrigation with STP water and look for mechanisms to maintain production, avoiding the negative effects of oxidative stress caused by STP irrigation.

In this sense, biostimulants, which are defined as substances whose function when applied to plants or in the rhizosphere is to stimulate natural processes to improve/facilitate absorption of nutrients, their efficiency, abiotic stress tolerance and crop quality (du Jardin, 2015), might be a good strategy to implement STP watering. The active compounds of biostimulants allow plant cells to establish different mechanisms to overcome the stresses to which they are subjected, acting on primary or secondary metabolism such as increasing antioxidant synthesis or improving photosynthetic efficiency, among others (Bulgari et al., 2019). After approval of its use by the European Fertilizer Regulation (EU) 2019/1009, and its classification and differentiation outside of the fertilizer category, its application has been studied in several crops (Liebig et al., 2020) such as pepper (Barrajón-Catalán, et al., 2020).

The use of 24-epibrassinolide (EBR), a brassinosteroid (polyhydroxylated steroid hormone) involved in plant growth and development, and also in response to abiotic stresses, has had advantageous effects in numerous studies for different crops of economic interest subjected to abiotic stress factors (Ahanger et al., 2020; Bajguz and Hayat, 2009; Jan et al., 2018). EBR has been approved for its use in Executive Regulation (EU) 2021/427 of Commission after being evaluated by the European Food Safety Authority (EFSA). The different effects of foliar application of brassinosteroids on several horticultural species were reviewed in Ali (2017), where an improvement in the photosynthetic rate, a reduction in ROS accumulation and an improvement in antioxidant systems, as well as production and total biomass, were highlighted among others in pepper plants. Precisely this capacity of the brassinosteroids to control numerous agronomic traits has promoted their study to be used as biostimulants in the agricultural sector.

This study hypothesizes that STP watering, with high content of salts, will elicit oxidative stress in the two different *C. annuum* genotypes and reduce quantity and quality of peppers. Moreover, we expect that the foliar application of a brassinosteroid (such as EBR) will improve the quality of pepper fruits and increase commercial production of both genotypes. Therefore, we propose that EBR can be used as a potentially biostimulant molecule to overcome the stress effects of STP watering and improve both harvested yield and quality of the peppers from the stressed plants promoting the use of STP non-drinkable water for agricultural purposes leading to a more sustainable world.

2. Materials and methods

2.1. Experimental design, plant material and STP water analysis

Two genotypes of *Capsicum annuum* with different capacity to tolerate salinity stress were chosen: the tolerant genotype was Red Cherry Small pepper (Salt Tolerant, ST) and the sensitive genotype was the Italian green pepper (Salt Sensitive, SS). Seeds from both genotypes were purchased in a seed company (Rocalba, SA., Girona, Spain), and the experiment was conducted in a greenhouse located in the Faculty of Biology at the University of Barcelona (Barcelona, NE Spain).

All plants were irrigated with half Hoagland's nutritive solution during their growth. In order to recreate the conditions under which this crop grows in the horticultural fields around Barcelona as accurately as possible, regenerated water was applied from the STP of Gavà (Barcelona, Spain) with several ion concentrations three times higher than tap water (Table S1). To analyze the STP water composition, measurements of pH, electrical conductivity and ionic concentration were performed. The sensor of pH and conductivity (HI 98129 Combo Waterproof, Hanna Instruments, Woonsocket, RI, USA) was used to adjust STP water pH to 6.5 with NaOH and measure its conductivity. Analysis of ions present in STP water were carried out with a multiparametric ion analyzer for the measurement of calcium, chloride, potassium, nitrate, magnesium, sodium and ammonium (Imacimus Multi Ion, El Catllar, Spain; Table S1). Three irrigation events with 1 L/pot were applied to irrigate controls (C, tap water) and STP (STP water) treatment. Three leaf samplings (1st, 2nd and 3rd) were performed two weeks after each irrigation event (Fig. S1).

To assess the ability to overcome the harmful effects of the stress caused by STP irrigation, EBR (Merck Life Science, S.L.U, Madrid, Spain) was applied on leaves by spray. EBR concentrations were chosen following previous studies (Li et al., 2015; Ali, 2017): E1, 0.1 μM EBR; E2, 0.5 μM EBR; E3, 1 μM EBR; E4, 2 μM EBR; and E5, 3 μM EBR. Solutions for exogenous EBR application were prepared by diluting pure EBR in de-ionized water. All solutions contained 0.015% of Tween-20 as a surfactant. De-ionized water with 0.015% Tween-20 was sprayed to the controls. Foliar applications of 1 L/plant were sprayed one week before each of the three irrigation events. Leaf samplings were performed two weeks after the 2nd irrigation event and fruit samplings were performed once in the ST genotype and twice in the SS genotype (Fig. S1).

Thus, two different controls were available, one corresponding to the optimal growth of the crop (C) and the other corresponding to the induction of stress (STP) with no EBR application, and five treatments corresponding to the different EBR concentrations (irrigated with STP water). Six biological replicates were used for each condition (treatment/genotype), with a total of 42 plants of each genotype.

2.2. Foliar stress measurements

2.2.1. Relative water content

To measure the RWC, fully-expanded young leaves were chosen. One leaf per plant was weighed taken directly from the plant to have the fresh weight (FW). After 24 h at 4 ºC, it was again weighed to obtain the turgid weight (TW). Finally, it was left to dehydrate at 60ºC for seven days (until constant weight) and the dry weight (DW) was obtained.

RWC was then calculated as: RWC (%) = 100 x (FW – DW / TW – DW).

2.2.2. Maximum efficiency of PSII

To perform chlorophyll fluorescence measurements, we used a MiniPam II fluorimeter (Heinz WalzGmbh, Effeltrich, Germany) and we chose fully-expanded young leaves. Maximum efficiency of PSII (F_v/F_m) was measured as an indicator of photoinhibition of the photosynthetic apparatus on leaves that were adapted to darkness at least 60 min

2.2.3. Methanol extractions

For analysis of hormonal content, tocochromanols, photosynthetic pigments and lipid hydroperoxides, fully-expanded young leaves were chosen in each leaf sampling (Fig. S1), which were immediately frozen into liquid nitrogen. For the quantification of phytohormones, tocochromanols and photosynthetic pigments, 100 mg of frozen leaves tissue were ground to powder, and extraction of different compounds was performed with 300 μL of cold methanol. After vortex, the extracts were ultrasonicated (Bransonic ultrasonic bath 2800, Emerson Industrial, Ferguson, MO, USA) at 4 ∘C for 15 min and centrifuged at 13000 rpm for 10 min at 4 °C (Centrifuge MR18–22, Jouan, France). To dispose of internal standards needed for hormonal content with subsequent chromatographic analysis, the corresponding labeled forms were incorporated during extraction. The supernatant was collected, and the pellet was re-extracted twice, pooling at the end all supernatants to proceed with measurements. To quantify lipid hydroperoxides (LOOH) in the sample, 100 mg of leaves tissue were ground to powder, and extraction was performed with methanol containing 0.01% w/v butylhydroxytoluene (BHT), following the same extraction procedure as described for phytohormones quantification without the addition of the phytohormones labeled forms.

