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# The Use of Ecological Hydromulching Improves Growth in Escarole (*Cichorium endivia* L.) Plants Subjected to Drought Stress by Fine-Tuning Cytokinins and Abscisic Acid Balance

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**Abstract:** Drought is considered as one of the major limiting factors to plant growth and productivity. Drought stress reduces stomatal conductance, affecting water relations and decreasing $CO_2$ assimilation rate and photosynthesis. Several strategies have been developed to alleviate the negative effects of drought in the agricultural industry. One of these strategies is the use of the mulching technology, which retains water in the soil surface. Knowing that hormones play a key role in plant growth and drought stress responses, we hypothesized that the use of a new ecological mulching technology called hydromulching would improve growth over bare soil under drought stress through changes in the hormonal balance. To test this hypothesis, escarole plants (*Cichorium endivia* L.) were grown in pots filled with coco fiber, non-covered (bare soil) or covered with polyethylene film (PE) and three types of hydromulches made up with recycled additives: wheat straw (WS), rice hulls (RH), and substrate used for mushroom cultivation (MS). Half of the plants were subjected to drought by reducing the volume of irrigation water to 70% of crop evapotranspiration. Despite drought stress impaired escarole growth-related parameters in all treatments, plants mulched with MS maintained significantly superior growth, due to improved plant water relations and photosynthetic function. This can be explained by an efficient interaction hydromulch/soil/plant in regulating the hormonal balance under water depletion. Indeed, the concentrations of the active cytokinins (CKs), *trans*-zeatin and isopentenyladenine, were higher in plants grown with MS treatment, associated with shoot growth-enhancing and photosynthetic rate maintenance under stress conditions. The concentrations of the stress-related hormone, abscisic acid (ABA), varied antagonistically to those of the active CKs. In this regard, ABA increased with drought but to a lower extent in MS plants thus regulating stomata opening, which, in crosstalk with the ethylene precursor 1-aminocyclopropane-1-carboxylic acid and salicylic acid, improved plant water relations. The results obtained demonstrate that hydromulching is an efficient and sustainable management strategy to ameliorate the drought effects on escarole plants through fine regulation of the CKs/ABA balance, which will be of utmost interest and applicability in the actual climate change scenario.

**Keywords:** escarole; hydromulching; drought stress; hormonal balance; cytokinins; abscisic acid

## 1. Introduction

With global climate change, the reduction in freshwater resources and the imbalanced distribution of water are becoming critical problems that limit global agricultural production [1]. In fact, modern agriculture has to increase yields and product quality while reducing environmental impacts. Thus, it is urgent to improve crop productivity with concomitant conservation of water resources and environmental safety to justify the demand for more yield per drop of water [2]. In the case of the Mediterranean region, this problem has been even more accentuated due to its location with extremely irregular rainfall regimes. Hence, there is a dire need to adopt appropriate technologies to conserve the water in the soil profile and its best possible utilization for plant growth [3,4].

Different agronomic management strategies have been conducted in order to enhance plant tolerance towards abiotic stresses. Mulching technology in horticultural crops is a very common practice to cope with the scarcity of water, apart from other agronomic benefits, such as soil temperature maintenance, weed control, reduction of soil erosion, etc. [5,6]. Recent studies have demonstrated that the use of plastic mulching is an effective strategy to alleviate water deficit in horticultural crops [7,8], and the responses of the plant to plastic mulching depend mainly on the environmental conditions, plant cultivar, and materials used. The reduction in evaporation from crop field by polyethylene mulches enhances both productivity and WUE [9]. The effects of plastic mulches have been investigated in different plants, such as eggplant [7], rice [10], wheat [11], and tomato [1,12]. Nevertheless, the use of plastic mulches in agriculture is associated with negative environmental impacts generating amounts of non-degradable and hardly recyclable wastes [13], which are likely to pollute the environment either through incineration emissions, landfill leaching, or microplastic residues. Thus, research efforts have been focused on the search for new mulching alternatives to the use of traditional polyethylene covers. One of these alternatives is the use of hydromulches, produced from a mixture of water with a lignocellulosic-type polymer, plus other additives, in such a way that a liquid film is obtained. Hydromulching is an innovative ecological mulching technology that has proven to be an efficient strategy for increasing yield in horticultural crops [14,15]. Claramunt et al. [16] reported some aspects of the composition of a set of hydromulches, as well as their mechanical properties from the point of view of resistance to traction and punching forces. Due to their intrinsic characteristics, hydromulches are able to decrease soil evaporation and increase soil water-holding capacity and mineral content by the decomposition of their components [17]. Some studies have shown the effects of inorganic, organic, and living mulches on soil quality and plant growth in horticultural crops [12,18,19]. Although there are not many studies on the use of hydromulching in different crops, some authors have demonstrated its effectiveness in the improvement of yield and fruit quality [15,20]. However, to the best of our knowledge, no attempts have been carried out so far to study the physiological mechanisms that explain the improved productivity associated with the use of hydromulching under water limitation.

Environmental stress induces a set of physiological and biochemical processes in plant metabolism that activate adaptive responses to reach a new cellular equilibrium [21]. Due to climate change, the limitation of natural water resources has become a dramatic problem for food production, and it is expected that water limitation will be even more accused during the next years [3,4,22]. Commonly, drought stress seriously restricts growth and plant development, affecting all the vegetative growth parameters, as plant height, leaf area, and shoot and root development [23,24]. Leaf gas exchange parameters are equally affected by drought, especially net photosynthetic rate, transpiration rate, and stomatal conductance [25]. Escarole (*Cichorium endivia* L.) belongs to the Asteraceae family and is an important and popular vegetable in salads with increasing interest worldwide due to its healthy properties and high nutritive value [26]. In the case of Spain, escarole can be grown all year round in all systems [27], and the total production was 79.9 thousand tons in 2020 with an average yield of 27.8 t $ha^{-1}$ in open field cultivation and 36.8 t $ha^{-1}$ in greenhouse cultivation [2]. Asteraceae species as escarole are well known as drought non-tolerant vegetable crops due to their shallow root system and high water content, consequently, their quality and yield can be affected by drought stress [28]. In response to drought conditions, plants have developed a series of mechanisms to maintain the water content through adaptive traits, which involve maintenance of cell turgor through osmotic adjustment and cellular elasticity, and increasing protoplasmic resistance [29]. These processes are regulated by numerous phytohormones which are the basic mediators to tolerate or avoid the negative effects of water shortage [30]. Indeed, phytohormones are known to play vital roles in the regulation of various phenomena in plants to adjust to varying drought environments [31]. Abscisic acid (ABA) is widely accepted as a stress-related hormone, since it enhances drought tolerance in plants through various physiological and molecular processes, including stomata regulation, root development, and initiation of ABA-dependent pathways [32]. Other hormones, such as ethylene, auxins, gibberellins (GAs) cytokinins (CKs), jasmonic acid (JA), salicylic acid (SA) are also very relevant to cope with the challenge of water stress [33]. Several studies have reported that, similar to ABA, JA induces drought tolerance in plants in various ways, including stomatal closure, ROS removal, and stomatal regulation in response to drought stress [24,34]. Other authors related stomatal closure through SA accumulation under drought stress in Arabidopsis [35]. Furthermore, recent studies have reported that both ethylene and auxins play an active role in regulating drought tolerance in plants by the expression of genes involved in drought stress in rice plants [36,37]. Importantly, CKs have been demonstrated to regulate growth, development, and acclimatization of plants to environmental stress [38,39]. Indeed, transgenic plants expressing an isopentenyltransferase gene (IPT), key in the CK-biosynthetic pathway, showed remarkable drought tolerance through delaying senescence by suppression of drought-induced leaf senescence [24]. Nevertheless, these hormones are usually crosstalking with each other to enhance the survival of plants in drought conditions [40]. Thus, the goal of this research was to analyze the physiological mechanisms that explain the use of novel hydromulching formulations on the growth and productivity of escarole plants subjected to drought stress, with an especial focus on hormonal crosstalk balance.

