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Evaluation of swiss chard (*Beta vulgaris* L. ssp. cicla) physiological and qualitative responses to water deficit and salicylic acid treatment

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ABSTRACT

Swiss chard is a rich dietary source of bioactive betalain pigments responsible of the red colour of the plant's organs and of phenolic compounds having recognized antioxidant properties. Vegetable colour and appearance, as the first characteristics perceived, are important quality attributes that influence the consumer's acceptance. Currently, elicitation based on growth plant regulator supply is considered in most cases a useful strategy to enhance the sensorial and nutritional value of dietary plants. Thus, this study aimed to assess the impact of salicylic acid application on Swiss chard (*Beta vulgaris* L. ssp. cicla) grown, isolated and under water shortage, in relation to physiological and qualitative parameters throughout cultivation and at harvest. Different effects were observed depending on the type and interaction of stress-inducing treatments. Positive effects consisted in highest chlorophyll (r.u.) (+19–40 %) carotenoids (+34–48 %), and phenolic contents (+2–42 %) in leaves when treatments were applied isolated or in combination, which also led to high sugar levels in plants (up to 380 %). Negative effects included the reduction of biomass production (−40 %), leaf size (−38 %), and betalain content (-7-27 %), especially with combined treatments. Nitrate accumulation was not affected but salicylic acid induced a reduction in proline levels probably due to increased carbohydrate and nitrate metabolisms in regulating water deficit.

1. Introduction

Swiss chard (*Beta vulgaris* L., var. cicla) is a biennial herbaceous leafy vegetable, originating from Mediterranean regions. Nowadays, Swiss chard is grown in different parts of the world, especially in North-Central America and Europe, and it is commercialized as ready-to-eat baby leaf salad. Its commercial success is due to the ease of cultivation, the adaptability to diverse environmental conditions, and its nutraceutical properties [1]. This vegetable contains abundant bioactive natural pigments and secondary metabolites, such as betalains, flavonoids, and other phenolic compounds which have strong antioxidant properties and the capability to counteract free radicals, which is related to multiple health benefits when consumed in the diet [2]. While these benefits are notable, Swiss chard remains relatively underexplored in comparison to other leafy greens.

Betalains are water-soluble nitrogen-containing pigments, specifically important for this crop as they are responsible for the typical red leaf colouration serving as substitutes for anthocyanin pigments in Caryophyllales plants [3]. These secondary metabolites, found in vacuoles, consist of a nitrogenous core structure, namely, betalamic acid [4-(2-oxoethylidene)-1,2,3,4-tetrahydropyridine-2,6-dicarboxylic acid]. Depending on the substituent residue of the betalamic acid, betalains are classified as red/purple betacyanins (conjugating it with *cyclo-Dopa* and hydroxycinnamic acid derivatives or sugars) and yellow-orange betaxanthins (condensation among betalamic acid and amines or amino acids residues). Among the bioactive compounds described in Swiss chard variety *cicla*, 20 % were classified as betalains being the largest category. Thirty-five betalains from both groups (betacyanins and betaxanthins) have identified in the petioles or stems of different varieties, being betacyanins the major one in the red/purple-coloured petioles [1].

Colour and appearance are features that define the sensory quality of food products, being the first attributes perceived and, frequently, the only criterion for consumer acceptance. In most dietary plants and

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vegetables, natural pigments are typically heterogeneously distributed across plant organs, which have distinct sizes and shapes. Currently, digital imaging is the best analytical tool to meet the requirements of objectively measuring their heterogeneous colour and appearance attributes. Additionally, imaging techniques have revolutionized the way quality is assessed in food products as they allow for the measurement of not only colour, but also other appearance-related features, such as morphological parameters, texture, and heterogeneity [4]. Beyond improving the visual appeal of agricultural foods, natural pigments and other secondary metabolites like colourless phenolics help plants to counteract the negative impacts of biotic and abiotic stresses, which are the primary constraints on productivity in agricultural systems [5]. Among potential abiotic plant stresses, water deprivation is one of the most common factors affecting plant physiology, growth, and composition. The reactive oxygen species (ROS) accumulation induced by water deficit causes elevated reactivity and toxicity as the balance between ROS generation and antioxidant activity is disrupted, which can lead to cell death in extreme cases [6]. However, depending on the water deprivation regime (moderate to severe) plants can respond to water stress at different levels through various defence mechanisms including stomatal closure, increased synthesis of bioactive compounds, activation of antioxidants enzymatic systems, or improved of osmotic adjustment, which in most cases helps to overcome the adverse environmental conditions [7,8]. All these physiological and biochemical changes are also reflected in the appearance and colour of plants. Thus, the application of controlled water stress is currently used in sustainable cultivation as a tool for the improvement of the sensory and nutritional quality of crops, and for a more rational use of water in agriculture [9]. Notwithstanding, the response of the plant to water deprivation may differ between different species, and the effectiveness of this agronomical strategy in improving quality traits may also depend on the type of secondary metabolite affected [7,8].