2.2.4. Phytohormones

From the obtained extracts, and after filtering with a 0.22 μm PTFE filter (Phenomenex, Torrance, CA, USA) we took 200 μL for analysis of the hormonal content using ultra high-performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry (MS/MS) as described by Müller and Munné-Bosch (2011) with slight modifications. Using this fraction of the extract, we analyzed the content of endogenous hormones, including abscisic acid (ABA), brassinosteroids, cytokinins (CKs), gibberelins (GAs), indolacetic acid (IAA), jasmonates, salicylic acid (SA) and brasionosteroids (castasterone, CAST; brassinolide, BL; epibrassinolide, EBR) and the labeled forms of the compounds d_6-ABA, d_6-2iP, d_6-IPA, d_5-Z, d_5-ZR, d_4-GA_1, d_2-GA_4, d_2-GA_7, d_5-IAA, d_5-JA, d_4-SA, d_3-CAST and d_3-BL were added as internal standards. The equipment consisted of an Aquity UPLC™ System (Waters, Milford, MA, USA) quaternary pump equipped with an autosampler. For the analysis of the extracts, a LUNA column C18 1.6 μm, 100 × 2.1 mm (Phenomenex Inc., Torrance, CA, USA) was used. Gradient elution was performed with water and 0.05% glacial acetic acid (solvent A) and acetonitrile with 0.05% glacial acetic acid (solvent B) at a constant flow rate of 0.5 mL min-1. The gradient profile for CKs and brassinosteroids was applied as follow (t (min), % A): (0, 99), (0.5, 99), (3.50, 0), (3.70, 0), (3.90, 99), (5, 99). The gradient profile for ABA, JA, SA, IAA, and GAs was applied as follow: (t (min), % A): (0, 99), (2, 0), (2.20, 0), (2.40, 99), (3, 99). MS and MS/MS experiments were performed on an QTRAP 4000 triple quadrupole mass spectrometer (PE Sciex, Concord, Ont., Canada). For the analysis of the extracts temperature was 400 °C, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 20 (arbitrary units), collision gas (N_2) 4 (arbitrary units) and the capillary voltage was 3.5 kV for CKs and brassinosteroids and − 3.5 kV for ABA, JA, SA, IAA, and GAs, respectively. Quantization was done considering the retention times of each display, calibration curves of standards and deuterated internal standards, using the MultiQuantTM 3.0.1 software (Ab Sciex S.L., Framingham, MA, USA).

2.2.5. Tocochromanols

From the same extract prepared for phytohormone analysis, the different homologues of vitamin E (tocopherols and tocotrienols) were separated by HPLC system (composed by Waters (Milford, MA, USA) 600 controller pump and Waters 717 plus auto-sampler) at room temperature using an Inertsil 100 A column (5 μm, 0.03 × 0.25 m, GL Sciences Inc., Tokyo, Japan) and quantified with a FP-1520 fluorescent detector (Jasco, Tokyo, Japan), as described by Amaral et al. (2005). A calibration curve was made with authentic standards (Sigma-Aldrich, Steinheim, Germany) for each of the tocopherols and tocotrienols analyzed.

2.2.6. Photosynthetic pigments

Finally, from the same extract used for phytohormones and tocochromanols, 50 μL were taken to dilute the extract 1/20 (v/v) with methanol and estimate pigments content spectrophotometrically (Cecil Aquarius CE7400, Cecil Instruments, Cambridge, UK). Chlorophyll content a, b, and total carotenoids were quantified by measuring absorbances at 470, 653, 666 and 750 nm, following the equations described by Lichtenthaler and Buschmann (2001).

2.2.7. Lipid hydroperoxides

The quantification of lipid hydroperoxides was performed spectrophotometrically by the ferrous oxidation-xylenol orange assay method described by Bou et al. (2008). Briefly 150 μL of the specific methanol extracts (0.01% BHT, w/v) were used for analyses using the Fox-2 reagent (consisting in a solution of 90% methanol [v/v] containing 25 mM sulfuric acid, 4 mM BHT, 0.25 mM iron sulphate ammonium (II) and 0.1 mM xylenol orange). Absorbances were measured at 560 and 800 nm using a plate spectrophotometer (xMarkTM Microplate Absorbance Spectrophotometer, Bio-Rad, Berkeley, CA, USA). A calibration curve using hydrogen peroxide at various concentrations was used for quantification.

2.3. Fruit quality

2.3.1. Italian green: total and commercial productivity and total soluble solids

During sampling, production was measured by the number of peppers produced by each plant, as well as by each fruit weight and length. Commercial peppers were considered when peppers weighed more than 30 g according to the Commission's Executive Board (EU) No 543/2011.

Fresh pepper tissue was ground using a grinder (StrongTitanium 19000, Cecotec, Valencia, Spain) to prepare extracts. The total soluble solids (TSS) value of the extracts was directly measured with a digital portable refractometer (HI 96800 Digital Refractometer, Hanna Instruments) evaluating 200 mL of extract to obtain ̊Brix (Boulton et al., 1999).

2.3.2. Red Cherry Small: total and commercial productivity, ascorbic acid and capsaicinoids

Production was estimated by the number of peppers produced by each plant, as well as by each fruit weight and diameter. Commercial peppers were considered when peppers diameter was greater than 2 cm.

For ascorbic acid and capsaicinoids extraction, three representative pepper fruits/plant were frozen into liquid nitrogen during the sampling and lyophilized and ground to powder.

For measurements of vitamin C contents, ascorbic acid (AsA) (reduced) and dehydroascorbate (DHA) (oxidized) were determined using ascorbate oxidase (AO) and dithiothreitol (DTT), respectively, as described in Queval and Noctor (2007) with some modifications. Briefly, 25 mg of lyophilized peppers were used for the extraction. Two extractions with 175 μL of extraction buffer (with 6% (w/v) meta-phosphoric acid and 0.2 mM diethylene triamine pentaacetic acid) were performed with 30 min of cold ultrasonication (Bransonic ultrasonic bath 2800, Emerson Industrial, Ferguson, MO, USA) and centrifugation at 13000 rpm for 10 min at 4 °C (Centrifuge MR18–22, Jouan, France). Both supernatants were pooled, the extracts were diluted before spectrophotometric readings and vitamin C determinations were performed in triplicate on a 96-well quartz microplate (Hellma Hispania SL, Badalona, Spain).

Capsaicinoid extraction was perfomed with 200 mg of the lyophilized peppers. Two extractions of 1000 µL methanol 80% were performed with 30 mins of ultrasonication (Bransonic ultrasonic bath 2800, Emerson Industrial, Ferguson, MO, USA) and centrifugation at 9000 rpm for 15 min at room temperature (Centrifuge 5804 R, Eppendorf, Hamburg, Germany). The filtrate was purified by passing through a 0,45 mm PTFE filter (Phenomenex, Torrance, CA, USA) before injection on the HPLC column.

Chromatographic separation of the capsaicinoids was performed on a Waters 2695 Separations Module and a Waters 2996 Photodiode Array Detector (Milford, MA, USA) HPLC system with a Mediterranean Sea18 column (25 ×0,46 mm, 5 µm particle size; Teknokroma, Sant Cugat, Spain). An injection volume of 5 µL was used from each sample to be separated in a gradient at constant flow (0,8 mL/min) with two solvents: Milli-Q water (solvent A) and acetonitrile (solvent B). The gradient used for the chromatographic separation of the capsaicinoids was: 0–10 min, 50% B; 10–15 min, increased to 100% B by linear gradient; 15–16 min decreased to 0% B by linear gradient; 16–22 min, 0% B to equilibrate and return to the initial concentrations. Calibration curves were built by injecting, in triplicate six increasing concentrations from 0,5 to 3,5 ppm of standard mixture (capsaicin and dihydrocapsaicin) prepared in 100% acetonitrile.

