

Article

The Use of Red Shade Nets Improves Growth in Salinized Pepper (*Capsicum annuum* L.) Plants by Regulating Their Ion Homeostasis and Hormone Balance

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Received: 15 October 2020; Accepted: 9 November 2020; Published: 12 November 2020



Abstract: The actual climate crisis scenario is aggravating the abiotic stress episodes that crop plants have to face. Salinity is one of the most important abiotic stresses directly impairing plant growth and productivity. Several strategies have been developed to minimize the negative effects of salinity in agricultural industry, mainly at the plant level, while management strategies, such as the control of microclimate conditions and light quality over plant canopy, have also been used. Indeed, shading plants with photoselective nets has been considered an efficient management strategy to modulate solar radiation to improve crop productivity. The aim of this work was to gain insights about the physiological factors underlying the salinity-alleviating effect of using red shading nets. For that, pepper plants (*Capsicum annuum* L.) were grown under control (0 mM NaCl) and moderate salinity (35 mM NaCl) conditions, with half of the plants covered with a red net (30% shading). The shoot growth impairment provoked by salinity was in part minimized by shading plants with red nets, which can be explained by their higher capacity to exclude Na⁺, control of K⁺ homeostasis and regulation of hormonal balance. Indeed, the concentrations of the most active cytokinin in pepper, *trans*-zeatin, as well as its metabolic precursor, zeatin riboside, increased in shaded plants, associated to shoot growth recovery and photosynthetic rate maintenance under salinity. Furthermore, the stress-related hormone abscisic acid (ABA) increased with salinity but in a lower extent in the plants shaded with red nets, suggesting a fine tune of stomata opening by ABA which, in crosstalk with salicylic acid increment, improved plant water relations. Likewise, the concentrations of gibberellins and the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid, also changed during salinity stress in shaded plants but those changes were uncoupled of growth responses as indicated by the principal component analysis and thus they seem to play a minor role. Our data demonstrate that shading pepper plants with red nets is an efficient management strategy to modulate microclimate conditions at crop level thus controlling the ion homeostasis and hormonal balance of the plant to cope with salinity stress. This is especially important due to the actual and expected changes of the global climatic conditions.

Keywords: pepper; red net; salinity; ion homeostasis; potassium; plant hormones; cytokinins; abscisic acid

1. Introduction

The need of feeding an increasing world's population together with the environmental threats provoked by the climate change, have changed the actual cultivation paradigm, entailing crop production in marginal soils with high salinity levels. This is contributing to maintain food supply but has a very high environmental cost, aggravating water and soil salinity problems [1], especially in the Mediterranean basin. In fact, salinity is one of the most important abiotic constraints in arid and semiarid areas [2], provoking a high yield gap worldwide [3,4]. Plants are affected at morphological, physiological and molecular levels during salinity stress, negatively affecting growth and development and limiting plant productivity [5–8]. Different strategies have been established at plant and/or agrotechnological levels to cope with salinity stress and improve crop productivity [9]. Shading has been used as an efficient technology to mitigate the extreme climatic fluctuations and pest incidence in arid and semiarid areas and to improve water use efficiency (WUE) while maintaining photosynthesis [10–13]. The use of novel shading nets, with specially-designed optical properties that can alter both the quality and the quantity of the light intercepted in addition to providing the desired protection, represents an efficient approach to manage solar radiation which has been demonstrated to improve crop production [14,15]. Different studies have dealt with colored nets in fruit crops (reviewed by Reference [13]) and vegetables (reviewed by Reference [15]). Colour shading nets provoke differential stimulation of some physiological processes that are directly regulated by light, such as photosynthesis and photomorphogenesis. Radiation characteristics are perceived by biological photoreceptors, mainly phytochromes, phototropins and cryptochromes, and their effects on different physiological and stress responses are largely driven by red/far-red (600–800 nm), green/yellow (500–600 nm) and UV-A/UV-B/blue (280–500 nm) wavelengths of the electromagnetic spectrum [16]. Phytochrome mutants in the model plant *Arabidopsis thaliana* have reduced biomass due to lower CO₂ assimilation but a higher adaptability to abiotic stress conditions [17,18]. Phototropins, activated by blue light and UV receptors have also shown to play important roles in abiotic stress responses [19–21]. The primary photosynthetic pigments in higher plants, chlorophylls *a* and *b*, have different absorption patterns but both with peaks in the blue (430–470 nm) and red regions (660–680 nm) and have very little absorption in the green region. Thus, photosynthesis and overall crop productivity could be enhanced by increasing the amount of blue and red light present in the growth environment [15,22,23].

Considering the different types of colored nets, black nets have been demonstrated to improve WUE and fruit quality in different fruit and vegetable crops, while pearl and yellow nets improved antioxidant activity, fruit yield and quality [13,15]. Likewise, the use of red nets induced greater fruit set in peach and apple, relative to other color nets and to un-netted controls [24], while a rapid development of the root system of banana plug transplants during hardening has been associated to the use of red net-covered plants when compared to the commercial black nets [25]. In 'Valencia' orange trees cultivated under top red netting, productivity was improved, as determined by fruit number, fruit size and overall yield [26]. In vegetable crops, plant productivity and quality have been also demonstrated to increase under shaded red nets [15,27,28]. In tomato, photosensitive red screen combined with optimum plant density promoted higher yield and commercial quality of the fruits [29,30]. Importantly, previous works testing sweet pepper under different photosensitive nets, have shown that red nets produced the highest intrinsic WUE and reproductive-to-vegetative ratio [14] and induced higher growth and development and whole productivity [28], while in bell pepper the use of red net improved yield [27] and physical quality characteristics and the antioxidant contents with respect to the black net [31].

Plant hormones regulate multiple physiological and regulatory processes in the plant as well as responses to biotic and abiotic stresses. Pioneer studies regarding the modulation of hormonal balance under salinity stress (apart from the "classical ABA") have shown a complex regulatory network among different hormonal classes in controlling growth and senescence [32–34]. Direct evidences of the role of plant hormones in improving growth and yield stability under salinity have appeared in the last years (reviewed by Albacete et al. [4]). For example, Ghanem et al. [35] demonstrated that enhancing

root cytokinin (CK) synthesis in tomato plants modified both shoot hormonal and ionic status, thus ameliorating salinity-induced decreases in growth and yield. Furthermore, the interaction of the active CK, *trans*-zeatin (tZ), with ethylene through its precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), explained the strong increase in fruit yield of transgenic plants overexpressing *CINI* gene, that encodes cell wall invertase, a key enzyme in controlling sucrose unloading in tomato fruits [36]. Recent studies have also demonstrated that gibberellins (GAs) play a critical role in regulating growth under salinity conditions [37], while jasmonic acid (JA) and salicylic acid (SA) have been described to play a role in the osmotic stress signaling responses, mainly in interaction with ABA [38,39].

By exogenous application of plant growth regulators, the plant-growth effect of photosensitive nets through the modulation of the hormonal balance have been evidenced. For example, Santos et al. [29] showed that spraying paclobutrazol, which belongs to the triazole family and have been shown to reduce GA and increase CK concentrations, impaired growth of tomato seedlings growing under red nets. Also, it has been considered that colored nets are able to replace the effect of plant regulators and other hormone analogues [40]. However, although controlling light intensity and quality through photosensitive nets could be an efficient management strategy to cope with salinity stress, so far, there are no direct evidences in literature of the physiological and regulatory mechanisms implicated. Furthermore, as stated before, shading red nets have been shown to be very effective in improving growth and yield in different fruit and vegetable crops. Therefore, the aim of this work was to determine whether red shading nets can improve growth and productivity under salinity stress in pepper plants, through the regulation of the ionic homeostasis and hormonal balance of the plant.