On the other hand, the supply of natural growth plant regulator is considered a useful strategy to promote plant growth and to improve plant tolerance to major abiotic stresses [10]. In this regard, the use of natural elicitors at non-toxic concentrations (isolated or in combination with plant stressing conditions) has also been shown to stimulate the secondary metabolites biosynthesis in different plant organs which in most cases increases the sensorial and nutritional value of dietary vegetables [11–13]. In comparison to chemical pesticides and fertilizers that have been extensively used in the past, the exogenous application of natural plant growth regulators represents a more sustainable tool for a safer future agriculture, plant protection, and growth promotion [10].

Salicylic acid, an endogenous phenolic phytohormone, is naturally produced by plants root cells in response to biotic or abiotic stresses, and it serves as a key regulator in governing various physiological processes such as ion uptake, photosynthesis, plant growth, or germination [14]. Plants typically contain a few salicylic acid micrograms per fresh weight gram, present in either free or various conjugated forms, such as glycosylated, methylated, glucose-esterified, or amino acid-conjugated forms [15]. Thus, salicylic acid could alleviate the adverse impacts of water stress on plants and influence the adjustment of cellular processes linked to stress tolerance, depending on its concentration. In general, low concentrations have a positive effect in the alleviation of abiotic stress damage; on the contrary, high concentrations induce oxidative stress [14]. As reported for other natural plant elicitors [16], salicylic acid application at non-toxic concentrations has been shown to activate secondary metabolism in red beetroot leading to a higher accumulation of betalains and phenolic compounds [12] and its application offers a low-impact solution that aligns with sustainable agricultural practices. However, its effect on the physiological response of Swiss chard plants to water stress, and especially its impacts on important bioactive molecules has been scarcely studied [13].

Therefore, the objective of the present study was to assess the impact of foliar application of salicylic acid on Swiss chard plants (cv. Jupiter F1) subjected to water deprivation, both as isolated factors and in combination. The hypothesis was based on the premise that salicylic acid is involved in various biological processes and could therefore play an important role in enhancing Swiss chard tolerance to water deprivation by stimulating chlorophyll and proline biosynthesis, as well as the production of bioactive pigments such as betalains and antioxidant phenolics during cultivation. Thus, investigating these dimensions could significantly improve both the drought resistance and nutritional value of this under-researched crop.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals used for physiological determinations (pigments, sugars, nitrate, MDA, and proline) were analytical grade (ACS-for analysis). 2-Thiobarbituric acid, sucrose, glucose, potassium nitrate, and methanol were purchased from CARLO ERBA reagents (Cornaredo, Italy). Anthrone was purchased from Fischer scientific (Segrate, Italy). Sulfuric acid, chloridric acid (37 %), 3,5-dinitrosalicylic acid (98 %), K-sodium tartrate, resorcinol, thiourea, glacial acetic acid, NaOH, salicylic acid, 5-Sulfosalicylic acid, toluene, proline, and TCA were purchased from Merck (Darmstadt, Germany).

Chromatographic solvents (acetonitrile, formic acid, and methanol) were HPLC-grade purchased from Merck (Darmstadt, Germany). All other chemicals (extraction and analytical solvents) were analytical grade and supplied by Panreac Química (Barcelona, Spain). Purified water was obtained from a NANOpure Diamond system (Barnsted Inc.). Commercial standards of gallic acid, ferulic acid, caffeic acid, sinapic acid, p-coumaric acid, (+)-catechin, and (−)-epicatechin were HPLC-grade purchased from Sigma-Aldrich (Madrid, Spain), and betanin standard from TCI, Tokyo Chemical Industry Co. (Tokyo, Japan).