2.4. Statistical analysis

Statistical analysis was carried out using the IBM SPSS Statistics 25 program (SPSS Inc., Chicago, IL, USA). An analysis of the variance (ANOVA) was used to compare mean values between samplings. The Duncan post-hoc test was used to determine differences between treatments. Significance levels of 95% ($P < 0.05$) are indicated in figure legends. Significantly different means are marked with different letters. Figures were built with SigmaPlot 10.0 (Systat software, Palo Alto, CA, USA).

3. Results

3.1. Stress effects on pepper

One of our main objectives was to understand the effect of using STP watering to reduce drinkable water use in crops. During the experiment, many changes were observed in different quantified parameters, such as in the quality and quantity of the fruits and in the plant physiological performance due to oxidative stress in both pepper genotypes: salt tolerant genotype (ST) and salt sensitive genotype (SS).

Comparing the control condition and stress condition, in the Italian green peppers we observed a 44% and 49% decrease in the total and commercial weight, respectively (Fig. 1 A). On the other hand, a reduction of 37% was found in the commercial weight of the Red Cherry Small peppers (ST), but no significant reduction of the total biomass (Fig. 1 B). Regarding the quality of the fruits, the Italian green peppers showed no significant differences on the total soluble solids (TSS) content (Fig. 1 C), while a major effect was found in the ST genotype, decreasing the quality of the fruits. The Red Cherry Small peppers under STP stress had 37% lower content of ascorbate, but the only form found in the fruits was the reduced form (AsA, Fig. 1 E), while the dehydroascorbate (oxidized form) was not present in the samples. The content of total capsaicinoids, as well as capsaicin and dihydrocapsaicin,

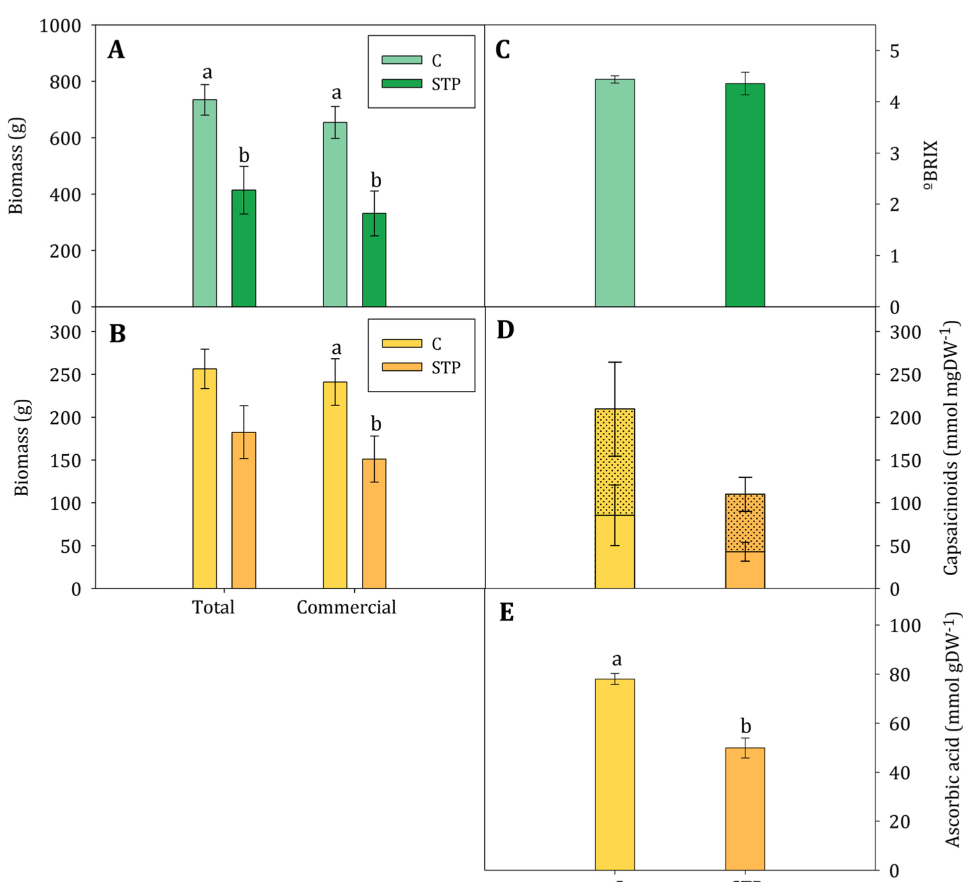

Fig. 1. Effect of sewage treatment plant (STP) irrigation in the quantity and quality of both pepper genotypes harvest: Italian green pepper (green panels; A and C) and Red Cherry Small (orange panels; B, D and E). Production (total weight, commercial weight and number of peppers; A – B), total soluble sugars (TSS, °BRIX; C), capsaicinoids (capsaicin (spotted pattern) and dihydrocapsaicin (plain pattern); D) and ascorbate (E). Data show the mean ± SE (n = 6). Different lowercase letters indicate significant differences between treatments with $P_{value} < 0{,}05$.

was reduced to a half (Fig. 1 D, Table S2), but no significant differences were found between C and STP watered plants. Therefore, harmful effects of stress produced by irrigation were observed in both the productivity and the quality of the fruits.

To realize if there was any stress due to the STP watering experimented by the plants, we analyzed the relative water content (RWC) and the lipid hydroperoxides content (LOOH). We found that the SS plants, showed a decrease to values lower than 75% in the RWC (Fig. 2 A) of 14% the 2nd second sampling with respect to the other samplings, indicating that plants were affected by stress caused by STP irrigation, while the ST plants managed to control the water content in the leaves (Fig. 2 F) and avoid any depletion by keeping the RWC values over 85% during the whole experiment. The consequence of this stress was also observed in the lipid hydroperoxides content (LOOH, Fig. 2 B), where the Italian green genotype showed higher LOOH of the STP watered plants in the first two samplings, showing a 3.3-fold increase in the 2nd sampling. The ST genotype also showed the strongest significant difference at the 2nd sampling on the LOOH content, as the STP plants experimented an increase of 105% compared to the C plants. In both genotypes, the values of lipid peroxidation decreased along the time of