2. Materials and Methods

2.1. Plant Material, Growth Conditions and Treatments

Seeds of the commercial pepper (*Capsicum annuum* L.) hybrid, Gacela F1 (Syngenta Seeds, USA), were germinated and, two weeks after sowing, transferred to 10 L plastic pots (50% sand, 30% peat, 20% vermiculite) and disposed in an arch-shaped multispan greenhouse covered with thermal polyethylene, located at the “Torreblanca” experimental field in Torre Pacheco, Murcia (latitude: 37°45' N; longitude: 0°59' W). Plants were distributed in rows, with a separation of 40 cm between plants and 100 cm between rows. A standard Hoagland nutrient solution was used to irrigate the plants and, 15 days after transplanting, half of the plants started to be irrigated with the Hoagland solution supplemented with 35 mM NaCl, reaching an electrical conductivity of 5.5 mS·cm⁻¹. We selected this salinity treatment on the basis of a previous experiment with different salinity levels, considering that pepper is a moderately sensitive crop to salinity [41,42]. Irrigation requirements were based on the weekly crop evapotranspiration (ET_c). The saline solution was drained (between 10% and 25% according to solar radiation and evapotranspiration) to maintain a constant salinity level throughout the experimental period.

At the time of the application of the salinity treatment, part of the assay area within the greenhouse was covered with a red net (ChromatiNet[®] Raschel type, 30% shading, Polysack Plastic Industries LTD, Nir-Yitzak, Israel) placed 50 cm over the plant canopy, that was periodically moved up as plants grew. The covered area with red net enclosed a half of the plants subjected to optimal nutrient irrigation and a half of the salinized plants. The assay lasted until all plants reached the fruiting stage. Four experimental blocks with 10 plants per treatment were evaluated. The air temperature and photosynthetically active radiation (PAR) in each experimental unit were monitored during the growing cycle using a Testo 177-T4 temperature data logger (Testo SE & Co. KGaA, Lenzkirch, Germany) and a quantum sensor (LI-COR Inc., Lincoln, NE, USA), respectively.

2.2. Plant Growth-Related Determinations

Plant growth-related parameters were recorded at the end of the experiment in 15 plants per treatment. Plant height was determined with a measuring tape and then root, shoot and leaves within

each shoot were separated to determine their fresh weight (FW). A part of each leaf and root was oven-dried (60 °C under constant weight) to determine dry weight (DW).

2.3. Gas Exchange Measurements

Gas-exchange was monitored in fully expanded leaves at the plant vegetative stage. Measurements were carried out at periodic time-points after starting the salinity stress treatment. Net CO₂ fixation rate (A_{max} , $\mu\text{mol CO}_2\cdot\text{m}^{-2}\cdot\text{s}^{-1}$), stomatal conductance to water vapor (g_s , $\text{mmol H}_2\text{O}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and transpiration rate (E , $\text{mmol H}_2\text{O}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) were measured in steady-state under conditions of saturating light (800 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) and 400 ppm CO₂ with a LI-6400 instrument (LI-COR, Lincoln, NE, USA).

2.4. Leaf Water Potential, Osmotic Potential and Relative Water Content

Water potential (Ψ_w) was determined in mature leaves using a Scholander type pressure chamber (model 3000, Soil Moisture Equipment Co., Goleta, CA, USA), while osmotic potential (Ψ_s) was analyzed in leaf extracts with an osmometer (model Vapro 5520, Wescor Inc., South Logan, UT, USA). The relative water content (RWC) was measured using leaf discs of 1 cm² and calculated according to the following equation: $RWC = [(FW - DW) / (TW - DW)] \times 100$, where FW is the fresh weight recorded during collection, TW is the turgid weight and DW is the oven dry weight (60 °C until constant weight). To determine TW, disc leaves were kept in distilled water in darkness at 4 °C to minimize respiration losses until they reached a constant weight (full turgor, typically after 24 h).

2.5. Chlorophyll Analysis

Chlorophylls were extracted from 1 g of frozen pepper leaves (−80 °C) with 25 mL of acetone/hexane (2/3, *v/v*) solvent. Samples were homogenized and centrifuged at 5000 g for 6 min at 4 °C. Subsequently, the optical density of the supernatant was measured spectrophotometrically at wavelengths of 663 and 645 nm. The contents of chlorophylls a and b were calculated according to the Nagata and Yamashita equations [43]:

$$\text{Chlorophyll a (mg}\cdot\text{100 mL}^{-1}\text{)} = 0.999\cdot A_{663} - 0.0989\cdot A_{645}$$

$$\text{Chlorophyll b (mg}\cdot\text{100 mL}^{-1}\text{)} = -0.328\cdot A_{663} + 1.77\cdot A_{645}$$

2.6. Ion Determinations

Mature leaves were oven-dried at 60 °C for 48 h and homogenized with a grinder. Fifty mg were digested at 80 °C in a HNO₃/H₂O₂ solution (5/3, *v/v*). Minerals were dissolved in 0.1 M HCl, diluted with de-ionized water and filtered. Cation concentrations were analyzed using an inductively coupled plasma optical emission spectrometer (ICP-OES, Vista-MPX, Varian, Belrose, Australia) calibrated with certified standard solutions.

2.7. Hormone Extraction and Analysis

Cytokinins (*trans*-zeatin, tZ, zeatin riboside, ZR and isopentenyl adenine, iP), gibberellins (GA₁, GA₃ and GA₄), indole-3-acetic acid (IAA), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA) and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) were analysed according to Albacete et al. [33] with some modifications. Briefly, 50 mg of freeze-dried plant material were dropped in 0.5 mL of cold (−20 °C) extraction mixture of methanol/water (80/20, *v/v*). Then, 10 μL of internal standard mix, composed of deuterated hormones ($[^2\text{H}_5]\text{tZ}$, $[^2\text{H}_5]\text{tZR}$, $[^2\text{H}_6]\text{iP}$, $[^2\text{H}_2]\text{GA}_1$, $[^2\text{H}_2]\text{GA}_3$, $[^2\text{H}_2]\text{GA}_4$, $[^2\text{H}_5]\text{IAA}$, $[^2\text{H}_6]\text{ABA}$, $[^2\text{H}_4]\text{SA}$, $[^2\text{H}_6]\text{JA}$, $[^2\text{H}_4]\text{ACC}$, Olchemim Ltd., Olomouc, Czech Republic) at a concentration of 1 $\mu\text{g}\cdot\text{mL}^{-1}$ each, was added to the extraction homogenate. Solids were separated by centrifugation (20,000 g, 15 min, 4 °C) and re-extracted for 30 min at 4 °C in additional 0.5 mL of the same extraction solution. Pooled supernatants were passed through Sep-Pak Plus C₁₈ cartridges (SepPak Plus, Waters, Milford, MA, USA) to remove interfering lipids and part of plant pigments and evaporated at 40 °C under vacuum to near dryness. The residue

was dissolved in 0.5 mL methanol/water (20/80, *v/v*) solution using an ultrasonic bath. The dissolved samples were filtered through 13 mm diameter Millex filters with 0.22 μm pore size nylon membrane (Millipore, Bedford, MA, USA).

Ten μL of filtered extract were injected in a U-HPLC-MS system consisting of an Accela Series U-HPLC (ThermoFisher Scientific, Waltham, MA, USA) coupled to an Exactive mass spectrometer (ThermoFisher Scientific, Waltham, MA, USA) using a heated electrospray ionization (HESI) interface. Mass spectra were obtained using the Xcalibur software version 2.2 (ThermoFisher Scientific, Waltham, MA, USA). For the quantification of the plant hormones, calibration curves were constructed for each analysed component (1, 10, 50 and 100 $\mu\text{g}\cdot\text{L}^{-1}$) and corrected for 10 $\mu\text{g}\cdot\text{L}^{-1}$ deuterated internal standards. Recovery percentages ranged between 92 and 95%.

2.8. Statistical Analysis

The data were tested first for homogeneity of variance and normality of distribution. Analysis of variance and principal component analysis (PCA) were performed using SPSS for Windows (Version 25.0, SPSS Inc., Chicago, IL, USA). Means of different graft combinations were compared using Tukey's test at 0.05 of confidence level and the Varimax method was used for PCA.