2.2. Experimental set-up

The experiment was conducted in a greenhouse of the University of Milan on a Swiss chard cultivar having green leaves and red veins (*Beta vulgaris* L., Subsp. *Cicla*, cv. Jupiter F1, Maraldi Sementi Srl), commonly used for baby leaf production. Plants were sown in 16 pots (6 seeds each pot) filled with a commercial substrate made up of green composted amendment, acidic peat moss, sand, and non-composted vegetable amendment (pH 6, 0.48 dS/m EC (Electrical Conductivity), dry bulk density of 87 kg/m^3).

Plants were divided into 4 groups and subjected to different conditions. Each treatment was applied in four replicates as follows.

- S: 0.01 mM salicylic acid treatment applied as foliar spray when leaves were fully expanded (30 days after sowing DAS). The solution was distributed to evenly cover both the upper and lower leaf surfaces (n = 4 pots; 6 seeds each pot).
- WS: water shortage applied by withholding water supply at 30 DAS (n = 4 pots; 6 seeds each pot).
- S + WS: 0.01 mM salicylic acid treatment + water shortage applied at 30 DAS (n = 4 pots; 6 seeds each pot).
- C: control plants were not subjected to any stressing conditions or treatment (n = 4 pots; 6 seeds each pot).

To avoid exclusive wetting effects on treated plants, plants grown under WS, and C conditions were sprayed with deionized water. As well, in order to maintain a constant water content in the substrate of control plants and to monitor an actual water decrease in WS plants, the weight of the pots was measured throughout the experimental trial the day of water deprivation (day 0) and after 1, 3, 5, 7, and 10 days (Fig. 1).

Non-destructive *in vivo* analyses were performed at 1, 3, 5, 7, and 10 days after the treatment or the water deprivation, while analytical determinations were performed on samples collected at harvest, 10 days after the salicylic acid application and the beginning of water deficit.

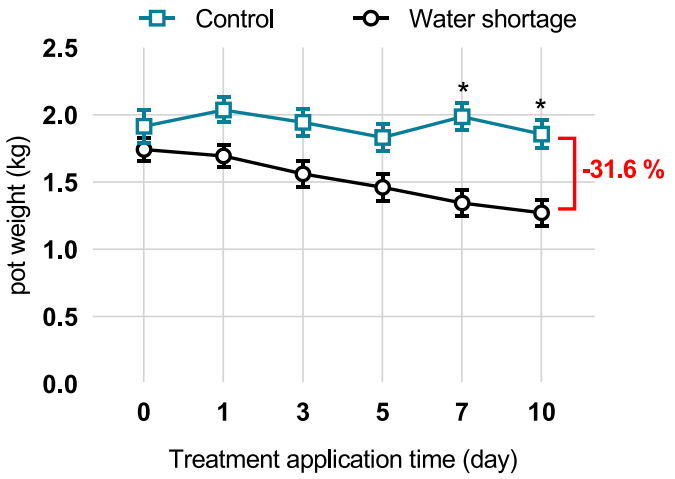

Fig. 1. Pots weight (kg) of plants grown under control and stress treatment (water shortage) applied for 10 days. Values are means ± SE (n = 3). Asterisks indicate significant ($p < 0.05$) differences among treatments each time point (Sidak multiple comparison test, ANOVA).

2.3. *In vivo evaluations*

2.3.1. *Chlorophyll a fluorescence*

In vivo, chlorophyll *a* fluorescence was measured using a portable fluorometer (Handy Plant Efficiency Analyzer (PEA) by Hansatech, UK) and for each treatment six leaves were randomly selected, and a leaf section has been kept in the dark for 30 min using leaf clips with a diameter of 4 mm. A light pulse of 3000 mol m^{-2} s^{-1} was then applied to the leaf to measure the re-emitted fluorescence. To evaluate the plant's performance and identify any stress conditions, a JIP analysis was conducted to calculate different parameters: the performance index (PI), which quantitatively reflects the functionality of the photosystem provides information about the plant's current performance state. The maximum quantum efficiency of photosystem II (Fv/Fm) represents the ratio between variable and maximum fluorescence. It indicates the likelihood of an electron captured by the antenna reaching the reaction centre by reducing the acceptor. For healthy herbaceous plant leaves, this parameter typically has a value equal to or greater than 0.83. Other parameters are reported in Supplementary Table S1.