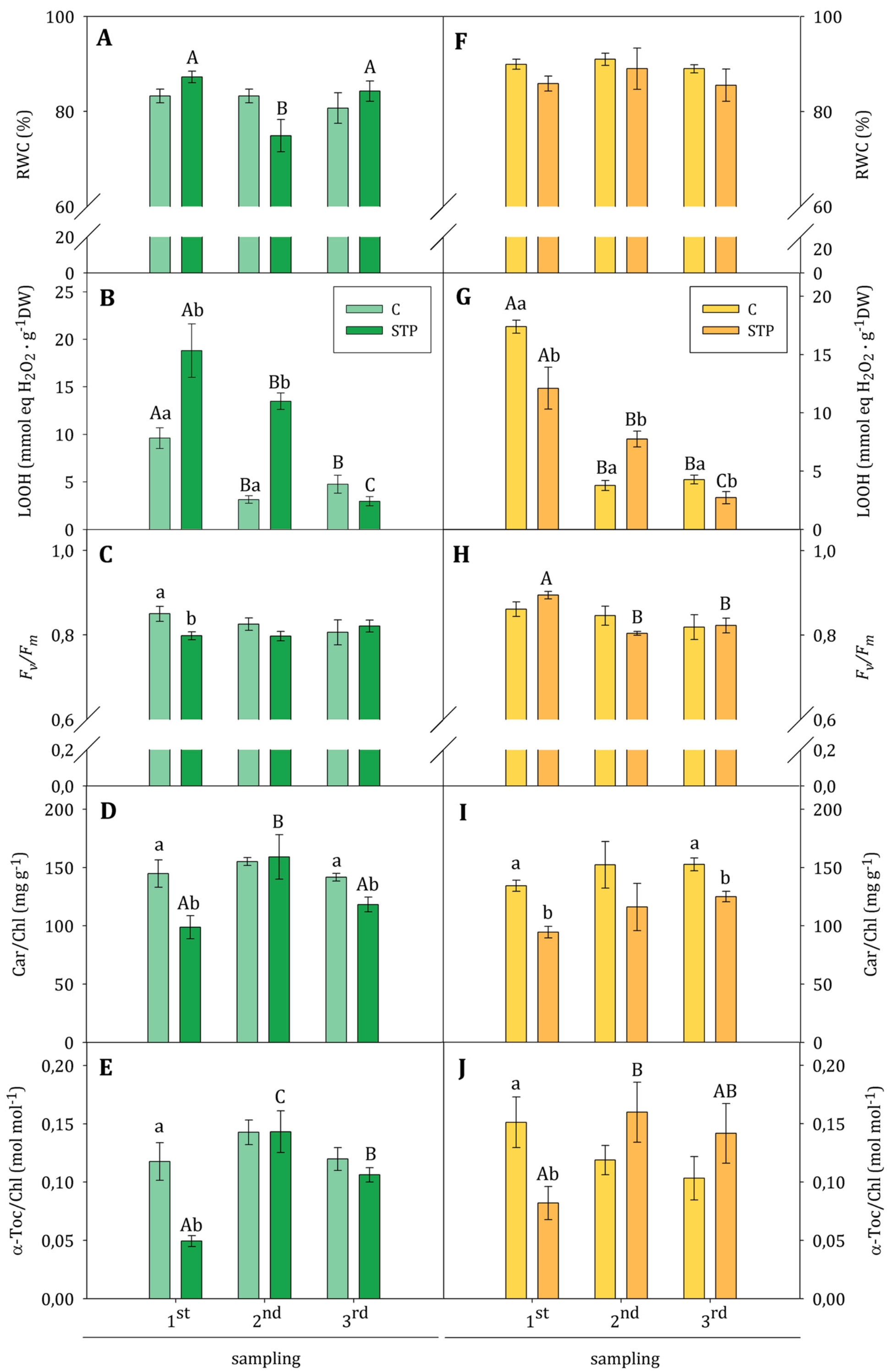

Fig. 2. Physiological responses to the sewage treatment plant (STP) irrigation along the three samplings of both pepper genotypes: Italian green pepper (green panels; A-E) and Red Cherry Small (orange panels; F-J). Relative water content (RWC; A and F), lipid hydroperoxides (LOOH; B and G), PSII maximum efficiency (F_v/F_m; C and H), carotenoids/chlorophylls (Car/Chl; D and I) and α-tocopherol/chlorophylls (α-Toc/Chl; E and J). Data show the mean ± SE (n = 6). Different lowercase letters indicate significant differences between treatments and different capital letters indicate significant differences between samplings with $P_{value} < 0,05$.

the experiment with less marked differences in the third sampling, indicating that plants were acclimating to the stress caused by the irrigation with regenerated water.

The effect of the STP watering stress was not severe into the chloroplasts. The maximum efficiency of PSII (F_v/F_m, Fig. 2 C and H), was over 0.79 in both genotypes in all samplings, and only a reduction of 6% was observed in the SS genotype due to the STP watering at the 1st sampling. To decipher the photoprotection strategies that pepper plants performed to counteract the suffered stress (Fig. 2 D, E, I and J), we quantified the relationship between carotenoids or tocopherols and chlorophylls (Car/Chl and α-Toc/Chl, respectively). In both genotypes, the maximum reduction in the content of lipophilic protection per chlorophyll was in the 1st sampling due to the STP water irrigation treatment. Moreover, both genotypes under STP water stress experimented an increase in these molecules at the 2nd sampling, indicating that plants were acclimating to the stress.

Finally, to deepen through the triggered mechanisms for the stress responses to STP watering, we quantified the levels of different stress related phytohormones (Fig. S2). The ABA content was reduced along the time in both genotypes with no clear pattern attributable to STP watering. On the other hand, SA content showed a 46% decrease on the stressed plants at the 1st sampling, but increased in both treatments, especially in the 2nd sampling. JA content also exhibited similar behavior in both genotypes, with increased content in the 2nd sampling. In both genotypes, the greater difference between treatments was shown at the 3rd sampling, with higher JA content in the STP watered plants of 1.85-fold on SS and 2.19-fold on ST. Regarding changes in growth related hormone levels, we quantified content of gibberellin 4 (GA_4), IAA, zeatin (Z) and isopentenyl adenine (IPA) (Fig. S3). No particular pattern was observed along the time, except for the auxin IAA, which tended to increase in both treatments and genotypes along the experiment.

In summary, the obtained results clearly pictured stress responses of both genotypes of peppers during STP watering and a moment of maximum stress could be observed during the second leaf sampling affecting the final harvest quantity and quality of both Italian green and Red Cherry Small peppers.

3.2. EBR effects on pepper fruit quantity and quality

After confirming the stress suffered by STP water irrigation, we explored the effects of the exogenous EBR application at different concentrations to comprehend if pepper plants could overcome the harmful consequences of the STP water stress on their productivity (Fig. 3 A and