3. Results

3.1. Microclimate Conditions

Shading pepper plants with red nets has a direct effect on microclimate conditions in the growing area, especially temperature and PAR. Our study show that during growing period, the average temperature in the area below the red net was 2.5 $^{\circ}\text{C}$ lower than in the non-shaded area at the central hours of the day (Figure 1a). The PAR over the plant canopy of one representative day between 13:00 and 14:00 h had a value of 1904 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ in the non-shaded area (Figure 1b). By shading the pepper plants with red nets, the PAR was lowered to 1425 $\mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$ (by 35%).

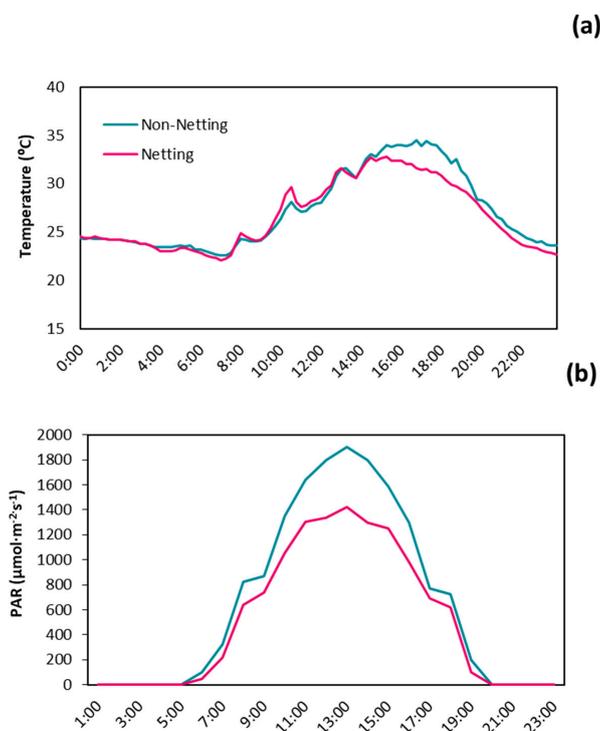


Figure 1. (a) Average temperature and (b) incident photosynthetically active radiation (PAR) during the growing period over the canopy of pepper plants cultivated under control (0 mM NaCl) and salinity (35 mM NaCl) conditions and covered or not with red nets.

3.2. Plant Growth Parameters

Plant growth parameters were significantly affected by the use of red nets and salinity application (Figure 2). Using red nets increased shoot FW under both control and salinity conditions but this increment was only significant in salinized pepper plants (by 40%, Figure 2a). Other shoot growth-related parameters were also affected. Leaf FW and plant height significantly increased (by 64% and 29%, respectively) when using red nets under control and salinity conditions (Figure 2b,c). In contrast, although salinity reduced root FW, the red nets did not have any significant effect in root growth (Figure 2d).

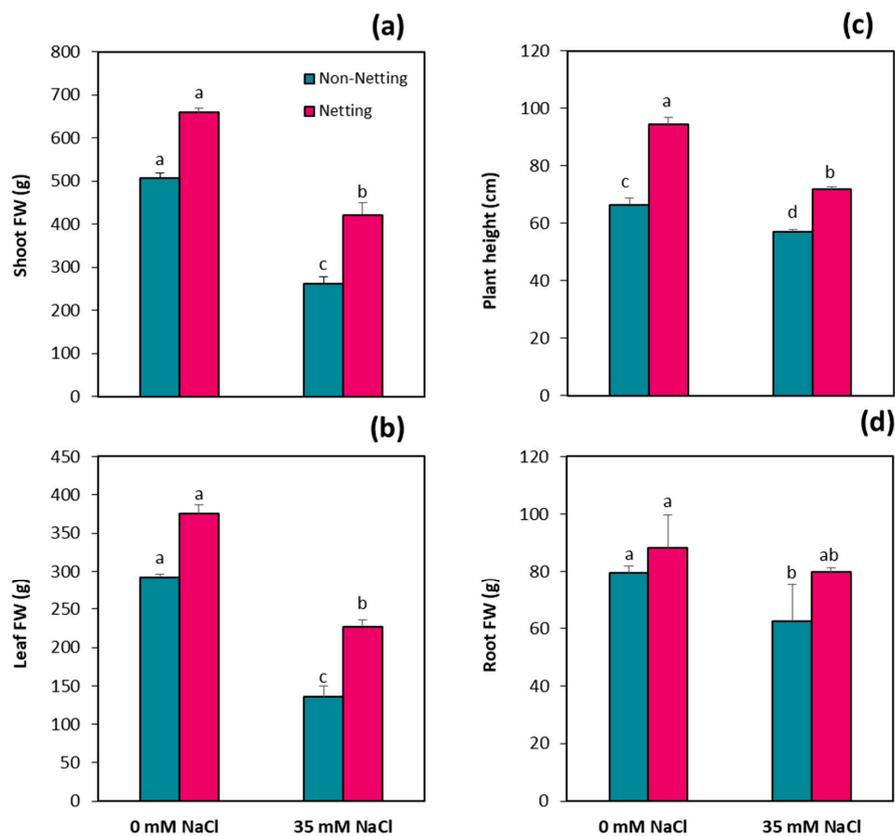


Figure 2. (a) Shoot fresh weight (FW), (b) leaf FW, (c) plant height and (d) root FW of pepper plants cultivated under control (0 mM NaCl) and salinity (35 mM NaCl) conditions and covered or not with red nets. Bars show the means of four plants with the standard error. Different letters among bars indicate statistical differences according to Tukey's test ($p \leq 0.05$).

3.3. Leaf Gas Exchange Parameters

In general, all leaf gas exchange-related parameters decreased over the salinity period, except intrinsic WUE, that is a derived parameter (Figure 3). Non-salinized pepper plants subjected to red net shading presented the highest net CO₂ assimilation over the whole experimental period, while non-shaded plants grown under salinity conditions showed the lowest photosynthetic rate (Figure 3a). Importantly, under salinity, the use of red nets maintained CO₂ assimilation between 35% and 50% higher than non-shaded pepper plants (Figure 3a).

Stomatal conductance also decreased over the experimental period. Interestingly, shaded plants under control conditions kept significantly higher conductance than the other treatments, except at the end of the assay (Figure 3b). A similar pattern was observed for the transpiration rate, but, in this case, although not significant, both control treatments (netting and not-netting) separated from the salinized ones (Figure 3c).

Concerning intrinsic WUE, calculated as the ratio between photosynthetic rate and transpiration rate, an increase over the assay was observed, with significant differences among treatments, especially apparent at the end of the experimental period (Figure 3d). In fact, 50 days after starting the salinity treatment, shaded plants under control and salinity conditions showed significantly higher WUE than non-shaded plants (by 40% and 2-fold, respectively). Importantly, shaded pepper plants subjected to salinity showed the highest intrinsic WUE (Figure 3d).