2.3.2. *Chlorophyll content estimation*

The chlorophyll content was assessed *in vivo*, using a chlorophyllmeter (CL-01, Hansatech, UK). This device determines the leaf greenness by measuring two specific wavelengths (620 nm and 940 nm). For each treatment and time point, 10 leaves were randomly selected for analysis. Results were expressed as an arbitrary unit (r.u.).

2.3.3. *Morphological and colour analysis by digital imaging*

Prior lyophilization for the extraction of bioactive compounds, the fresh Swiss chard leaves were individually analysed at harvest for the morphological and colour characteristics. Thus, 20 leaves per treatment (5 leaves/pot; n = 4 pot/treatment) and a total of 80 Swiss chard leaves were individually analysed by digital imaging.

The DigiEye® imaging system used comprises an illumination cabinet (VeriVide Ltd., Leicester, UK) with fluorescent lamps that reliably simulate the CIE Standard Illuminant D65. A digital camera (Nikon D-80) receives and stores the images in 48-bit TIFF format (3872 x 2592 pixels). For the colour measurements, fresh leaves samples were appropriately placed into the cabinet using a white background that ensures an adequate segmentation process of sample image. Fig. 2 shows the leaves selected from the pot replicate 1 of all treatments.

For each image, a 500-pixel fixed area was taken aleatory. By means of the certified colour chart DigiTizer (VeriVide Ltd., Leicester, UK), the software transforms from RGB to CIELAB colour spaces by applying advanced non-linear multivariate models, which allows obtaining a colour matrix (500 colour pixels) of each leave based on the CIELAB colour parameters (L*, a*, b*, C^*_{ab}, and h_{ab}) estimated according to the method of Rodriguez-Pulido et al. (2013) [17] with the software Matlab.

The CIELAB colour space is based on the opponent color model of human vision and defines colour based on three colour coordinates [4]: L*, a*, and b*. Coordinate L*represents lightness, the vertical axis ranging from black (L* = 0) to white (L* = 100). The a* and b* values represent the chromaticity scalar coordinates (orthogonal to the achromatic lightness axis), which in turn represent opponent red-green and blue-yellow scales: a* (which takes positive values for reddish colours and negative values for greenish ones) and b* (positive for yellowish colours and negative for the bluish ones).

From a* and b*, two polar coordinates are defined in the CIELAB space: the hue angle (h_{ab} = arctan (b*/a*)), and the chroma (C^*_{ab}= $((a^*)^2 + (b^*)^2)^{1/2}$). Hue angle ($h_{ab}$) is the attribute according to which colours have been traditionally defined as red, green, etc; values of h_{ab} = 0° (or 360°), 90°, 180°, and 270° indicate red, yellow, green, and blue colour, respectively. Chroma (C^*_{ab}, the intensity of the colour) represents the distance from the coordinate's origin (a* = 0, b* = 0) to a determined point within the CIELAB space for any L* value. In addition, colour differences in the CIELAB colour space are determined by means of the CIE76 colour difference parameter (ΔE^*_{ab}). It was calculated as the Euclidean distance between two points in three-dimensional space defined by L*, a*, and b*: $\Delta E^*_{ab} = ((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2}$.

Measuring colour of each pixel, it is possible to have an estimation of the colour heterogeneity, which is obtained by using the Mean Colour Difference from the Mean (MCDM) [17]. The percentage of red area in leaves (veins) was also calculated based on a segmentation process.

Morphological characteristics were also obtained by digital imaging: length (diameter along major axis of the leaves, expressed in cm), width (diameter along the axis perpendicular to the major axis of leaves, expressed in cm), area (expressed in cm^2), and perimeter (expressed in