## 2. Materials and Methods

### 2.1. Plant Material and Experimental Design

The experiment was conducted in a climate-controlled greenhouse located at the "Torreblanca" experimental field in Torre Pacheco, Murcia, Spain (latitude: 37° 45′ N; longitude: 0° 59′ W). Escarole seeds (*Cichorium endivia* L. var. Brillantes) were transplanted to 15 L round black containers filled with coconut fiber. Plants were distributed in rows, with a separation of 10 cm between plants and 2 m between rows. A standard nutrient solution (NPK) for escarole was applied through a drip irrigation system at a depth of 10 cm, with emitters of 2.2 L/h. The mulching treatments were installed on top of the substrate just before the transplanting. Three ecological hydromulching formulations with different recycled additives were used, wheat straw (WS), rice hulls (RH), and substrate used for mushroom cultivation (MS), as well as two control treatments, non-covered substrate (C), and polyethylene (PE). The first control treatment (C) consisted of conventional cultivation without any substrate cover, while the second control treatment (PE) was applied by covering the substrate with a plastic film made of low-density polyethylene. The thickness of all types of hydromulching covers was 2 cm, while the polyethylene mulch had a thickness of 400 gauges. When plants were well established (15 days after transplanting, DAT), a water depletion treatment consisting of 70% of crop evapotranspiration (ETc) was applied to half of the plants assayed throughout the whole experimental period (71 DAT). The substrate moisture was monitored at a depth of 10 cm during the whole experimental period using ECH2O moisture sensors (Decagon Devices, Pullman, WA, USA). Three cultivation blocks with 30 plants per treatment were established.

### 2.2. Plant Growth-Related Determinations

Plant growth-related parameters were recorded at the end of the experiment in 4 plants per treatment. The shoot and root fresh weights (*FW*) were determined and the total leaf area (*LA*) was quantified using a LI-COR leaf area meter (Model LI-3100C; LI-COR, Lincoln, NE, USA). The dry weight (*DW*) was obtained after the samples were oven-dried at 65 °C for 72 h.

*2.3. Plant Water-Related Parameters*

Osmotic potential (Ψs) was analyzed in leaf extracts with an osmometer (model Vapro 5520, Wescor Inc., South Logan, UT, USA). The percentage of leave water content (LWC) was obtained as the difference between FW and DW of the leaf, while leaf succulence (*LS*) was calculated as the ratio between leaf water content and leaf area (*LA*) as follows [41]:

$$LS\left(gH_2O{\cdot}cm^{-2}\right) = \frac{FW_{leaf} - DW_{leaf}}{LA}$$

*2.4. Gas Exchange Parameters*

Gas exchange was monitored in fully expanded leaves during the active growing period (40 DAT). Net $CO_2$ fixation rate ($A$, μmol $CO_2$ $m^{-2}$ $s^{-1}$), stomatal conductance to water vapor ($gs$, mmol $H_2O$ $m^{-2}$ $s^{-1}$), and transpiration rate ($E$, mmol $H_2O$ $m^{-2}$ $s^{-1}$) were measured in a steady-state under conditions of saturating light (800 μmol $m^{-2}$ $s^{-1}$) and 400 ppm $CO_2$ with a LI-6400 instrument (LI-COR, Lincoln, NE, USA). The intrinsic water use efficiency (*WUEi*) of leaf gas exchange was calculated as the ratio between the carbon assimilated through photosynthesis and the stomatal conductance: $WUE_i = \frac{A}{gs}$

*2.5. Chlorophyll Content and Fluorescence*

Chlorophylls were extracted from 1 g of frozen escarole leaves (−80 °C) with 25 mL of acetone 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 [42]. On the leaf used for gas exchange, the dark-adapted maximum fluorescence ($F_m$) andminimum fluorescence ($F_o$), and the light-adapted steady-state chlorophyll fluorescence (F) and maximum fluorescence ($F_m{}^{\circ}$) were measured to calculate the maximum potential quantum efficiency of photosystem II ($F_v/F_m$) with a portable modulated fluorometer, model OS-30P (Opti-Science, Hudson, NY, USA). A special leaf clip holder was allocated to each leaf to maintain dark conditions for at least 30 min before reading.

*2.6. Leaf Mineral Content*

The youngest full-sized leaves of each plant (four plants per treatment) were freeze-dried for 72 h at −55 °C (Christ Alpha 1-2 LDplus, Osterode am Harz, Germany). Anions were extracted with de-ionized water and were subsequently measured with an ion chromatograph (METROHM 861 Advanced Compact IC, Herisau, Switzerland), using a METROHM Metrosep CARB1 150/4.0 mm column. The cations were extracted from freeze-dried leaves (0.1 g) by acid digestion, using an ETHOSONE microwave digestion system (Milestone Inc., Shelton, CT, USA), and analyzed by inductively coupled plasma optical emission (ICP-OES, Varian Vista MPX, Palo Alto, CA, USA). The total nitrogen was analyzed in freeze-dried leaves using a combustion nitrogen determinator (LECO FP-528, Leco Corp., St. Joseph, MI, USA). Analyses were carried out in four replicates.

*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 analyzed according to Albacete et al. [32] and Großkinsky et al. [43] with some modifications. Briefly, 50 mg of freeze-dried escarole leaves were dropped in 0.5 mL of cold (−20 °C) extraction mixture of methanol/water (80/20, $v/v$). Then, 10 μL of an internal standard mix, composed of deuterated hormones ([$^2H_5$]tZ, [$^2H_5$]tZR, [$^2H_6$]iP, [$^2H_2$]GA1, [$^2$H2]GA3, [$^2H_2$]GA4, [$^2H_5$]IAA, [$^2H_6$]ABA, [$^2H_4$]SA, [$^2H_6$]JA, [$^2H_4$]ACC, Olchemim Ltd., Olomouc, Czech Republic) at a concentration of 1 μg $mL^{-1}$ each, were 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 $C_{18}$ 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 0.2 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). For each sample, 10 µL of filtered extract was 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 analyzed component (1, 10, 50, and 100 µg $L^{-1}$) and corrected for 10 µg $L^{-1}$ deuterated internal standards. Recovery percentages ranged between 92% and 95%.

### *2.8. Statistical Analyses*

The data were tested first for homogeneity of variance and normality of distribution. Analysis of variance (ANOVA) and principal component analysis (PCA) were performed using the SPSS software (Version 25.0, SPSS Inc., Chicago, IL, USA). The significance ($p < 0.05$) of the differences between mean values was tested by Tukey's honestly significant difference (HSD). The varimax rotation method was used for loading-PCA while score-PCA was graphically plotted as a Bi-Plot score.