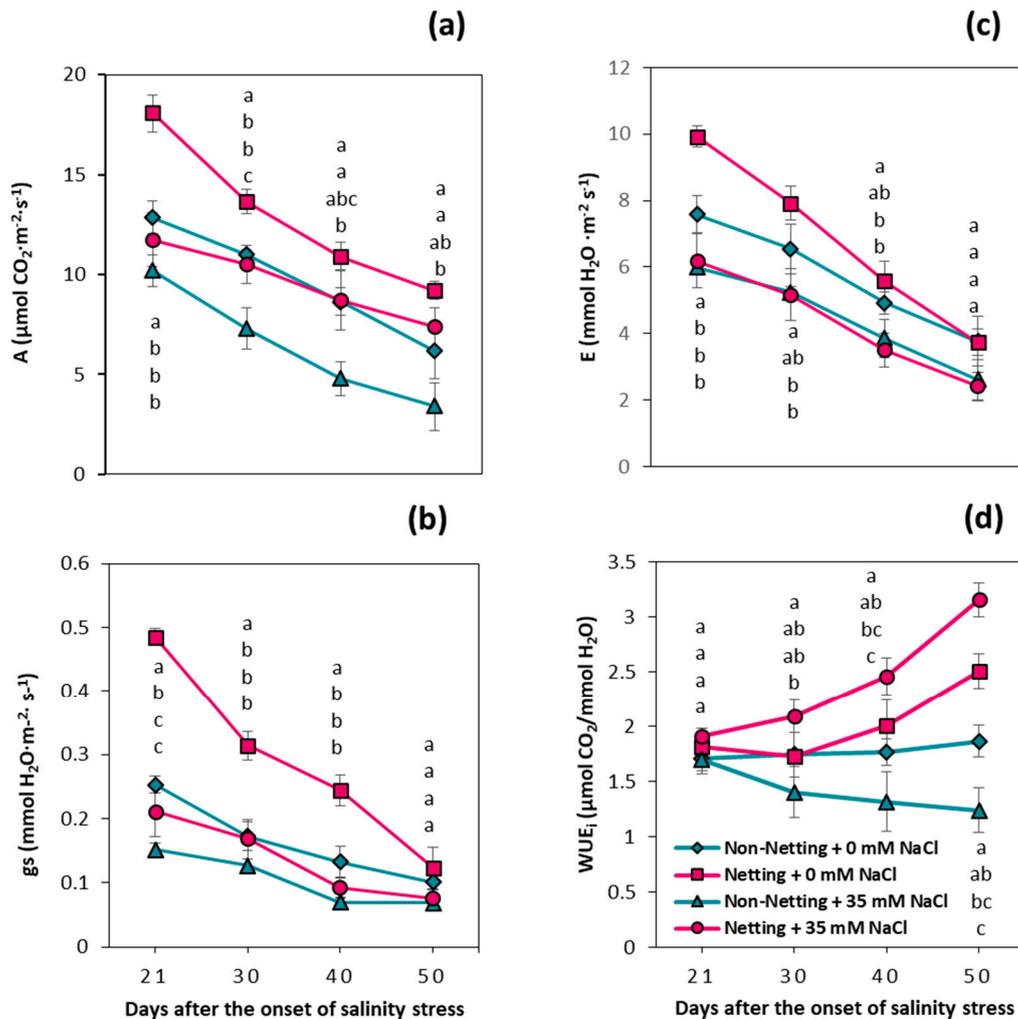


Figure 3. (a) Evolution of photosynthetic rate (A), (b) transpiration rate (E), (c) stomatal conductance (g_s) and (d) intrinsic water use efficiency (WUE_i) in pepper plants cultivated under control (0 mM NaCl) and salinity (35 mM NaCl) conditions and covered or not with red nets. Data are means of four plants \pm standard error. Different letters within each time-point indicate statistical differences according to Tukey’s test ($p \leq 0.05$).

3.4. Plant Water Relations

Leaf water potential decreased with salinity in both shaded and non-shaded plants (Table 1). However, plants grown under red nets showed higher leaf water potential than non-covered pepper plants under both control (by 27%) and salinity conditions (by 28%). Similarly, osmotic potential decreased with salinity, being significantly lower in non-netted plants under salinity conditions with respect to the other treatments (Table 1). Importantly, despite relative water content also decreased with salinity, salinized pepper plants grown under red nets presented significantly higher values (Table 1).

Table 1. Leaf water potential (Ψ_w), osmotic potential (Ψ_s) and relative water content (RWC) in pepper plants cultivated under control (0 mM NaCl) and salinity (35 mM NaCl) conditions and covered or not with red nets.

Cover ¹	Salt Treatment ¹	Ψ_w (Mpa)	Ψ_s (Mpa)	RWC (%)
Non netting	0 mM	−0.74 ab	−1.76 b	88.80 a
	35 mM	−0.94 b	−2.04 a	87.98 ab
Netting	0 mM	−0.53 a	−1.64 b	89.33 a
	35 mM	−0.68 ab	−1.77 b	88.17 b
ANOVA ²				
Cover		*	*	*
Salinity		**	**	*
C × S		*	*	ns

Data are means of four plants. ¹ Different letters within a column indicate significant differences among treatments ($p \leq 0.05$). ² Analysis of variance: ns, not significant; * $p \leq 0.05$; ** $p \leq 0.01$.

3.5. Chlorophyll Content

Salinity significantly decreased chlorophyll *a* content only in non-shaded pepper plants, while the concentrations of this pigment in salinized plants grown under red net were maintained at similar levels to those of control conditions (Figure 4a). However, chlorophyll *b* and total chlorophyll content ($a + b$) were significantly reduced by salinity in both shaded- and non-shaded-grown plants (Figure 4b,c). Interestingly, this reduction was lower in plants grown under red net (by 33% and 15% for chlorophyll *b* and total chlorophyll content, respectively) than in non-shaded pepper plants (45% and 35%, respectively), thus shaded plants maintaining significantly higher chlorophyll *a* and total chlorophyll concentrations under salinity (Figure 4b,c).

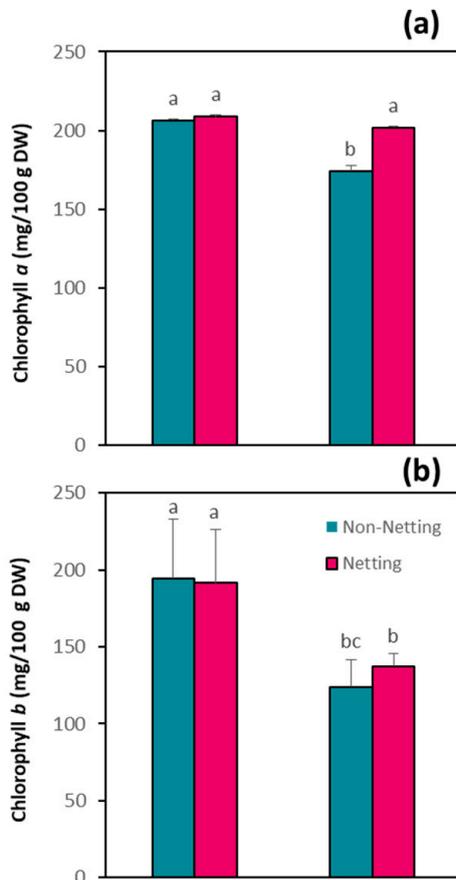


Figure 4. Cont.

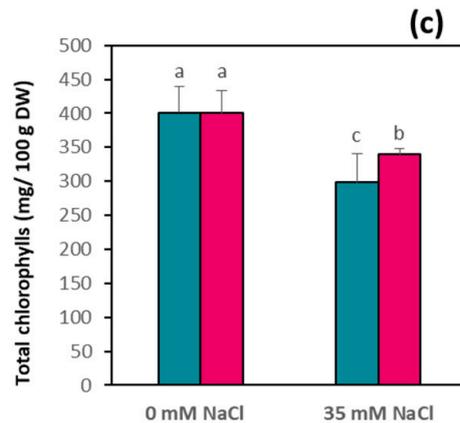


Figure 4. (a) Chlorophyll *a*, (b) chlorophyll *b* and (c) total chlorophyll concentrations in leaves of pepper plants cultivated under control (0 mM NaCl) and salinity (35 mM NaCl) conditions and covered or not with red nets. Bars show the means of four plants with the standard error. Different letters among bars indicate statistical differences according to Tukey's test ($p \leq 0.05$).