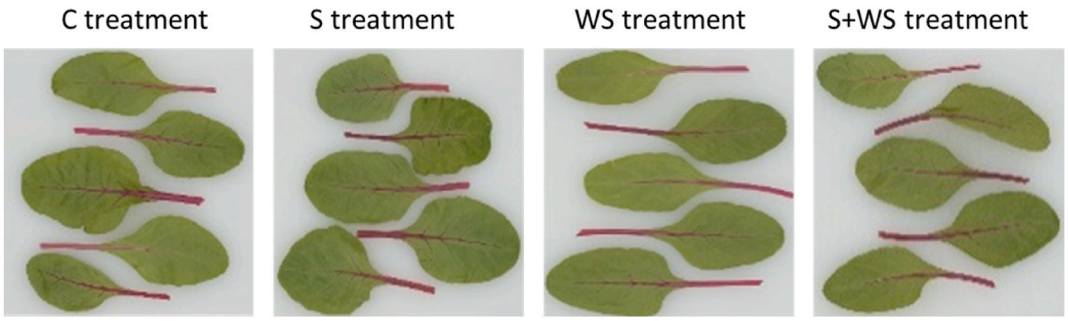

Fig. 2. Digital images of fresh Swiss chard leaves taken at harvest (pot replicate 1). Four pots for each treatment were considered as replicates and 5 leaves were selected per pot. Treatments: control: (C), 0.01 mM salicylic acid (S), water shortage (WS), and combination of 0.01 mM salicylic acid and water shortage (S + WS).

cm) [17].

2.4. Analytical determinations

2.4.1. Betalains

For each treatment, 5 fresh leaves were randomly selected from each pot at harvest, considering 4 pots for each treatment as replicates. Samples were further freeze-dried (lyophilizer Cryodos-80, Telstar Varian DS 102) and ground until extracted. The betalains extraction was carried out by adding 3 mL of methanol:water (50:50) containing 50 mM sodium ascorbate to 0.05 g of lyophilized leaf sample. Solutions were stirred at 200 rpm for 10 min in darkness. Supernatants were separated by centrifugation at 12000*g* at 10 °C for 5 min. Subsequent extractions with the same solvents were carried out to achieve a complete discolouration of the extract, and finally with 100 % methanol. The extracts were then concentrated in vacuum (30 °C) until around 3 mL, adjusting volume until 4 mL with purified water [18]. All analysis were carried out in triplicate. Separation, identification, and quantification of betalains were carried out in a Hewlett-Packard 1100 series liquid chromatograph (Agilent Technologies, Palo Alto, USA), equipped with a quaternary pump, a UV–Vis diode-array detector, an automatic injector, and ChemStation software. The separation of betalains was performed in a Zorbax C18 column (250 × 4.6 mm, 5 μm particle size) thermostatic at 25 °C, using 1 % formic acid in water (v/v, eluent A) and methanol (eluent B), at a flow rate of 1 mL/min [18]. Quantification of betacyanins and betaxanthins was determined at 535 nm and 482 nm, respectively. For identifying each chromatographic peak, the visible spectral characteristics and retention times, based on our previous results [18,19], were used. Once determining by spectrophotometry, the actual content of betanin in the standard (1 mg g^{-1}). For betacyanins quantification (535 nm), a betanin curve calibration was used (0.2–110 mg/L; y = 387.16x–129.87, R^2 = 0.9976). Similarly, for betaxanthins quantification (482 nm), a betanin curve calibration was used (0.2–10 mg/L; y = 279.64x– 59.811, R^2 = 0.9910). Results were expressed as μg g^{-1} of dry weight (DW).

2.4.2. Phenolic compounds

For the phenolic extraction, approximately 0.03 g of the homogeneous lyophilized leaf sample (each replicate considering 5 leaves per pot; n = 4 pot/treatment) was extracted with 75 % methanol (1 % 1N HCl) with occasional agitation and sonication. Supernatants of each replicate were separated by centrifugation (12000 g, 5 min, 10 °C) and the leaf residue was submitted to subsequent extractions until complete the phenolic extraction. Supernatants were combined, concentrated in vacuum (30 °C) and the volume was adjusted to 2 mL with purified water. High performance liquid chromatography (HPLC) was applied for the phenolic content determination by direct injection of the samples, previously filtered through a 0.45 μm Nylon filter, in an Agilent 1200 liquid chromatograph (Palo Alto, CA), equipped with quaternary pump, UV–Vis diode-array detector, automatic injector, and the ChemStation software. All analysis were carried out in triplicate. The separation, identification, and quantification of phenolics was performed following a modification of the method described in Gordillo et al. [20]. Phenolic compounds were separated on a Zorbax C18 column (250 × 4.6 mm, 5 μm particle size) maintained at 38 °C. Acetonitrile-formic acid-water (3:10:87) as eluent A, and acetonitrile-formic acid-water (50:10:40) as eluent B were used. The elution profile used was: 0–10 min with 6 % B; 10–15 min with 30 % B; 15–25 min with 40 % B; 25–35 min with 45 % B; 35–40 min with 50 % B; 40–42 min with 60 % B; and 42–43 min with 6 % B. The flowrate was 0.8 mL/min and the injection volume was 50 μL. UV–Vis spectra were recorded from 200 to 800 nm with a bandwidth of 2.0 nm. The wavelengths of detection were 280 nm (benzoic acids and flavonoids) and 320 nm (hydroxycinnamic acids and their derivatives). Identification of phenolics was performed according to the spectra features and retention times with those of the available pure standards and our data library of the standards. The quantification of identified compounds was estimated from a calibration curve by using commercial standards of gallic acid (0–80 mg/L; y = 178.57x + 57.07, R^2 = 0.997) and *p*-coumaric acid (0–80 mg/L; y = 421.94x + 362.02, R^2 = 0.996), and quercetin (0–100 mg/L; y = 270.27x + 40.54, R^2 = 0.998) purchased by Sigma-Aldrich (Madrid, Spain). The concentration of compounds was expressed as μg g^{-1} DW. Total benzoic acids, total cinnamic acids, total flavonoids, and total phenolics were calculated as the sum of individual phenolic compounds identified by HPLC.