## 3. Results

### *3.1. Plant Growth Parameters*

Plant growth parameters were significantly affected by both the use of hydromulching and water stress application (Figure 1). Plants grown with MS treatment showed a significant increase in shoot FW under control conditions and water stress conditions by 43% and 37%, respectively, in comparison with non-covered plants (Figure 1a). Similarly, root FW, and thus TFW, significantly augmented in the MS-treated plants grown under both optimal and drought stress conditions (Figure 1b,c). Leaf area, which is a developmental parameter related to the gas exchange capacity of the plant, significantly increased in plants grown with MS under both well-irrigated and drought-stressed conditions by 43% and 41%, respectively (Figure 1d).

### *3.2. Plant Water Relations*

Osmotic potential rose with drought in all treatments, although it was significant only in non-mulched plants (Table 1). Under control conditions, mulching did not produce any effect on the osmotic potential, while drought stress provoked a significant reduction in plants grown on bare soil and mulched with MS with respect to the other treatments. Interestingly, despite leaf water content decreasing with drought, it was significantly higher in plants grown with MS compared to the other mulching treatments and the bare soil (Table 1). Mulching treatments produced a significant effect in leaf succulence only under optimal conditions. Indeed, plants grown in soil covered with MS presented significantly higher leaf succulence than the other treatments, but no differences among treatments were observed under drought stress (Table 1).

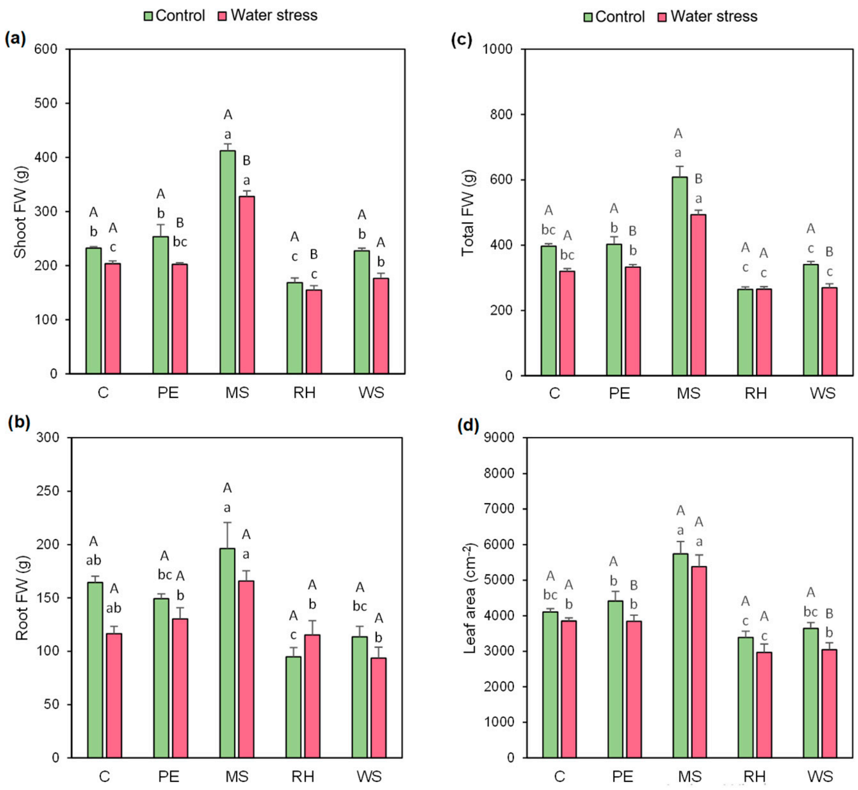


**Figure 1.** (**a**) Shoot FW, (**b**) root FW, (**c**) total FW, and (**d**) leaf area of escarole plants of the commercial variety "Brillantes" non-mulched or subjected to different mulching treatments and cultivated under control (well-watered) and water stress (70% ETc) conditions. Bars show the means of five plants ± standard error. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey's test ($p \leq 0.05$). Abbreviations used: non-covered substrate (C), polyethylene mulch (PE), mushroom substrate-based hydromulch (MS), rice hulls-based hydromulch (RH), and wheat straw-based hydromulch (WS).

**Table 1.** Osmotic potential (Ψs), leaf water content (LWC), and leaf succulence (LS) of escarole plants of the commercial variety "Brillantes" non-mulched or subjected to different mulching treatments and cultivated under control (well-watered) and water stress (70% ETc) conditions.

| Irrigation | Mulch | Ψs (MPa) | LWC (%) | LS (g $H_2O$ cm$^{-2}$) |
|---|---|---|---|---|
| Control | C | −0.97 ± 0.05 A a | 91.72 ± 0.24 A a | 0.05 ± 0.002 A bc |
| | PE | −1.04 ± 0.05 A a | 90.13 ± 0.25 A a | 0.05 ± 0.002 A bc |
| | MS | −1.11 ± 0.07 A a | 92.19 ± 0.42 A a | 0.07 ± 0.003 A a |
| | RH | −0.93 ± 0.01 A a | 88.94 ± 0.16 A b | 0.04 ± 0.001 A c |
| | WS | −1.08 ± 0.07 A a | 91.09 ± 0.10 A a | 0.06 ± 0.002 A b |
| Stress | C | −1.27 ± 0.06 B b | 84.69 ± 0.07 B c | 0.04 ± 0.001 B a |
| | PE | −1.08 ± 0.03 A ab | 85.52 ± 0.14 B bc | 0.05 ± 0.003 A a |
| | MS | −1.26 ± 0.07 A b | 85.94 ± 0.33 Ba | 0.05 ± 0.002 B a |
| | RH | −1.01 ± 0.07 A a | 85.50 ± 0.39 B bc | 0.04 ± 0.002 A a |
| | WS | −1.02 ± 0.01 A a | 85.57 ± 1.13 B b | 0.05 ± 0.004 A a |

Data are the means of five plants ± standard error. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey's test ($p \leq 0.05$). Abbreviations used: non-covered substrate (C), polyethylene mulch (PE), mushroom substrate-based hydromulch (MS), rice hulls-based hydromulch (RH), and wheat straw-based hydromulch (WS).

### 3.3. Gas Exchange Parameters

Under control conditions, all treatments presented similar values of net photosynthetic production. Drought stress significantly reduced net photosynthesis except for MS plants, which maintained significantly higher values than non-mulched (by 44%) and the other mulched plants (by 83%, Figure 2a). In contrast to net photosynthesis, the reduction in stomatal conductance provoked by drought stress was not significant. However, PE-, MS-, and RH-plants presented significantly higher stomatal conductance than plants grown on bare soil (by 10%, Figure 2b). Regarding transpiration rate, this parameter was not affected by either drought stress or mulching treatment, with the only exception of plants mulched with WS that showed lower (not significant) values than the other treatments (Figure 1c). The combination of different mulching treatments and the water stress produced significant effects on the intrinsic water use efficiency (WUEi). Despite WUEi decreasing with the stress, plants mulched with MS presented significantly higher WUEi than the other hydromulching treatments and the controls (by 80%), matching the WUEi values under non-stressed conditions (Figure 1d).