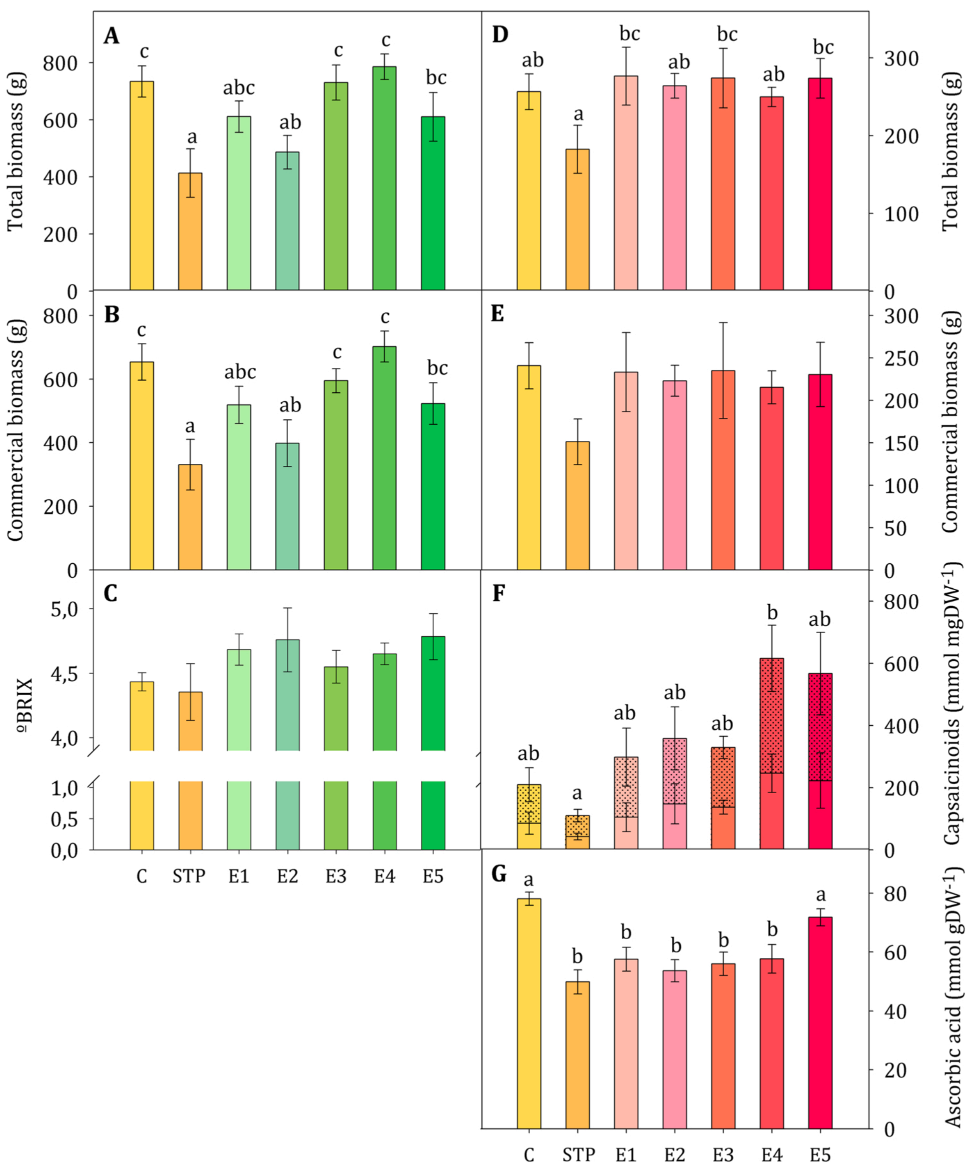

Fig. 3. Effect of exogenous application of EBR at increasing concentration (from E1 to E5) in the harvest quantity and quality of sewage treatment plant (STP) irrigated pepper genotypes: Italian green pepper (green panels; A-C) and Red Cherry Small (red panels; D-G). Production (total weight and commercial weight; A-E), total soluble sugars (TSS, ºBRIX; C), capsaicinoids (capsaicin (spotted pattern) and dihydrocapsaicin (plain pattern); F) and ascorbic acid (AsA, G). Data show the mean ± SE (n = 6). Different lowercase letters indicate significant differences between treatments with $P_{value} < 0{,}05$.

D). When quantizing the peppers biomass produced per plant in the SS genotype, the results showed that E3, E4 and E5 concentrations were able to improve production despite irrigation with STP water. The maximum increase (90%) with respect to the STP watered plants with no EBR application (STP) was with the E4 concentration (2 μM), which could also recover green pepper biomass to the values obtained for tap water. The tolerant genotype also showed an improvement of the production with respect to the STP plants with the application of EBR at concentrations E1, E3 and E5 (0,1, 1 and 3 μM respectively, with increases around 50%). Finally, measuring the commercial weight taking into account the morphological criteria of marketing in both genotypes (Fig. 3 B and E), no differences in production are observed on the ST, while in the SS genotype, the conditions E3, E4 and E5 lead to an improvement in production when comparing to the STP treatment, keeping the production occurring when plants are not subjected to any stress source (control treatment), with maximum increase of 112% in E4 (2 μM) with respect to STP treatment.

Regarding the quality parameters for the Italian green peppers, although no significant differences were observed for TSS, a clear tendency was observed to accumulate more sugars on the EBR-treated fruits (Fig. 3 C). In the Red Cherry Small peppers an increase in the capsaicinoids content (Fig. 3 F) was observed with the EBR application reverting the reduction experimented by the STP watering. The two studied capsaicinoids (capsaicin and dihydrocapsaicin) showed the same pattern with more than double content than controls and 5-fold higher than STP in the E4 and E5 treatments (Table S2). Moreover, the reduced ascorbate (Fig. 3 G) was also recovered to the control content, but differences were only significant when the maximum EBR concentration (E5, 3 μM) was applied, with a 44% increase compared to STP treatment.

3.3. Brassinoesteroids changed by the exogenously applied EBR

First, the verification of the brassinosteroid entrance to the leaves was necessary to confirm the cause of all the observed improvements. Therefore, the effect of the treatment with EBR on the content in CAST (precursor of the brassinoesteroids synthesis pathway), BL and EBR was quantified at the maximum stress sampling (Fig. 4). In terms of CAST, in the SS genotype we can observe how condition C and STP showed higher content of the precursor, while EBR treatments were lower. In the ST genotype, we found that the treatments C, E4 and E5 (2 and 3 μM) were higher than the other treatments, indicating a decrease due to the suffered stress. BL levels were not significantly different between C and STP treatments, in either of the genotypes. However, in the SS genotype an increase in BL content was detected in E2, E3 and E4 treatments with respect to the C plants, and in treatments E3 and E4 with respect to the STP treatment, with a maximum difference of 159% between treatments E3 and C. Similarly, the genotype ST has differences in BL content between E3 and E5 treatments in the EBR application at 1 and 3 μM, with a maximum difference of 90% increase between E3 and C. Finally, the EBR levels, directly related to the exogenous application of the biostimulant, confirm EBR's entrance, so in both pepper genotypes the basal levels seen in C and STP treatments increased parallelly to the concentration of the applications, reaching maximum values in E4 and E5 treatments.

3.4. EBR facilitated the stress overcoming

Regarding the effects of EBR treatment on different variables related to stress at the foliar level we wondered which mechanisms were activated by the application of EBR and if they were efficient to overcome the harmful effects of stress on their productivity for the two genotypes.

On one hand, in the SS genotype, RWC values were increasing gradually (Fig. 5 A), as the applied EBR concentration was increasing