3.6. Mineral Composition

Regarding macronutrients, salinity significantly reduced leaf K^+ , P^{5+} and Mg^{2+} concentrations but no effect of netting was observed in P^{5+} and Mg^{2+} , whereas Ca^{2+} was not affected by either salinity or netting (Table 2). Furthermore, netting did not have any effect on K^+ concentrations under control conditions; however, under salinity, shaded plants presented significantly higher K^+ concentrations (by 40%) than non-shaded plants. For micronutrients, differential trends were observed among ion concentrations. Fe^{2+} and B^{3+} concentrations in leaf decreased with salinity, while Cu^{2+} concentrations were increased by netting under both control and salinity conditions but were not affected by netting (Table 2). Surprisingly, Mn^{2+} and Zn^{2+} concentrations increased with salinity in both net treatments but a positive effect of shading on leaf Mn^{2+} concentrations were observed under salinity. As expected, leaf Na^+ concentrations significantly increased with salinity in both shaded and net-grown pepper plants (Table 2). Importantly, pepper plants covered with red nets presented significantly lower Na^+ concentrations (by 27%) than non-netted plants under salinity conditions. Therefore, K^+/Na^+ ratio, despite decreased under salinity, it was significantly higher in pepper plants grown under red nets, especially in salinized plants (Table 2).

Table 2. Cation composition (macronutrients and micronutrients) in leaves of pepper plants cultivated under control (0 mM NaCl) and salinity (35 mM NaCl) conditions and covered or not with red nets.

Cover ¹	Salt treatment ¹	Macronutrients (mg g ⁻¹ DW)				Micronutrients (mg g ⁻¹ DW)						
		K ⁺	P ⁵⁺	Ca ²⁺	Mg ²⁺	Fe ²⁺	B ³⁺	Cu ²⁺	Mn ²⁺	Zn ²⁺	Na ⁺	K ⁺ /Na ⁺
Non netting	0 mM	42.370 a	6.903 a	42.93 a	17.020 a	0.439 a	0.11 a	0.002 a	0.076 b	0.016 b	0.363 c	142.540 b
	35 mM	24.617 c	3.403 b	42.85 a	14.522 c	0.321	0.09 a	0.002 a	0.079 b	0.033 a	7.365 a	3.441 a
Netting	0 mM	42.938 a	6.957 a	44.88 a	17.168 a	0.47 a	0.094 a	0.001 a	0.070 b	0.017 b	0.309 c	165.687 a
	35 mM	34.728 b	3.464 b	40.58 a	12.978 bc	0.32 b	0.089 a	0.001 a	0.101 a	0.0300 a	5.762 b	9.582 b
ANOVA ²												
Cover		*	ns	ns	ns	ns	ns	ns	ns	ns	*	*
Salinity		***	***	ns	*	ns	ns	ns	*	**	**	***
C × S		*	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Data are means of four plants. ¹ Different letters within a column indicate significant differences among treatments ($p \leq 0.05$). ² Analysis of variance: ns, not significant; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

3.7. Hormone Concentrations

Salinity significantly decreased the active CK, tZ, in both shaded and non-shaded plants, while its metabolic precursor, ZR, decreased with salinity only in plants shaded pepper plants and the other active CK, iP, was not affected by salinity (Figure 5a–c). Given that absolute concentrations of tZ and ZR were much higher than those of iP, total CK concentrations, calculated as the sum of the three CKs analyzed, decreased with salinity in both netting treatments (Figure 5d). Importantly, the concentrations of the three CKs and thus total CKs, were significantly superior in netted plants under both control and salinity conditions (by 90 and 20%, respectively, as total CK concentrations, Figure 5a–d).

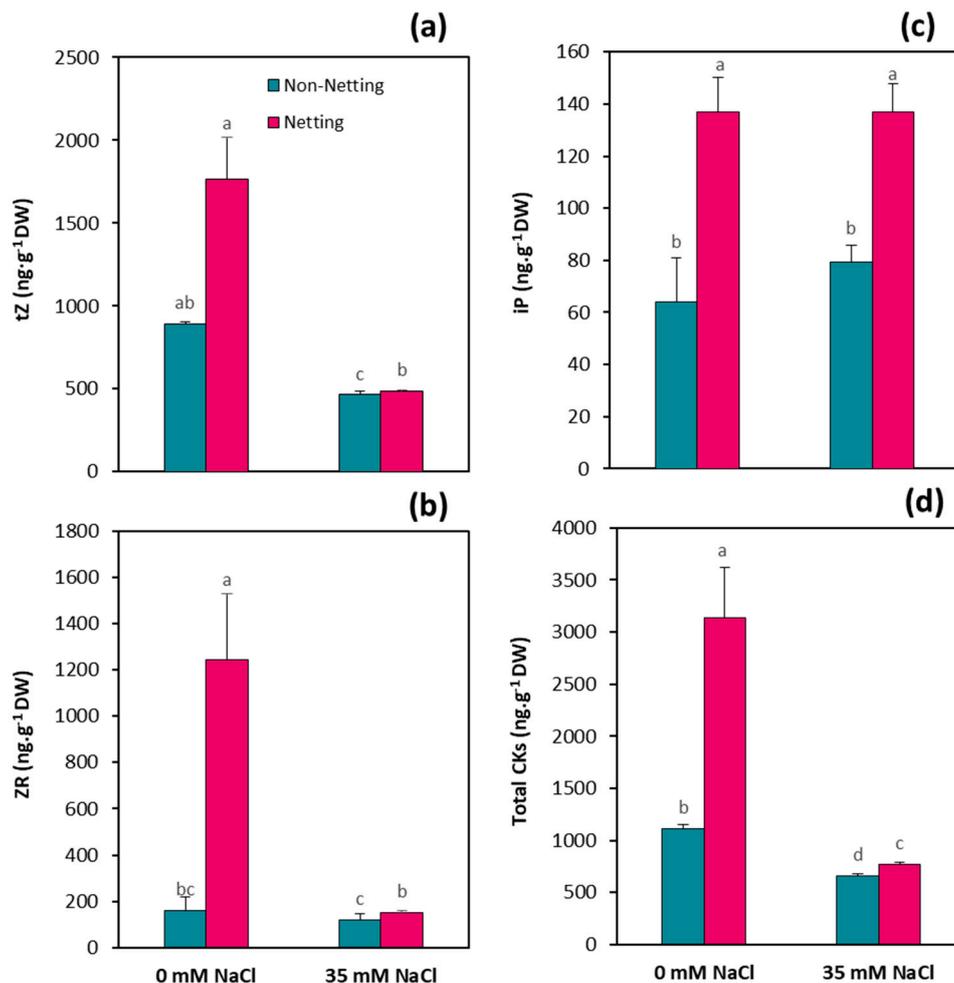


Figure 5. (a) *Trans*-zeatin (tZ), (b) zeatin-riboside (ZR), (c) isopentenyladenine (iP) and (d) total cytokinin (CKs) concentrations in leaves of pepper plants cultivated under control (0 mM NaCl) and salinity (35 mM NaCl) conditions and covered or not with red nets. Bars show the means of four plants with the standard error. Different letters among bars indicate statistical differences according to Tukey's test ($p \leq 0.05$).

Another important hormonal class, the group of GAs, was also differentially affected in pepper plants by salinity and netting. GA_1 and GA_3 decreased with salinity, particularly in non-netted plants (Figure 6a,b), whereas GA_4 significantly increased with salinity in netted plants (by 6-fold, Figure 6c) but their absolute concentrations were lower. Therefore, total GAs, significantly decreased with salinity in pepper plants grown without netting but did not change in plants grown under red nets (Figure 6d). Interestingly, all GAs analyzed and thus total GAs, increased in netted plants, with the only exception of GA_4 in plant grown under control conditions (Figure 6a–d).