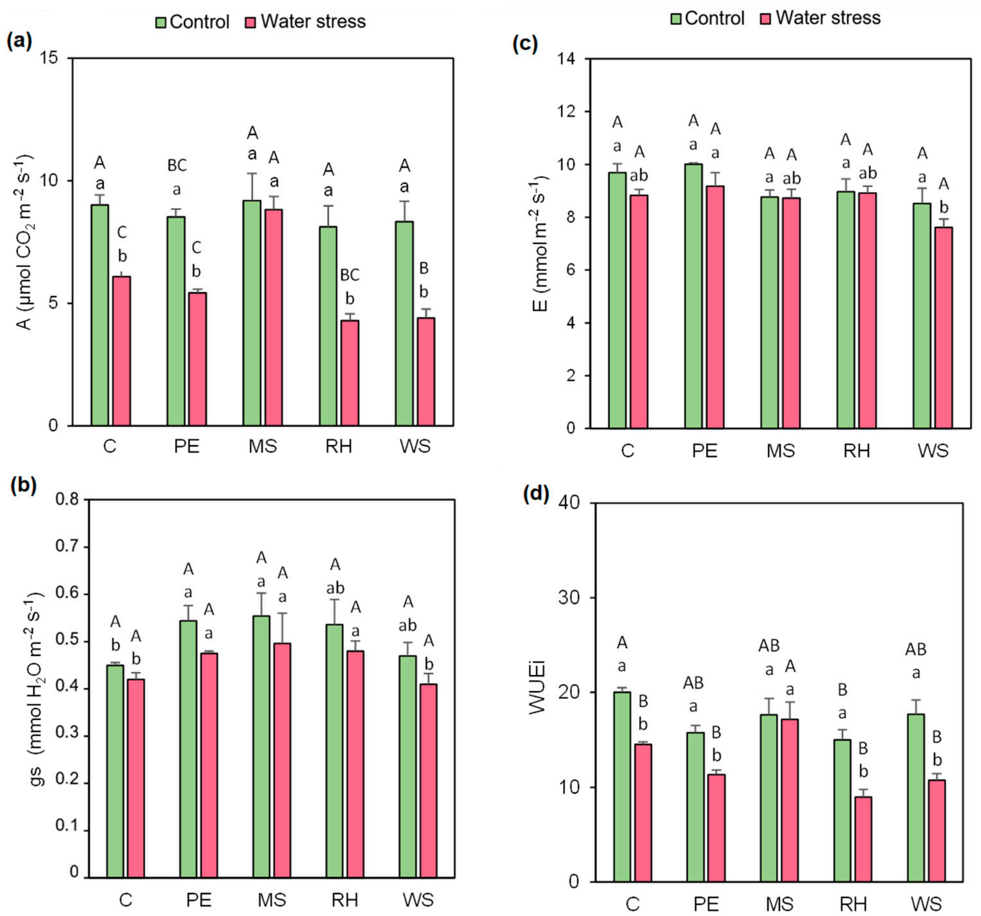


**Figure 2.** (**a**) Photosynthetic rate (A), (**b**) stomatal conductance (gs), (**c**) transpiration rate (E), and (**d**) intrinsic water use efficiency (WUEi) of escarole plants of the commercial variety "Brillantes" non-mulched or subjected to different mulching treatments and cultivated under control (well-watered) and water stress (70% ETc) conditions. Bars show the means of five plants ± standard error. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey's test ($p \leq 0.05$). Abbreviations used: non-covered substrate (C), polyethylene mulch (PE), mushroom substrate-based hydromulch (MS), rice hulls-based hydromulch (RH), and wheat straw-based hydromulch (WS).

### 3.4. Chlorophyll Fluorescence and Content

Chlorophyll fluorescence (Fv/Fm), which indicates the efficiency of photosystem II, was significantly affected by the drought stress only in plants grown on bare soil (Figure 3a). Despite the fact that chlorophyll a was not affected by drought stress, non-mulched plants showed a significantly lower concentration of chlorophyll a than the mulched plants (Figure 3b). However, chlorophyll b was affected by both the mulching treatment and drought stress. Indeed, chlorophyll b concentrations significantly decreased with drought stress in plants grown on bare soil, whereas PE- and MS-plants presented significantly higher concentrations than non-mulched plants under drought stress (by 60%, Figure 3c). Therefore, total chlorophyll concentrations, calculated as the sum of chlorophyll a and chlorophyll b concentrations, were also significantly reduced by drought in non-mulched plants, whereas increased significantly in PE- and MS-plants under water stress with respect to the non-covered plants (by 35%, Figure 3d).

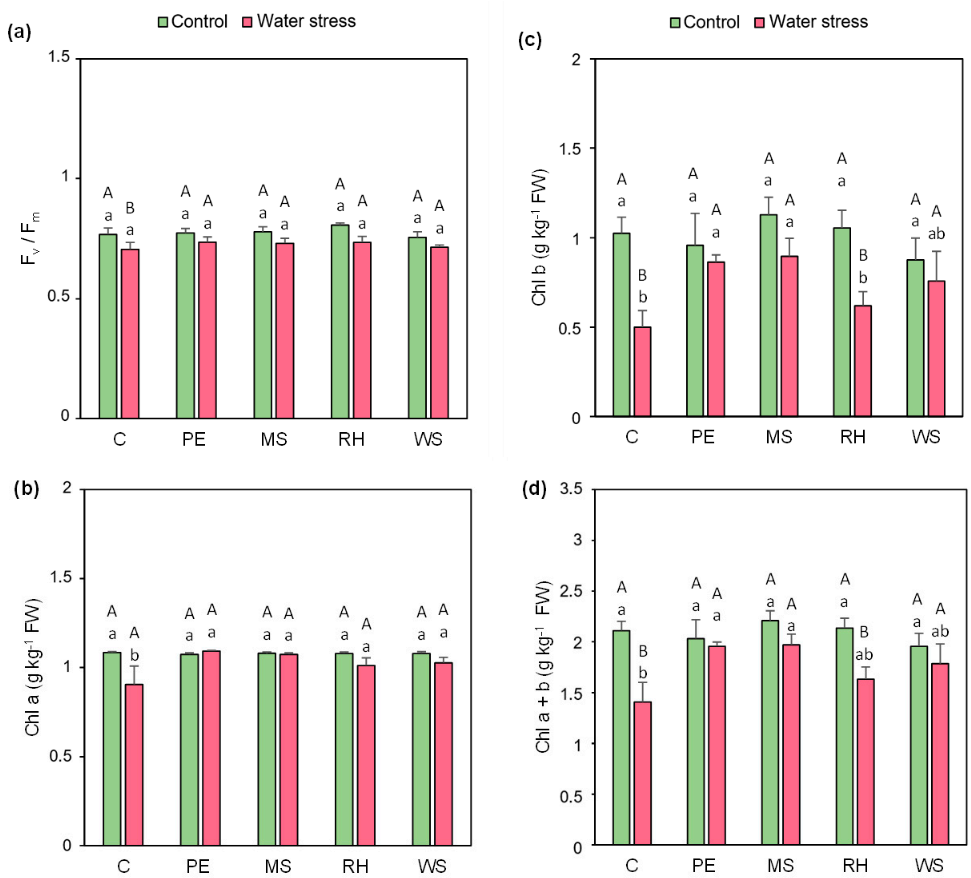


**Figure 3.** (**a**) Chlorophyll fluorescence ($F_v/F_m$), (**b**) chlorophyll a, (**c**) chlorophyll b, and (**d**) total chlorophyll (a + b) in the leaves of escarole plants of the commercial variety "Brillantes" non-mulched or subjected to different mulching treatments and cultivated under control (well-watered) and water stress (70% ETc) conditions. Bars show the means of five plants ± standard error. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey's test ($p \leq 0.05$). Abbreviations used: non-covered substrate (C), polyethylene mulch (PE), mushroom substrate-based hydromulch (MS), rice hulls-based hydromulch (RH), and wheat straw-based hydromulch (WS).