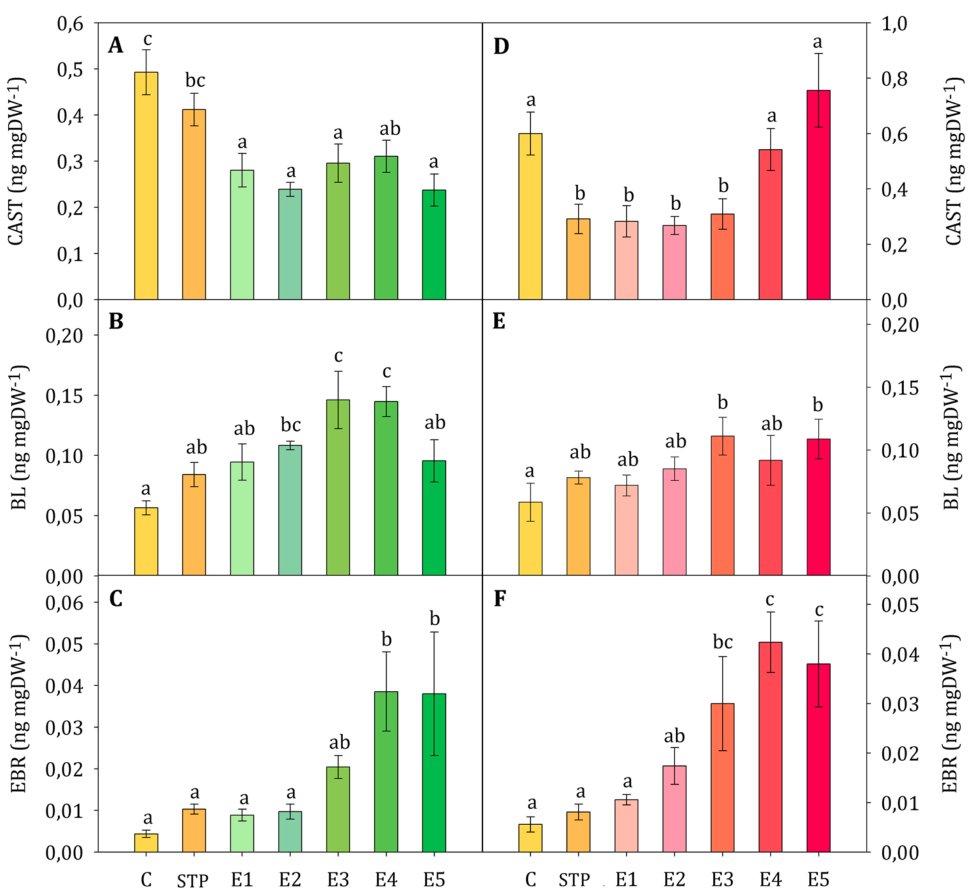

Fig. 4. Effect of exogenous application of EBR at increasing concentration (from E1 to E5) in the phytohormones concentration of sewage treatment plant (STP) irrigated pepper genotypes: Italian green pepper (green panels; A-C) and Red Cherry Small (red panels; D-G). Castasterone (CAST; A and D), brassinolide (BL; B and E) and epibrassinolide (EBR; C and F). Data show the mean ± SE (n = 6). Different lowercase letters indicate significant differences between treatments with $P_{value} < 0,05$.

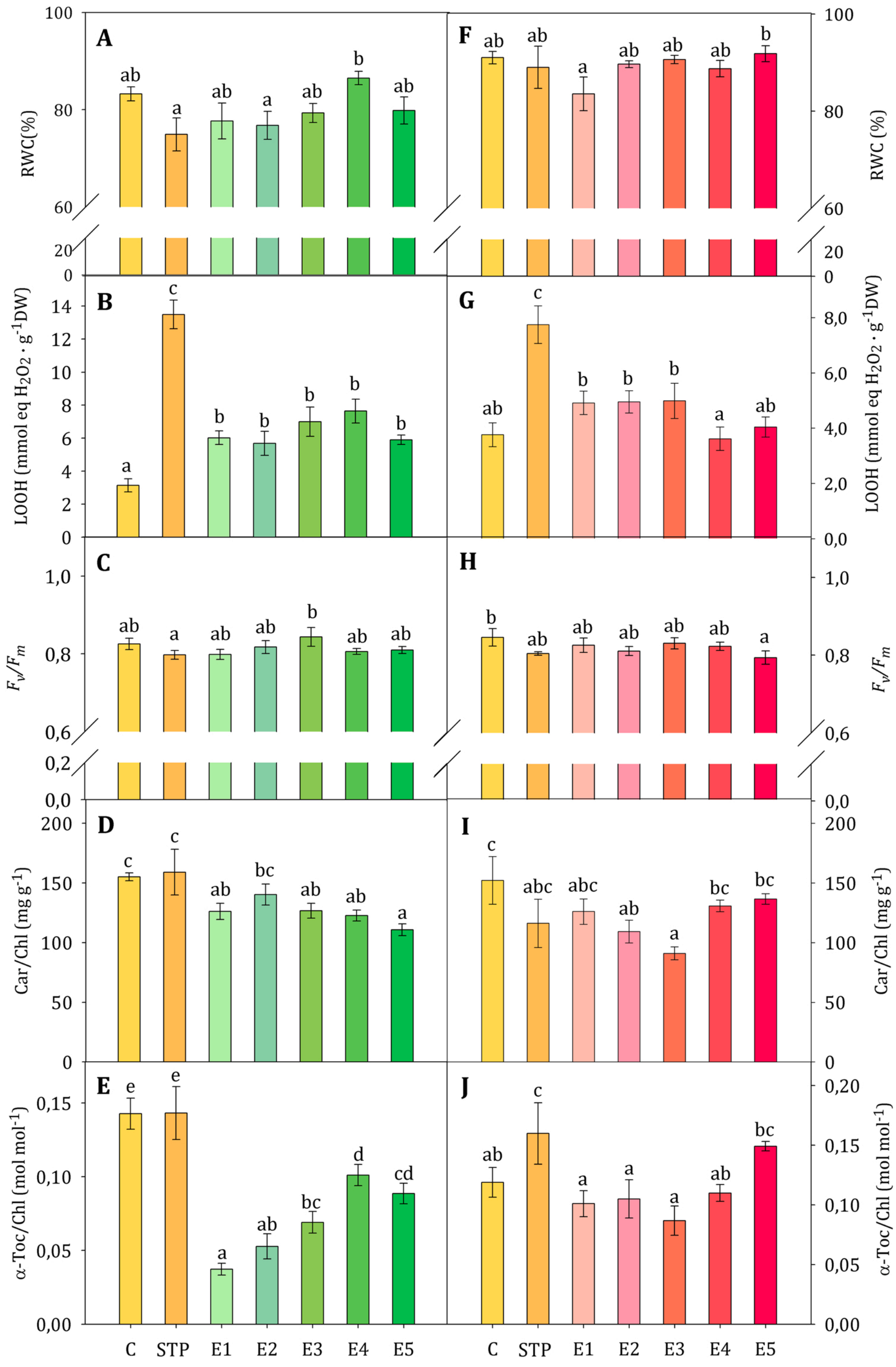

Fig. 5. Effect of exogenous application of EBR at increasing concentration (from E1 to E5) in the physiological responses of sewage treatment plant (STP) irrigated pepper genotypes: Italian green pepper (green panels; A-E) and Red Cherry Small (red panels; F-J). Relative water content (RWC; A and F), lipid hydroperoxides (LOOH; B and G), PSII maximum efficiency (F_v/F_m; C and H), carotenoids/chlorophylls (Car/Chl; D and I) and α-tocopherol/chlorophylls (α-Toc/Chl; E and J). Data show the mean ± SE (n = 6). Different lowercase letters indicate significant differences between treatments with $P_{value} < 0,05$.

until E4 treatment, which resulted in an increase (15%) compared to STP watered plants. At the same time, LOOH content (Fig. 5 B) showed the effect of oxidative stress on plants, but the EBR application was able to decrease lipid peroxidation to a half of the STP treatment with all the concentrations. F_v/F_m values (Fig. 5 C) were above 0.8 in all treatments, but E3 (1 μM) application showed higher values than the STP treatment. The Car/Chl ratio (Fig. 5 D) slightly decreased as the applicated EBR concentration increased, with a decrease in the of 30% compared to the STP treatment at 3 μM (E5) indicating that the plants with EBR required lower level of protection in the photosystems. Finally, if we consider the relationship between α-Toc/Chl (Fig. 5 E), a particular trend was observed: a progressive increase from E1 with increasing concentrations of EBR, but all of them were lower than both C and STP treatments.