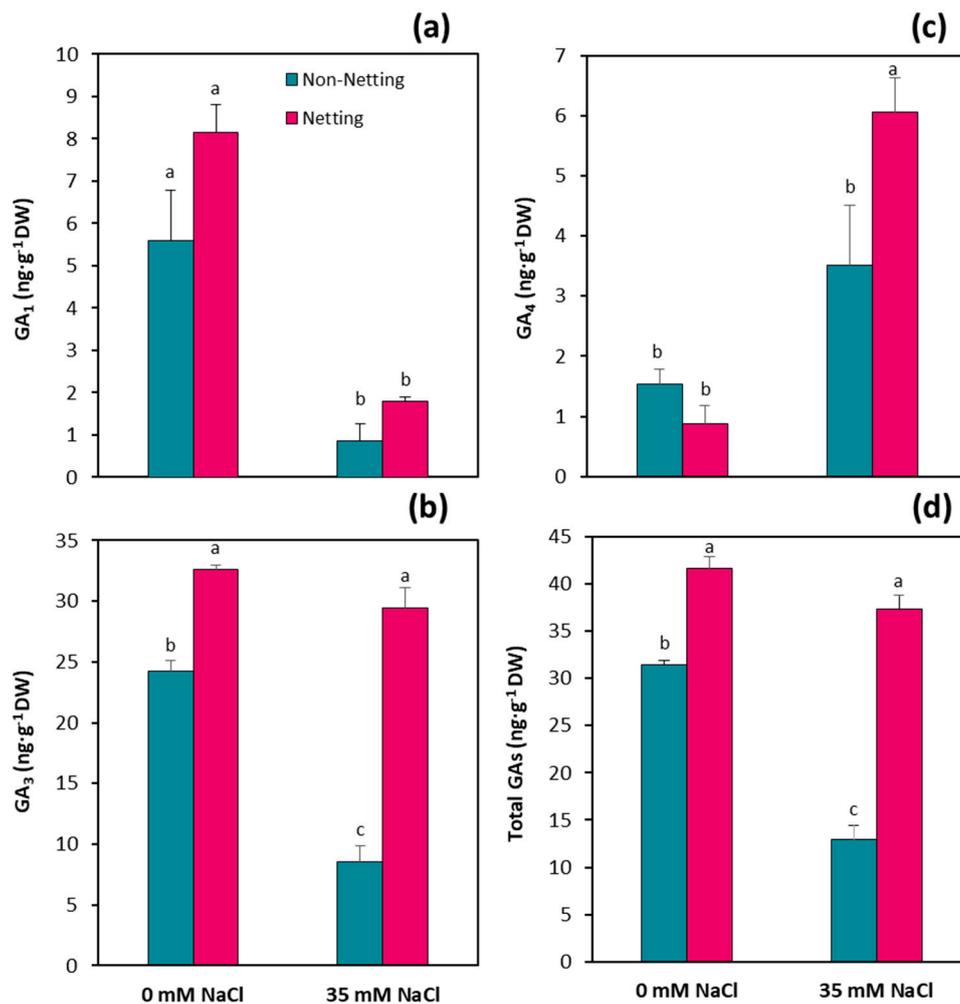


Figure 6. (a) Gibberellin A1 (GA₁), (b) gibberellin A3 (GA₃), (c) gibberellin A4 (GA₄) and (d) total gibberellin (GAs) concentrations in leaves of pepper plants cultivated under control (0 mM NaCl) and salinity (35 mM NaCl) conditions and covered or not with red nets. Bars show the means of four plants with the standard error. Different letters among bars indicate statistical differences according to Tukey's test ($p \leq 0.05$).

The most active auxin form, IAA, was also analyzed and their concentrations significantly increased by netting but were not affected by salinity (Figure 7a). In contrast, the classical abiotic stress-related hormone, ABA, significantly augmented with salinity in both shaded and non-shaded plants but it was kept at significantly lower levels in shaded plants (Figure 7b). The concentrations of the ethylene precursor, ACC, also long considered as a "stress hormone," dramatically increased with salinity in plants grown without (by 12-fold) and with red nets (by 22-fold, Figure 7c). However, netting did not affect ACC concentrations. We also analyzed, two hormones long associated to biotic stresses, JA and SA. SA did not change by salinity treatment but increased in netted plants under both control and salinity conditions (Figure 7d). In contrast, JA significantly decreased with salinity but was not affected by the netting treatment (Figure 7e).

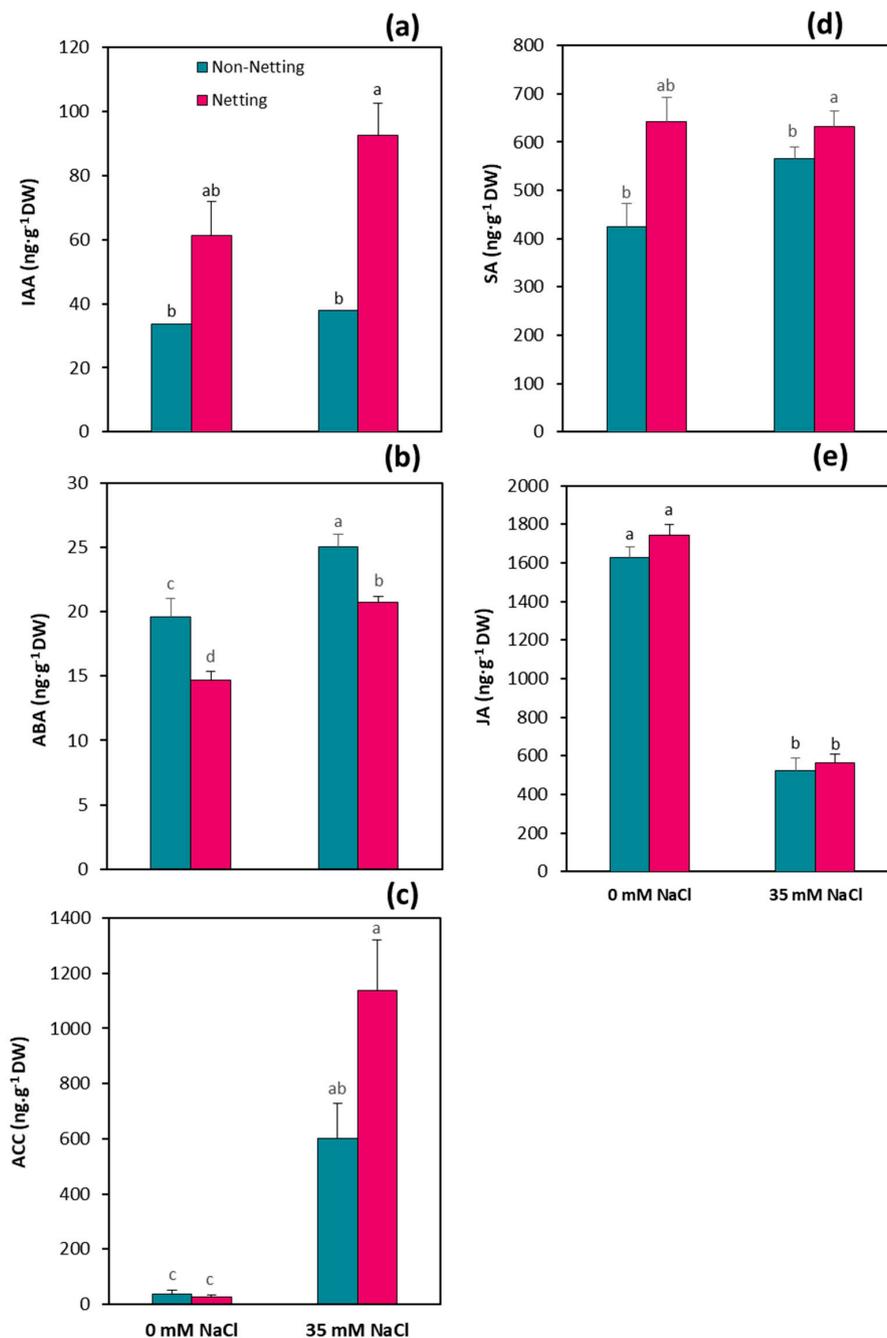


Figure 7. (a) Indole acetic acid (IAA), (b) abscisic acid (ABA), (c) 1-aminocyclopropane-1-carboxylic acid (ACC), (d) salicylic acid (SA) and (e) jasmonic acid (JA) concentrations in leaves of pepper plants cultivated under control (0 mM NaCl) and salinity (35 mM NaCl) conditions and covered or not with red nets. Bars show the means of four plants with the standard error. Different letters among bars indicate statistical differences according to Tukey's test ($p \leq 0.05$).