### 3.5. Leaf Mineral Content

Table 2 exhibits the concentrations of mineral nutrients in escarole leaves. Under control conditions, N, K, Zn, and Cl concentrations presented significant differences among mulching treatments. Importantly, MS-treated plants presented significantly higher concentrations of K, Zn, and Cl than plants grown with PE or on bare soil (Table 2). In general, drought stress increased leaf mineral content due to, in part, a concentration effect as a consequence of reduced leaf water content and leaf succulence of stressed leaves. The mulching treatment affected the concentrations of P, K, $SO_4^{-2}$, and Zn under water stress, from which leaf K concentrations of MS-mulched plants stood out, with 46% higher leaf K concentrations than bare soil.

**Table 2.** Macronutrient and micronutrient concentrations in leaves of escarole plants of the commercial variety "Brillantes" non-mulched or subjected to different mulching treatments and cultivated under control (well-watered) and water stress (70% ETc) conditions.

| Irrigation | Mulch | N (mg $g^{-1}$ DW) | P (mg $g^{-1}$ DW) | K (mg $g^{-1}$ DW) | Mg (mg $g^{-1}$ DW) | Ca (mg $g^{-1}$ DW) | $SO_4^{-2}$ (mg $g^{-1}$ DW) |
|---|---|---|---|---|---|---|---|
| Control | C | 14.89 A a | 8.70 A a | 23.60 B b | 2.61 A a | 7.57 A a | 2.54 A a |
| | PE | 16.94 A ab | 8.63 B a | 31.33 A ab | 2.39 A a | 7.38 A a | 2.90 A a |
| | MS | 13.43 B ab | 7.90 B a | 37.70 A a | 2.25 A a | 7.12 A a | 3.62 A a |
| | RH | 16.18 A ab | 8.33 B a | 29.29 A ab | 2.68 A a | 7.62 A a | 3.00 A a |
| | WS | 14.25 A b | 7.29 B a | 29.99 A ab | 2.47 A a | 7.08 A a | 3.09 A a |
| Stress | C | 15.35 A a | 9.23 A b | 27.99 A b | 2.77 A a | 8.37 A a | 2.89 A ab |
| | PE | 17.51 A a | 9.82 A ab | 28.49 A b | 2.85 A a | 9.18 A a | 2.36 A b |
| | MS | 19.51 A a | 9.92 A ab | 41.24 A a | 2.31 A a | 8.89 A a | 3.96 A a |
| | RH | 16.82 A a | 11.11 A a | 30.62 A b | 3.06 A a | 9.51 A a | 3.82 A a |
| | WS | 15.66 A a | 10.76 A ab | 30.80 A b | 2.59 A a | 9.05 A a | 3.46 A ab |
| **Irrigation** | **Mulch** | **Cu (mg $kg^{-1}$ DW)** | **Mn (mg $kg^{-1}$ DW)** | **Zn (mg $kg^{-1}$ DW)** | **B (mg $kg^{-1}$ DW)** | **Cl (mg $g^{-1}$ DW)** | **Na (mg $g^{-1}$ DW)** |
| Control | C | 1.31 A a | 26.26 A a | 8.75 B b | 25.82 A a | 29.68 A b | 12.46 A a |
| | PE | 3.02 A a | 32.09 A a | 7.81 A b | 28.18 A a | 26.13 A b | 11.00 A a |
| | MS | 2.86 A a | 22.86 A a | 19.76 A a | 25.79 B a | 36.99 A a | 12.92 B a |
| | RH | 2.07 A a | 18.41 A a | 18.43 A ab | 26.33 A a | 26.73 B b | 10.56 A a |
| | WS | 1.84 A a | 25.77 A a | 12.37 B a | 26.04 A a | 30.31 A b | 11.88 A a |
| Stress | C | 1.66 A a | 29.42 A a | 15.84 A b | 24.46 A a | 28.99 A a | 12.67 A a |
| | PE | 1.30 A a | 27.68 A a | 12.02 A b | 27.05 A a | 28.25 A a | 13.45 A a |
| | MS | 2.68 A a | 30.23 A a | 24.36 A a | 30.08 A a | 40.63 A a | 15.49 A a |
| | RH | 1.59 A a | 22.08 A a | 17.55 A ab | 25.23 A a | 35.48 A a | 12.58 A a |
| | WS | 1.81 A a | 30.41 A a | 17.60 A ab | 25.85 A a | 32.20 A a | 13.89 A a |

Data are the means of five plants. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey's test ($p \leq 0.05$). Abbreviations used: non-covered substrate (C), polyethylene mulch (PE), mushroom substrate-based hydromulch (MS), rice hulls-based hydromulch (RH), and wheat straw-based hydromulch (WS).

### *3.6. Hormonal Profiling*

Three of the most active CKs in higher plants, tZ, RZ, and iP, were analyzed in escarole leaves, but only tZ and iP were detected (Figure 4). Water stress provoked a significant decrease in CKs in all mulching treatments. Even though no significant differences between mulching treatments were found in control conditions, plants grown with MS mulching presented the highest levels of tZ (Figure 4a). Similarly, the concentrations of tZ in stressed plants were significantly higher in MS mulching with respect to the other mulching treatments and, especially, to the non-covered plants (by 2.2-fold). A similar pattern was observed in iP concentrations, with 3-fold higher iP concentrations in MS-covered plants than non-covered plants under water stress conditions (Figure 4b). Therefore, plants grown with MS mulching presented the greatest concentrations of total CKs, calculated as the sum of concentrations of the two active CKs detected, under control conditions, which turned significant under stress conditions (2.3-fold higher than plants grown on bare soil, Figure 4c).

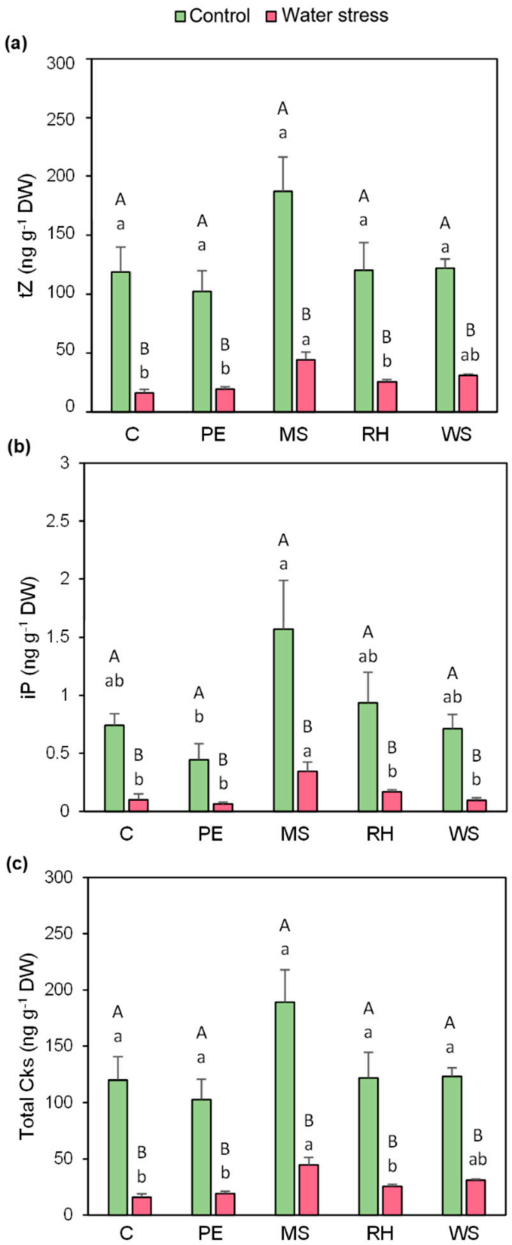


**Figure 4.** (**a**) Trans-zeatin (tZ), (**b**) isopentenyladenine (iP), and (**c**) total cytokinin (CKs) concentrations in leaves of escarole plants of the commercial variety "Brillantes" non-mulched or subjected to different mulching treatments and cultivated under control (well-watered) and water stress (70% ETc) conditions. Bars show the means of five plants ± standard error. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey's test ($p \leq 0.05$). Abbreviations used: non-covered substrate (C), polyethylene mulch (PE), mushroom substrate-based hydromulch (MS), rice hulls-based hydromulch (RH), and wheat straw-based hydromulch (WS).