On the other hand, the ST genotype, less variations were observed, as it is a very tolerant genotype. Both RWC and F_v/F_m values (Fig. 5 F and Fig. 5 H, respectively) did not show relevant variations from the C or STP treatments, with values above 80% in the RWC, and above 0.80 in the F_v/F_m. Regarding the LOOH content (Fig. 5 G), as similarly observed in the SS genotype, a strong reduction of the lipid peroxidation (e.g. 56% in E1 compared to STP) was derived from the biostimulant molecule effectiveness on stressed plants, reaching values of the non-STP-treated plants (Controls). Finally, Car/Chl ratio (Fig. 5 I) and α-Toc/Chl

(Fig. 5 J) showed similar pattern on slightly decrease their ratio in E3 treatment and increasing again in E4 and E5. Interestingly, plants with EBR application decreased α-Toc/Chl ratio from the STP treatment, but as found in SS genotype, the ratio was increasing progressively as the applied EBR concentration increased.

We also analyzed the levels of different stress-related hormones (Fig. S4) in the second sampling of the two pepper genotypes, to prove the effect of the EBR application. First, in both genotypes we can see how ABA levels did not differ significantly, while SA decreased under STP watering, recovering control values with the maximum EBR applied concentration. Finally, on the SS genotype the previously observed a 33% JA decrease in pepper plants watered with STP irrigation was recovered with the EBR application at any concentration.

Regarding the changes in growth hormone levels (Fig. S5) both genotypes presented similar patterns. In the Italian green pepper genotype, EBR application on STP treated plants reduced to control values the hormonal content of Z and GA4 (under E2 and E3 concentrations). The IPA content showed no significant differences among treatments in none of the genotypes. Finally, the auxin IAA showed no differences in the ST genotype, while was clearly affected by the EBR application in the SS genotype. Particularly, the highest concentration of EBR (E5, 3 μM) showed a 1.4-fold increase content in IAA compared to STP plants without EBR application.

4. Discussion

4.1. STP watering provoked a misfunction on pepper plants

The accumulation of salts on agricultural soils caused by irrigation results in a decline in soil water potential, which might induce osmotic stress on the cultivated plants, and may also result in a toxic effect (Munns and Tester, 2008). However, watering crops with STP water will be necessary on the near-future agricultural practices due to drinkable water scarcity (Lu et al., 2019), although the effect of salts on soil can vary depending on other factors such as concentration of these salts, the frequency of irrigation, and local climatic conditions, among others. This is the first study that analyzed how foliar application of a biostimulant molecule improved the performance of two genotypes of pepper regarding productivity and quality under STP watering treatment (Fig. 6).

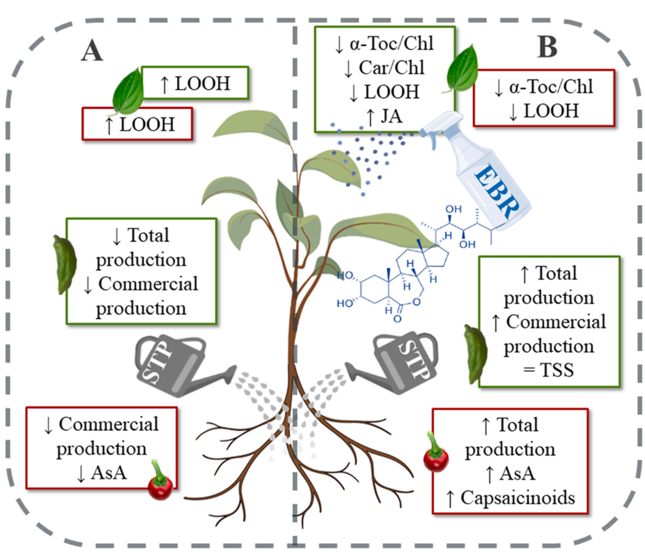

Fig. 6. Brief summary of plant responses in leaves or fruits of Italian green pepper (green boxes) and Red Cherry Small pepper (red boxes) under STP watering (A) or STP watering with EBR foliar application (B). STP: sewage treatment plant; EBR: 24-epibrassinolide; LOOH: lipid hydroperoxides; α-Toc/Chl: α-tocopherol/chlorophylls; Car/Chl: carotenoids/chlorophylls; TSS: total soluble sugars; JA: jasmonic acid; AsA: ascorbic acid.

The Italian green pepper genotype (sensitive to salt stress, SS) has been affected at the water relationships as it has experienced a decrease in RWC due to irrigation with STP water, while the Red Cherry Small (more tolerant to salt stress, ST) managed to maintain RWC during the whole experiment. Lower osmotic potential due to STP watering could cause stomatal closure adjustment, triggering oxidative damage that plant mechanisms might be unable to counteract. In this situation, ROS contribute to molecular and structural damage, due to the oxidative stress to which the plant is subjected (Pintó-Marijuan and Munné-Bosch, 2014). In membranes, the structural damage taking place is induced by ROS that trigger lipid peroxidation. For this reason, SS culture shows an increase in LOOH when comparing controls and STP treatments, in the first two samplings. In the third sampling, the LOOH content of the STP treated plants decreased to control values, indicating the crop managed to finally acclimate to the salt stress.

An increase in salt concentration in the soil can affect photosynthetic process and decrease chlorophyll and carotenoids content (Acosta-Motos et al., 2017) inducing newly generated ROS, which will increase the likelihood for LOOH production from membrane lipids. To counteract the stress consequences and protect lipid membranes from oxidative stress, carotenoids and other lipophilic antioxidants, such as tocopherols, are activated cellular response mechanisms as they deactivate singlet oxygen, by physical quenching and/or chemical scavenging (Polle and Rennenberg, 1994; Falk and Munné-Bosch, 2010). In the two studied genotypes of pepper, a decrease on the Car/Chl ratio suggested photosynthetic consequences to the stress, while tocopherol accumulation was activated from the 2nd sampling on the STP watered plants highlighting the need to protect photosystems. In pepper ST genotypes, antioxidant mechanisms that are activated differ from those respective to SS crops. Salinity tolerance has been correlated with higher content of certain antioxidant enzymes, of the hydrophilic antioxidant system (ascorbic acid and glutathione), and an increase in protein synthesis to set osmotic balance (Aktas et al., 2012; Acosta-Motos et al., 2017).

In consequence, the stress caused by STP water also provoked loses in peppers production depending on the genotype sensitivity, as plant response to oxidative stress depends on its state of development, growth conditions and their degree of tolerance, which can vary between crops within the same species (Munns and Gilliham, 2015). The two genotypes responded to the stress caused by the STP irrigation with a decrease in production parameters (SS genotype reduced total weight and commercial weight, while ST genotype reduced the commercial weight), as seen in previous studies with other crops (Giordano et al., 2021).

The specific characteristics that define the nutritional value for each genotype were highlighted (Uarrota et al., 2021). Regarding the two selected genotypes for this study: the Italian green peppers are nutritionally important for their sweetness, and the Red Cherry Small peppers are distinguished for their pungency and vitamin C content (Uarrota et al., 2021). In this study, no reduction in the TSS was observed due to the stress in the SS genotype, while in the ST plants under STP watering experimented reduction in quality, which was mainly caused by strong decrease on the ascorbic acid. The decline of the ascorbic acid content as salinity increased was already observed by Azuma et al. (2010) with the Caballero cultivar, very similar to the ST genotype, and water availability or salt stress has been proved to affect capsaicinoid production in several pepper genotypes (Jeeatid et al., 2018; Uarrota et al., 2021). So far, we have confirmed the stress caused by irrigation with STP water influencing the oxidative responses and mechanisms established to deal with it, in particular in the SS genotype. Yet one of our main objectives and one of the most agronomic concerns is to disentangle if the damage caused by this type of irrigation can be overcome using biostimulant molecules.