3.8. Principal Component Analysis

To identify important parameters associated to the variability factors used in this study, salinity and red netting, in relation to pepper plant productivity, we performed a loading-PCA with all variables evaluated and leaf mineral and hormonal traits analyzed (Figure 8a), as well as a score-PCA showing the scores in the transformed coordinates of the 4 biological replicates for each treatment (Figure 8b). The loading-PCA uses an orthogonal transformation to convert the evaluated physiological parameters with high autocorrelation into a set of values of linearly uncorrelated variables called

principal components (PCs). PC1 represented a 49% and PC2 a 15% of the population variability, thus accounting both PCs for the largest part of the total variability (64%). The prevailing productivity patterns, namely shoot FW and CO₂ assimilation (A), are represented by eigenvectors indicating the strength and direction of the parameter relative to both PCs (Figure 8a). Most growth-related parameters and the photosynthetic rate clustered together with important ionic (K⁺ and K⁺/Na⁺ ratio) and hormonal (tZ, ZR and total CKs, GA₁ and JA) factors. In another cluster were associated those parameters which co-varied in opposite direction to the productivity parameters, also involving ionic (Na⁺, Mg²⁺, Fe²⁺, Mn²⁺ and Zn²⁺) and hormonal (ABA and ACC) leaf traits. (Figure 8a). Importantly, the score-PCA clearly grouped the 4 biological replicates of each treatment combination obtained from the two experimental factors (netting and salinity), leading to four differentiated clusters (Figure 8b).

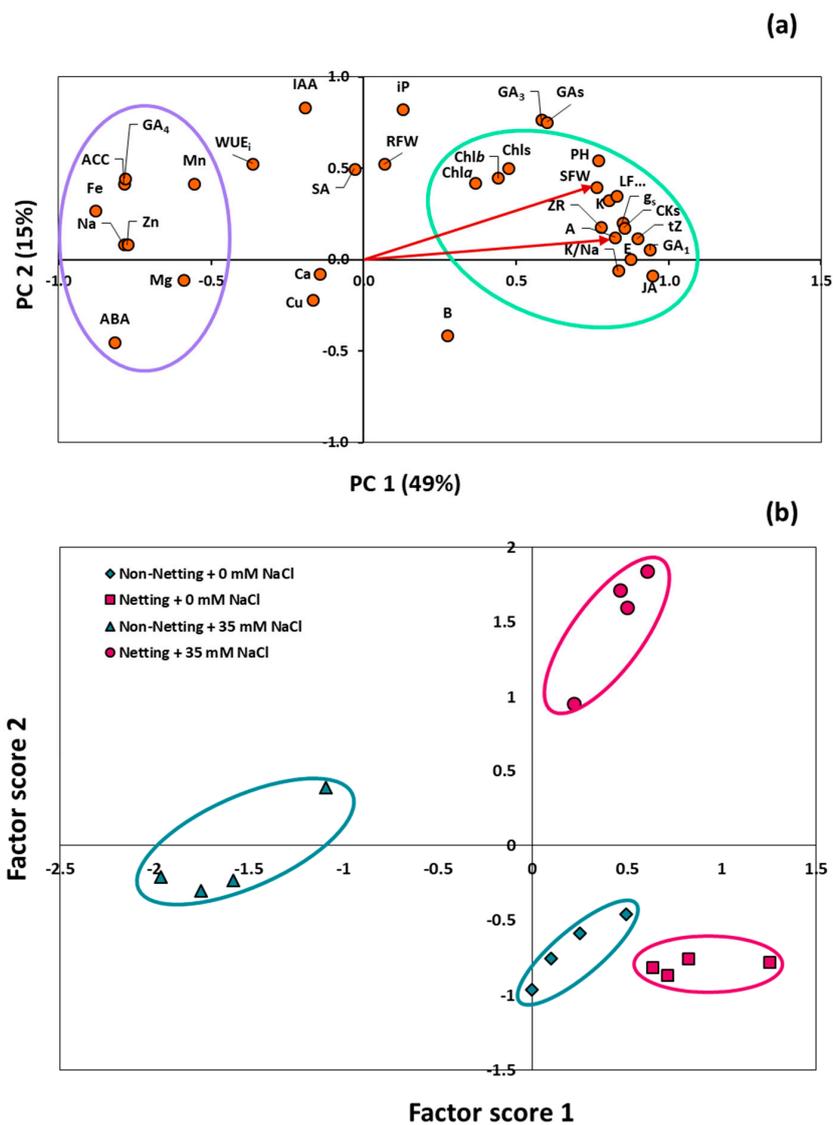


Figure 8. (a) Two axes of a principal component (PC1, PC2) analysis showing the productivity trait vectors (shoot FW, SFW and photosynthetic rate, A) and the loadings of various growth-related, ionic and hormonal variables (denoted by abbreviations) of pepper plants cultivated under control (0 mM NaCl) and salinity (35 mM NaCl) conditions and covered or not with red nets. (b) Bi-Plot representing the score values of each biological replicate in the four treatment combinations. Arrows indicate eigenvectors representing the strength and direction of the trait relative to the two PCs and circles enclose those variables/scores which cluster together. SFW: shoot fresh weigh; LFW: leaf fresh weight; PH: plant height; Chla: chlorophyll a; Chlb: chlorophyll b; Chls: total chlorophylls.

4. Discussion

The agriculture industry is frequently affected by salinity which strongly limits plant productivity. Salinity has been found to disrupt several physiological processes, namely CO₂ assimilation, leading to reduction in vegetative growth [44,45]. As stated previously, some agro-technological strategies, such as protective and photosensitive netting, have been used to cope with abiotic stresses in fruit and horticultural crops and to improve growth performance [26,46,47]. Our study shows that salinity reduced shoot growth-related parameters but shading plants with red nets significantly alleviated salinity effects (Figure 2a–c). This can be in part explained by the reduced temperature and PAR incidence under red nets in the central hours of the day during the growing period (Figure 1). In fact, photosensitive netting has proven to modify the micro-climate conditions (temperature, humidity and wind velocity) leading to plant growth and productivity effects [48]. Low light intensities increased stem elongation in tomato plants [49], whereas leaf area, plant growth and photosynthesis have been found to increase with increased shade level in bell pepper due to the reduced leaf and root zone temperatures [50,51]. Importantly, Rajapakse and Shahak [25] suggested a shading rate of 30% or less as a way to limit thermal impacts on vegetable development, as we used in this study. Furthermore, the use of shading screen alleviated heat stress in ‘Herminio’ sweet pepper in a winter–summer crop cycle as reflected by better plant vegetative growth and leaf water status [46]. Light regime through photosensitive filtration by red netting can also explain the positive growth effects observed in the pepper plants evaluated [25,30].

Additionally, shaded leaves generally have larger total chlorophyll content than leaves from control plants, which can be associated to better photosynthetic status and improved growth. Chlorophyll pigments absorb in the blue (~430 nm) and red regions (~660 nm) and thus red netting would increase leaf chlorophyll status and capacity to capture light [16,22]. In this study, despite salinity reduced chlorophyll concentrations, salinized pepper plants subjected to red net shading were less affected than non-shaded pepper plants (Figure 4). In previous studies with lettuce, a strong positive correlation of leaf total chlorophyll content with light deficiency was detected [52] and in interaction with nitrogen nutrition [53]. As stated by Beneragama and Goto [54], in a study performed with the shade tolerant algae *Euglena gracilis*, although shade-grown photosynthetic organs are not directly exposed to sunlight, they produce additional chlorophyll *a* and *b* to capture diffuse radiation to produce the carbohydrates needed for the plant to grow. Thus, the extent of modulation of these pigments depends on the species, variety, light conditions and temperature, influencing growth and productivity [15]. Sunlight captured by chlorophyll provides the energy for photosynthesis, thus higher content of chlorophyll of shaded pepper plants under salinity conditions is directly associated to a better performance of the photosynthetic apparatus, as demonstrated by their significantly higher CO₂ assimilation with respect to non-shaded plants (Figure 3a). This can be explained by the temperature reduction induced by shading, which might bring net CO₂ assimilation rate closer to its optimal temperature range, thus vapor pressure deficit at the leaf surface will decrease. This would lead to increased photosynthesis and stomatal conductance, as proposed in the regulation of drought and salinity stresses [10,45]. Indeed, protective netting have been demonstrated to improve leaf-level photosynthetic light use efficiency, thus reducing photoinhibition symptoms, in ‘Honeycrisp’ apple under heat stress [47]. Furthermore, light quality manipulation through red nets would help to adjust photosynthesis to stress conditions [55]. Accordingly, sweet pepper leaves of the same age and position were found to respond to light quality manipulation via red netting by increasing net assimilation and stomatal conductance [23].