Figure 5 shows the GA profile in the leaves of escarole plants. From the three GAs analyzed (GA1, GA3, and GA4), only GA1 and GA4 were detected. GA1 concentrations decreased significantly with drought stress in all mulching treatments. Importantly, under control conditions, the concentrations of GA1 of plants mulched with MS were higher than those of the other treatments, despite only showing significant differences with the PE treatment (Figure 5a). However, the mulching treatment did not provoke any significant change in GA1 under water stress. The concentrations of GA4 in plants mulched with MS under control conditions were notably higher than those of the other treatments

(14-fold higher than in PE-treated plants, Figure 5b). As in GA1, the mulching treatment did not provoke significant changes in the concentrations of GA4 under stress. Therefore, total GA concentrations were significantly reduced by the water stress, but the mulching treatment only significantly increased GA concentrations in MS-covered plants with respect to PE-covered plants under non-stressed conditions (Figure 5c).

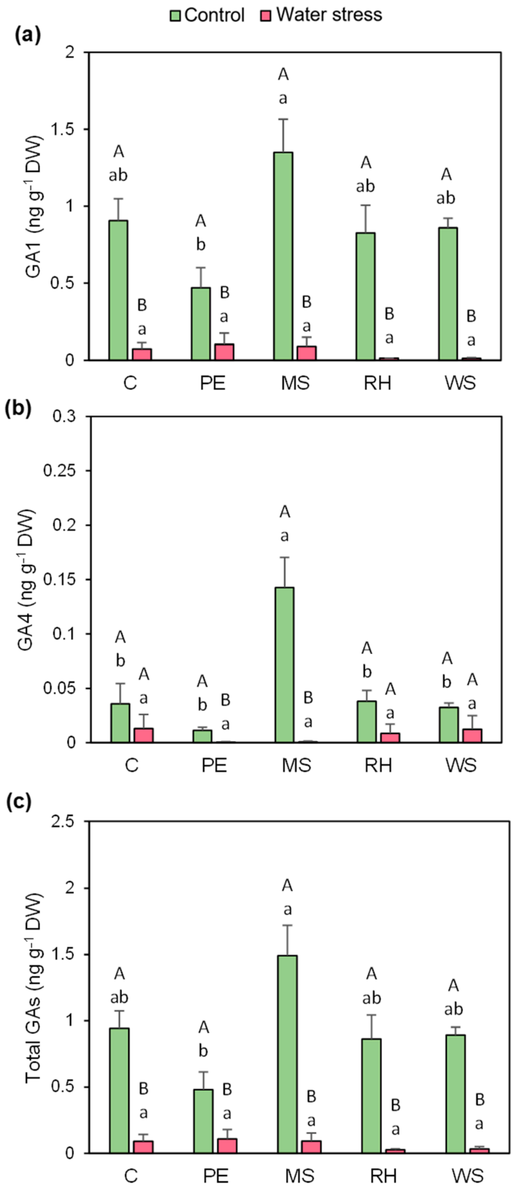


**Figure 5.** (**a**) Gibberellin A1 (GA1), (**b**) gibberellin A4 (GA4), and (**c**) total gibberellin (GAs) concentrations in leaves of escarole plants of the commercial variety "Brillantes" non-mulched or subjected to different mulching treatments and cultivated under control (well-watered) and water stress (70% ETc) conditions. Bars show the means of five plants ± standard error. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey's test ($p \leq 0.05$). Abbreviations used: non-covered substrate (C), polyethylene mulch (PE), mushroom substrate-based hydromulch (MS), rice hulls-based hydromulch (RH), and wheat straw-based hydromulch (WS).

ABA has been classically associated with abiotic stress responses, especially through its role in regulating stomatal closure during drought stress. In this study, ABA concentrations significantly augmented under drought stress conditions (Figure 6a). However, this increase was less apparent in plants covered with MS, which showed significantly lower ABA concentrations than the other treatments (by 59% compared to non-covered plants). The important role of ethylene as a stress-related hormone is well known, and the concentrations of ACC, its direct precursor, dramatically increased in stressed conditions (Figure 6b). Importantly, this augmentation was less evident in hydromulched plants, despite no significant differences among mulching treatments were observed under drought stress. Regarding auxins, the most important hormone of this class is IAA, which was affected by the mulching treatment in non-stressed plants, with a significant increase in IAA concentrations in the plants covered with MS with respect to the other treatments (2.5-fold higher than non-covered plants, Figure 6c). Despite the fact that SA and JA have been classically associated with biotic stress, their role in abiotic stress responses has also been described, and we thus resolved to analyze them. Under control conditions, SA concentrations were similar in all mulching treatments (Figure 6d). When plants were subjected to drought stress, SA concentrations generally increased, especially in non-covered plants (2.5-fold). Under stress, the lowest concentrations of SA were found in MS plants, which were significantly lower than those of non-covered plants (by 40%). In contrast, JA decreased with drought stress, especially in RH plants, but no differences due to the mulching treatment were observed under stress (Figure 6e).