4.2. EBR application improved pepper plants performance under STP watering

First, in this study we aimed to confirm that foliar applications of EBR could enhance pepper plants performance under STP and also characterize its effect on other molecules key for the brassinosteroids metabolism such as CAST and BL. The content of detected EBR and BL in the leaves increased proportionally as the concentration of the foliar application was higher, but it is lower than the concentration of the applied EBR due to the time past between the application of the biostimulant molecule and the time at which sampling was performed, indicating either that biostimulant was applied at foliar level is quickly removed to other parts of the plant, or it does not end up penetrating entirely due to the physical barrier of the leaf cuticle (Janeczko et al., 2011). On the SS genotype, we proved that brassinoesteroids were not responding to the suffered stress, while in the ST genotype, CAST content decreased under stress and only recovered C content of the precursor when E4 and E5 EBR concentrations were applied (2 and 3 μM). In ST genotype, we cannot contrast the idea that EBR acts as an inhibitor of synthesis of the precursor, but we can correlate the CAST recovery to C values with the strongest reduction on the LOOH content under E4 and E5 treatments, indicating that higher concentrations of EBR might be leading to an overprotection process (Tanveer et al., 2018). With these results, and bearing in mind that the EBR synthesis pathway is not described in all of its steps (Tanveer et al., 2018), we can hypothesize that there is a negative feedback in which from the CAST precursor BL is synthesized and an increase in BL levels increase EBR that, in turn, inhibits the synthesis of the precursor, in the same way as is known in the case of the exogenous BL application, which has a negative feedback effect on different enzymes in the biosynthesis pathway of brassinoesteroids (Zhao and Li, 2012).

Therefore, we wondered if the assimilated EBR at different concentrations might effectively provide responses to counteract the harmful effects of irrigation with STP water. At the leaf level, we proved the effect of the EBR ameliorating parameters that were affected due to the oxidative stress. Foliar EBR application showed positive results in different pepper cultivars to amend salt stress with NaCl on shoots of *Capsicum annuum* L. cv. Beldi (Houimli et al., 2008) or increasing the proline content to face NaCl stress in *Capsicum annuum* var. Frutescens and *Capsicum annuum* var. Baccatum (Abbas et al., 2013). In the SS genotype, EBR strongly decreased LOOH content under all the applied concentrations, indicating that 0.1 μM (E1) is enough to deal with the effects of stress and prevent the formation of lipid hydroperoxides in the Italian green pepper. Moreover, E1 concentration also decreased the content in α-Toc and Car (in the ST genotype), suggesting that protection using lipophilic antioxidants was not necessary to reduce the oxidative consequences of the stress due to the activation of enzymatic and non-enzymatic (especially glutathione and ascorbate) antioxidants as reviewed by Tanveer and colleagues (Tanveer et al., 2018). However, as EBR concentration progressively increased, the content of α-Toc (in both genotypes) also increased indicating that, under elevated concentrations, EBR is able to promote an overprotection of the crop without affecting the final productivity. This set of results supported that EBR can activate hydrophilic antioxidant defense and, consequently, less investment is needed in carotenoids and tocopherols to protect photosystems (Tanveer et al., 2018). A hypothesis for the mechanisms that trigger EBR for the response to face the stress consequences can be by increasing the content in JA, as we have seen in SS crop and as seen in other studies carried out in *Arabidopsis thaliana*, where treatment with brassinoesteroids showed increasing expression of the OPR3 gene, involved in the JA biosynthesis pathway (Müssig et al., 2000; Bajguz and Hayat, 2009). Thus, EBR could influence stress responses by stimulation of JA synthesis, which is derived from lipid peroxidation and bound to the activation of the chloroplast antioxidant system and the accumulation of different antioxidants such as tocopherols (Munné-Bosch et al., 2007; Casadesús et al., 2021).

Fruits production, as a consequence of the plants suffered stress, was also decreased by the STP watering. In this study, we proved that the EBR application is capable of improving the productivity of different pepper genotypes. In the SS genotype, treatments E3, E4 and E5 (1, 2 and 3 μM, respectively) were able to increase the total and commercial production. Total pepper production in the ST genotype was also higher after EBR application. Despite irrigation with STP water led to oxidative stress due to an increase in the available salts in the root surroundings, the use of EBR is useful to prevent the harmful effect of this stress on the plants' productivity (Ali, 2017). By comparing the two genotypes, we've seen the SS genotype, which is more stress-sensitive, needed a higher minimum concentration of EBR necessary to counter stress, 1 μM. Instead, the ST genotype required a lower concentration of 0.1 μM, to show differences with the STP treatment, as it is less stress-sensitive and less affected by stress.

EBR is a plant phytohormone that shares structural similarity with animal steroidal hormones, and some brassinosteroids can act using the same cell receptors, but there is yet no evidence that EBR can be recognized by human steroidal receptors (Kohli et al., 2020). In our experiment, the content of EBR in controls was around 1.2 mg EBR/100 g of FW, values that are consistent with the previous literature. After E5 treatment, 8.3 mg EBR/100 g of FW were quantified, not even reaching the levels in coriander leaves (18.7 mg EBR/100 g of FW) as described by Kohli et al. (2020). Moreover, the therapeutic effects that brassinosteroids could have in human health are currently being studied because of their anticancer (Ma et al., 2022), antiproliferative and antiviral properties, among others.

The quality of the recollected Red Cherry Small peppers was also ameliorated by the EBR application in both pungency and antioxidant activity. Several publications demonstrated that EBR application activates different enzymatic and non-enzymatic antioxidants under salt stress (Tanveer et al., 2018) and the effect of foliar applied brassinosteroids or brassinosteroid analogues was proved in different pepper cultivars with positive results on productivity and quality of the fruits (Serna et al., 2012). In the Italian green genotype, only a tendency to accumulate more sugars (TSS) was observed after EBR application, but differences were not significant, as already observed on *Capsicum annuum* L. cv. Orlando with different brassinosteroids application (Serna et al., 2012). Therefore, the quality of the harvested peppers from the STP watered plants was ameliorated by the EBR application in relevant parameters on both genotypes.

5. Conclusions

In this study, we proposed the use of STP water as a solution for the future drinkable water restrictions which concurs with several United Nations' Sustainable Development Goals (SDGs): the 6th (Clean Water and Sanitation), the 11th (Sustainable Cities and Communities) and the 12th (Responsible Consumption and Production). We studied the impact of STP irrigation in the production of two genotypes of one of the most cultivated horticultural crops: *Capsicum annuum*. STP water led to important reductions of commercial weight together with losses of organoleptic and nutritional quality induced by increases of oxidative stress in both tolerant and salt sensitive pepper genotypes. To mitigate the effects of STP water we proposed foliar application of a brassinosteroid (EBR) and proved an endogenous increase that elicited in lipid hydroperoxides content, by activating antioxidant defense mechanisms, guided by a higher JA synthesis, in particular for the salt-sensitive genotype. Our data demonstrated that exogenous EBR induced higher production of both Italian green and Red Cherry Small peppers counteracting the STP watering. Moreover, the quality of Red Cherry Small peppers was also improved by enhancing capsaicin, dihydrocapsaicin and ascorbic acid content with EBR application. Therefore, our results reveal a great advance to overcome current and future deficiencies in the agricultural field due to climate change, ensuring the maintenance of production despite the use of STP non-drinkable water, becoming of important economic and environmental relevance following circular economy actions.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests, Sergi Munne-Bosch reports financial support was provided by Catalan Institution for Research and Advanced Studies.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.plantsci.2023.111764.

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