Increased photosynthetic rate under salinity conditions in shaded pepper plants while stomatal conductance was unaffected resulted in an important improvement of intrinsic WUE (Figure 4a,b,d). Previously, shaded young lemon and apricot trees have been shown to improve WUE through a better control of leaf gas exchange parameters and water relations [10,12,56]. It has been long demonstrated that nets reduce turbulence and produce a humid blanket, which contribute to decreasing environmental evaporative demand, thus influencing WUE, which is especially relevant under drought and salinity

conditions [57]. In fact, our study demonstrated that shading plants with red nets improved leaf water and osmotic potentials as well as relative water content, especially under salinity conditions (Table 1). This can be explained by a higher decoupling of the shaded plants from the atmosphere, particularly under high evaporative demand [12], as shown in apple and peach orchards grown with protective netting, which improved stem water potential with respect to non-shaded orchards [24].

The most detrimental ionic effect of salinity stress is the accumulation of Na^+ in the plant tissues, provoking severe ion imbalances and thus significant physiological disorders [58]. Although Na^+ concentration increased under salinity conditions in the leaves of plants subjected to both netting treatments, thus leading to growth inhibition (Table 2, Figures 1 and 8), shaded pepper plants presented significantly lower leaf Na^+ levels than the non-shaded ones. This can be explained by an efficient Na^+ -exclusion mechanism in the roots [59] and/or a dilution effect because of the better water status (Table 1) of pepper plants grown under red nets. Furthermore, high Na^+ concentration inhibits the uptake of K^+ , which is an essential mineral nutrient for plant growth and development [58]. This salinity-induced ion imbalance seems to be cushioned by shading salinized plants with red nets, since K^+ concentrations and the K^+/Na^+ ratio were significantly higher than those of non-shaded plants (Table 2). Therefore, the increased shoot growth observed in shaded plants under salinity could be partially explained by improved ion homeostasis, as suggests the close association of K^+ and K^+/Na^+ with the growth related parameters in the PCA (Figure 8). Previously, it has been demonstrated, using a recombinant tomato population, that productivity-related parameters under salinity were associated to a better regulation of the ion balance of the plant [34], whereas increased K nutrition has been proven to have ameliorative effects on salinized pepper plants [60,61].

In addition, the reduction in plant growth under salinity conditions could be an outcome of altered hormonal balance. In fact, leaf growth inhibition and premature senescence in salinized tomato plants have been previously associated to decreased bioactive CK concentrations and increased ABA and ACC contents [32,33], as observed in this study. Thus, strategies to increase CK concentrations have been considered to be effective to recover growth and productivity under salinity conditions. For example, engineering tomato rootstocks with a gene encoding the key enzyme of CK biosynthesis, isopentenyl transferase, thus increasing CK concentrations, improved growth and productivity of salinized tomato plants [35]. Importantly, our results show that shading pepper plants significantly increased the concentrations of all bioactive CKs analyzed (Figure 5), closely associated to shoot growth improvement, as demonstrated by their strong linkage within the PCA (Figure 8). This is especially relevant under saline conditions since CK-induced growth and productivity of salinized tomato plants have been previously associated to improved ion homeostasis, particularly of K^+ and K^+/Na^+ ratio, stomatal regulation and CO_2 assimilation [34,35], as also indicates the close association of these factors with the CK content (Figure 8). GAs have been also studied in relation to their role in regulating growth under salinity stress. In fact, exogenous GA application have been shown to improve growth of tomato plants subjected to salinity through crosstalk with other hormones, particularly CKs [37]. However, in the present study, although GA concentrations of pepper plants showed an important increment by shading, especially under salinity conditions (Figure 6), this was partially uncoupled of shoot growth responses as indicated by the PCA (Figure 8).

The classical stress hormones, ABA and ethylene, have been associated to growth impairment under drought and salinity [62–64]. In fact, our results show an increment in the concentrations of ABA and the ethylene precursor, ACC, in pepper plants grown under salinity conditions (Figure 7b,c) linked to growth reduction (Figure 2). However, while shading increased leaf ACC concentrations under salinity, decreased ABA. This could explain the improved water status, gas exchange regulation and thus efficiency in the use of water of salinized peppers plants grown under red nets, since ABA has been long demonstrated to control stomatal opening and water relations under stress [65]. Furthermore, SA and JA, despite classically implicated in the defense response of plants against pathogen attack, they have been also considered to play an important role in controlling salinity and drought stress responses in interaction with other hormones, namely ABA [38,39]. Our data revealed that SA

accumulated in shaded plants under salinity conditions (Figure 7d), which could be an adaptive response to salinity thus limiting ABA accumulation, as previously demonstrated by using tomato mutants impaired in hormone biosynthesis [39]. However, SA did not cluster with shoot growth parameters within the PCA (Figure 8), which limits their role in controlling growth under salinity conditions. Concerning JA, an important drop was observed under salinity (Figure 7e), which would explain their clustering very close to the shoot growth in the PCA (Figure 8). However, JA's role in controlling growth of shaded plants under salinity seems to be marginal, since its concentrations did not vary by growing pepper plants under red nets (Figure 7e).

5. Conclusions

Salinity stress decreased shoot growth of pepper plants associated with leaf FW and plant height reduction, whereas shading plants with red nets improved growth, especially under salinity conditions. The growth recovery of shaded plants under salinity has been associated to better performance of the photosynthetic apparatus due to improved chlorophyll content and CO₂ assimilation. Likewise, stomatal conductance, water relations and thus WUE notably improved by shading. The positive effect of photoselective netting, particularly in salinized plants, can be explained by an efficient mechanism of Na⁺ exclusion, control of ion homeostasis and hormonal balance regulation. A particularly relevant role can be given to CKs, which have been associated to growth control and ion regulation and also to ABA, a master regulator of stomatal opening, which, in crosstalk with SA, lead to a better regulation of water relations in shaded plants. Our results show for the first time that shading plants with red nets modifies plant ion homeostasis and hormonal balance, which could be a strategy to improve growth and productivity under salinity conditions. This is especially important and urgent in the actual scenario of climate crisis, since the most important horticultural areas of the world are suffering severe stress episodes due to reduced quality of both water and soil.

Author Contributions: Conceptualization: A.A., F.M.d.A. and J.L.-M.; methodology: A.G., A.A. and J.L.-M.; formal analysis: A.G. and A.A.; resources: A.A., F.M.d.A. and J.L.-M.; data curation: A.A. and J.L.-M.; writing—original draft preparation: A.A.; writing—review and editing: A.G., A.A., F.M.d.A. and J.L.-M.; supervision: A.A. and J.L.-M.; funding acquisition: F.M.d.A. and J.L.-M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by European Regional Development Fund (ERDF) 80%—Región de Murcia (FEDER 1420-08).

Acknowledgments: The authors thank Carmen Maxi Rodríguez for technical assistance with field experiments and José Sáez-Sironi and María del Puerto Sánchez-Iglesias for technical assistance with ion and hormone analyses.

Conflicts of Interest: The authors declare no conflict of interest.

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