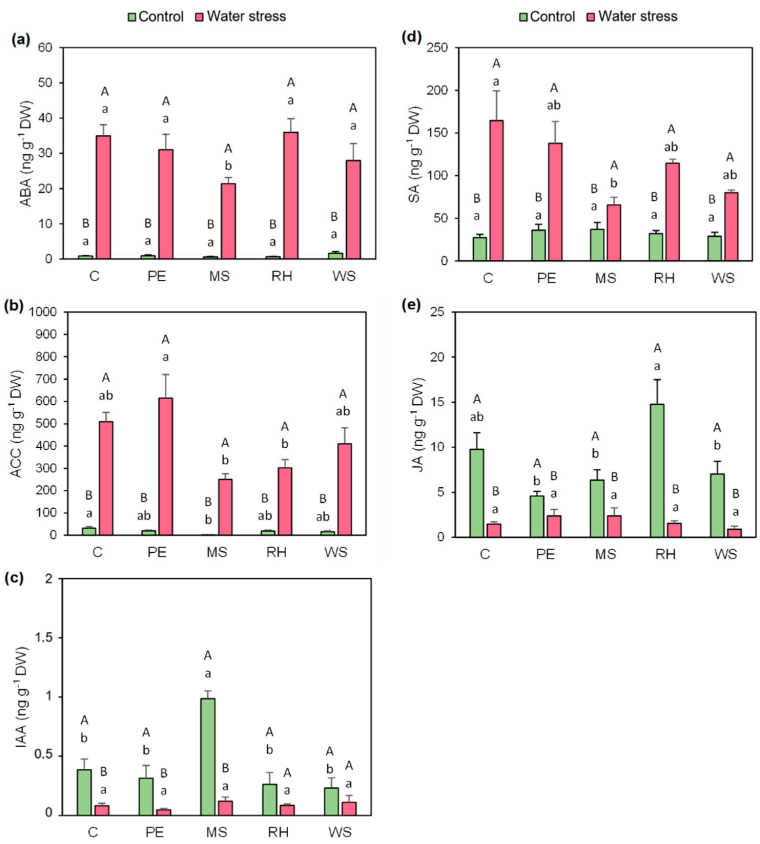


**Figure 6.** (**a**) Abscisic acid (ABA), (**b**) 1-aminocyclopropane-1-carboxylic acid (ACC), (**c**) indole acetic acid (IAA), (**d**) salicylic acid (SA), and (**e**) jasmonic acid (JA) concentrations in leaves of escarole plants of the commercial variety "Brillantes" non-mulched or subjected to different mulching treatments and cultivated under control (well-watered) and water stress (70% ETc) conditions. Bars show the means of five plants ± standard error. Different capital letters indicate significant differences due to the water stress treatment while different small letters show significant differences among mulching treatments according to Tukey's test ($p \leq 0.05$). Abbreviations used: non-covered substrate (C), polyethylene mulch (PE), mushroom substrate-based hydromulch (MS), rice hulls-based hydromulch (RH), and wheat straw-based hydromulch (WS).

### *3.7. Principal Component Analysis*

To statistically test whether existed differential patterns of the mulching treatments assayed under control and drought conditions, the set of data was subjected to a score-PCA (Figure 7a). This statistical test converts the normalized data into transformed coordinates of the four biological replicates of each group treatment through multiple dimension rotation that describes the major patterns of variation associated with the two experimental factors. The score-PCA revealed a clear separation between the scores of the plants assessed under control and drought conditions and among those of the different mulching treatments but only under stress conditions (Figure 7a). Importantly, the scores of plants grown with MS treatment were clearly separated from the rest of the treatments under both control and stress conditions.

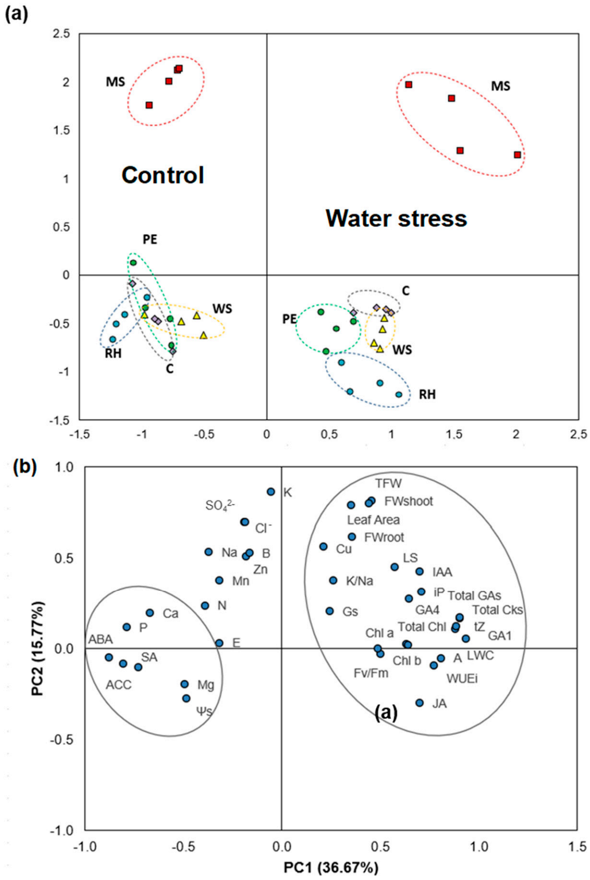


**Figure 7.** (**a**) Bi-Plot representing the score values and (**b**) two axes of a principal component (PC1, PC2) analysis showing the loadings of various growth-related, ionic and hormonal variables (denoted by abbreviations) of the escarole commercial variety "Brillantes" non-mulched or subjected to different mulching treatments and cultivated under control (well-watered) and water stress (70% ETc) conditions. Circles enclose those variables/scores which cluster together in loading PCA and score PCA. Abbreviations used: boron (B), calcium (Ca), copper (Cu), iron (Fe), potassium (K), magnesium (Mg), manganese (Mn), sodium (Na), phosphorus (P), chloride ($Cl^-$), sulfate ($SO_4^{2-}$), root fresh weigh (FWroot), shoot fresh weight (FWshoot), leaf area (LA), total fresh weight (TFW), chlorophyll a (Chl a), chlorophyll b (Chl b), total chlorophylls (Total Clh), net $CO_2$ fixation rate (A), stomatal conductance (gs), transpiration rate (E), intrinsic water use efficience (WUEi), osmotic potential (Ψs), leaf succulence (LS), leaf water content (LWC), abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (ACC), indole acetic acid (IAA), salicylic acid (SA), jasmonic acid (JA), gibberellin A1 (GA1), gibberellin A4 (GA4), total gibberellins (GAs), trans-zeatin (tZ), isopentenyladenine (iP), total cytokinins (CKs), non-covered substrate (C), polyethylene mulch (PE), mushroom substrate-based hydromulch (MS), rice hulls-based hydromulch (RH), and wheat straw-based hy-dromulch (WS).

Additionally, a loading-PCA was performed to identify important parameters associated with the variability factors used in this study, mulching and drought stress, in relation to escarole growth and productivity (Figure 7b). 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). This mathematical algorithm allows the identification of physiological traits regarding productivity and growth behavior under drought stress in escarole. The loading-PCA revealed a clear association between the growth-related parameters (shoot FW, root FW, total FW, and leaf area) and the photosynthesis-related parameters (chlorophyll a and b, total chlorophylls, chlorophyll fluorescence, and net photosynthesis). Those growth and productivity traits were positively associated with important hormonal factors, namely tZ, iP, total CKs, GA1, GA3, total GAs, IAA, and JA, whereas ABA, ACC, and SA were associated in an opposite cluster (Figure 7b). Notably, Cu was the only mineral nutrient that was positively associated with the growth and productivity parameters.

## 4. Discussion

Several efforts have been directed toward the search for technological strategies seeking to more efficiently use water resources in food production [44]. Plastic mulching is an effective technology used to conserve soil moisture and to improve crop growth and productivity [5], but, as stated before, presents negative environmental impacts. Therefore, hydromulching has been proposed here as a sustainable alternative to plastic mulching. Our results revealed that plants grown with the MS hydromulch significantly increased the growth-related parameters (shoot FW, root FW, and total FW) under both optimal irrigation conditions and under water limitation (Figure 1). Regarding water relations, leaf succulence under control conditions and leaf water content under stress were superior in plants grown with MS than in the other treatments (Table 1). Enhanced escarole growth with the MS hydromulching might be partly explained by better water availability due to soil moisture conservation, especially under water stress, as suggests their association in the loading-PCA (Figure 7b). Covering the soil with hydromulch could be useful for both delaying water evaporation and controlling drought stress due to its physical properties. Claramunt et al. [16] and Verdú et al. [17] showed that MS hydromulch delayed the process by which the liquid water is converted into vapor and removed from the surface. Indeed, several authors have demonstrated the boost of yield and leaf area using mulches, due to their direct effect on better conservation of soil water [45–47]. Furthermore, root growth improvement associated with the use of MS hydromulch (Figure 1b) could enhance water and nutrient absorption capacity, thereby increasing yield [46,48]. Leaf area is commonly used to measure the ability of a plant to photosynthesize, use water efficiently, and accumulate dry matter [49]. In conditions of water deficit, the water potential of the cells is reduced, and the leaf stomata close, reducing transpiration. Despite reducing water loss, stomatal closure prevents the entry of $CO_2$, negatively affecting photosynthetic rates and plant productivity [50]. It has been previously reported that mulching significantly increased net photosynthesis and leaf area, with a subsequent improvement of crop yield [51,52]. In our study, leaf area improved with the MS treatment (Figure 1d), while, in general, the photosynthetic rate was strongly reduced in all treatments by stress conditions except for MS treatment, in which plants were able to maintain a similar rate of photosynthesis to that of optimal conditions during the stress period (Figure 2a). Furthermore, MS-covered plants presented higher chlorophyll concentrations than non-covered plants under drought stress (Figure 3) linked to a better performance of the photosynthetic apparatus. Notably, improved photosynthetic capacity of MS-treated plants while maintaining stomatal conductance under water stress (Figure 2c) resulted in a remarkable increase in water use efficiency (Figure 2e). Indeed, drought resistance has been shown to be regulated by stomatal characteristics to improve integral water use efficiency and photosynthetic performance [53].

In this study, a relevant effect of the drought stress imposed was the concentration of several mineral nutrients in the leaf tissues of escarole plants, mainly K, Ca, P, Mn, and Zn (Table 2), as a consequence of reduced leaf water content (Table 1). The mulching treatment also affected leaf mineral composition, particularly in plants grown on soil mulched with MS, which presented the highest concentrations of N, K, Cu, Cl, and Zn under water depletion, but only K concentrations were statistically significant (Table 1). In the leaf, K allows an efficient osmotic adjustment within the cells, which is a key process to retain water. Efficient stomatal closure prevents excessive water loss that is achieved by K release from guard cells [54]. Regarding leaf Cl, the elevated concentrations found in all treatments, but especially in the MS treatment under drought stress, could be explained owing to the experimental conditions, as stated in previous studies done under similar field conditions [55] and geographical areas [56]. Indeed, recent studies have shown a positive physiological meaning of Cl, with important roles in plant development, cell osmoregulation, and photosynthetic performance [57–59]. This may suggest that the growth increase observed in the MS-treated plants could be partially explained by improved ion homeostasis.

However, none of the mineral nutrients analyzed clustered with plant growth-related parameters in the loading-PCA except for Cu (Figure 7b), thus uncoupling mineral nutrient regulation from growth improvement under water stress. Cu is a micronutrient involved in many essential biological processes, such as photosynthesis and respiration, thus playing an important role in the regulation of plant growth and development and the stress responses [60,61]. However, in our study Cu seems to have a marginal function in the mulching-associated growth changes observed under drought stress, since its concentrations did not vary significantly with the mulching treatment (Table 2).

Considering that plant hormones play key roles in the physiological responses to drought [62,63], the regulation of the hormonal balance has been proposed here as a mechanism of growth modulation in mulched plants under drought conditions. IAA downregulation under drought stress has been extensively shown in different studies, while JA regulation varies depending on the level of stress and/or the plant species [64,65]. This differential regulation permits the plant to adapt its physiology to the harmful conditions. Specifically, water stress has been shown to produce an impact on polar auxin transport in rice with the consequent decrease in IAA and increase in JA [64], while enhanced drought tolerance in rice has been reported via the downregulation of IAA [66]. We found that GAs, auxins (IAA), and JA clustered with growth and productivity traits in the PCA (Figure 7b), but their concentrations, despite decreasing under water stress, did not show mulched-associated changes (Figures 5 and 6c,e), thus indicating a marginal role of these hormones in the control of plant growth. Importantly, some authors have reported the interaction between IAA and CKs under water limitation [67]. Despite the active CKs, tZ and iP, and the total CK concentrations depleted during the drought period, they were significantly higher in plants mulched with MS than in the other mulching treatments and in the bare soil (Figure 4), and associated with growth- and photosynthesis-related parameters in the loading-PCA (Figure 7b). In this regard, the first mechanistic demonstration of the direct implication of the CKs in plant productivity improvement under abiotic stress was carried out by Ghamen et al. [68]. By using tomato plants overexpressing the IPT gene, which codes a key enzyme for CK biosynthesis (isopentenyltransferase), these authors showed that enhanced root CK synthesis modified shoot hormonal status, thus ameliorating salinity-induced decreases in growth and yield [68]. Other authors also highlighted the important role of CK homeostasis towards the more active forms for growth maintenance in tomato plants subjected to a high evaporative demand caused by water limitation [69].Interestingly, leaf ABA, ACC, and SA concentrations augmented with the water stress, but to a lower extent in escarole plants mulched with MS, although no statistical differences were observed in the concentrations of ACC and SA with respect to plants grown on bare soil or mulched with PE (Figure 6b,d). This suggests a secondary role of ACC and SA in growth control under water stress, as also stated by other authors [70,71]. However, ABA was significantly lower in MS-mulched plants under water stress compared to the other treatments (Figure 6a) and grouped in an opposite cluster to that of the growth and gas exchange and photosynthetic parameters (Figure 7b), thus buffering photosynthesis limitation associated with ABA-induced stomatal closure under water stress [72,73]. Therefore, the modulation of plant growth under drought conditions could be an outcome of altered hormonal balance, mainly the balance between CKs and ABA [74,75]. Indeed, CK signaling has been demonstrated to be antagonistic to ABA signaling, which is critical when assessing plant responses to abiotic stresses [76]. Recently, Gálvez et al. [77] found that shoot growth recovery and photosynthetic rate maintenance under moderate salinity (mainly considered as an osmotic stress) in pepper plants shaded with red nets were associated with a decrease in bioactive CK concentrations and with an increase in ABA and ACC contents. Likewise, in the present study, CKs seem to have an antagonistic effect with ABA (and secondarily also with ACC and SA) in controlling stomatal behavior of escarole plants mulched with MS in response to drought stress, therefore modulating growth through the regulation of photosynthetic rate, stomatal conductance, and water use efficiency, as reported previously [69,78].

## 5. Conclusions

In this study, we have tested a commercial escarole variety affected by both (hydro) mulching and water stress factors. Growth was significantly improved under drought stress in plants hydromulched with MS, explained by a specific effect on plant water relations and photosynthesis-related parameters. The regulation of the hormonal balance has been demonstrated here underlying the growth improvement. Indeed, CKs antagonistically interact with ABA to regulate water relations and photosynthesis under stress through fine-tuning stomata opening to maintain growth in MS plants. For the first time, this work shows that specific hydromulching formulations (MS) improve growth under water depletion by hormonal crosstalk regulation. The results of this study are of special interest as hydromulching has proven to be a sustainable alternative to plastic mulching for drought stress tolerance and maintaining food production within the upcoming climate crisis scenario.

**Author Contributions:** Conceptualization: A.A., F.M.d.A. and J.L.-M.; methodology: M.R.-M., A.G., A.A., M.C.P. and J.L.-M.; formal analysis: M.R.-M., A.G., M.C.P. 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: M.R.-M., A.G., A.A., M.C.P., 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.

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