2.4.3. Total chlorophylls and total carotenoids

For each treatment, 9 fresh leaves were randomly selected from each pot at harvest, considering 4 pots for each treatment as replicates.

To extract chlorophyll and carotenoids, 5 mL of 99.9 % methanol (CH_3OH) was added to 30–50 mg of fresh leaf disc tissue obtained with a cork borer. The mixture was incubated overnight at 4 °C in darkness, then the solution was analysed using a spectrophotometer (Evolution 300 UV–Vis Thermo Scientific, UK), with readings taken at 665.2 nm for chlorophyll *a*, 652.4 nm for chlorophyll *b*, and 470 nm for total carotenoids. The concentrations of the pigments were determined using Lichtenthaler's formulas (Chlorophyll *a* (Ca) = 16.72 $Abs_{665.2}$–9.16 $Abs_{652.4}$ (μg/mL solution), Chlorophyll *b* (Cb) = 34.09 $Abs_{652.4}$–15.28 $Abs_{665.2}$ (μg/mL solution), Total carotenoids = (1000 Abs_{470} – 1.63 Ca – 104.96 Cb)/221 (μg/mL solution)) [21]. Results were expressed as μg mg^{-1} of FW. Analysis was conducted in biological triplicate.

2.4.4. Total sugars

For each treatment and time point, leaves were randomly gathered. Approximately 1 g of leaf tissue was ground in 4 mL of distilled water and the resulting homogenate was subjected to centrifugation at 4000 rpm for 15 min (PK 130 Centrifuge, ALC International SRL, Milano, Italy). The anthrone method was employed to determine the total sugar content of the obtained extract [22]. Subsequently, 1 mL of the anthrone reagent was added to 0.2 mL of extract in Eppendorf tubes, resulting in a blue and yellow phases. The tubes were then placed on ice for 5 min, vigorously shaken, and heated for 5 min at 95 °C in a thermostatic Dubnoff bath (M428-BD, tecnovetro S.r.l., Monza, Italy). Afterward, the tubes were cooled to RT. The absorbances were measured using a spectrophotometer (Evolution 300 UV–Vis Thermo Scientific, UK) at 620 nm, and the total sugar content was calculated based on a calibration curve using a glucose standard solution (glucose 0–2.0 mM, y = 0.837x + 0.0094, R^2 = 0.996). Results were expressed as mg g^{-1} of FW. Analysis was conducted in biological quadruplicate.

2.4.5. Reducing sugars

The sample preparation was the same as described for total sugar determination. The reagent used was prepared by dissolving 2.5 g of DNS (3,5-dinitrosalicylic acid) in 150 mL of distilled water and then combined with a solution of K-sodium tartrate in 4 N NaOH in a beaker, brought to a temperature of 50 °C, and stirred until the powders were dissolved. Finally, the reagent was filtered (Whatman filter paper 0.45 μm) and used for analysis. The concentration of reducing sugars was calculated by preparing a calibration curve (glucose 0–4.0 mM, y = 0.1714x - 0.0171, R^2 = 0.997) using a glucose standard solution. Into the test tubes, 200 mL of extract and 200 mL of DNS reagent were added. The closed tubes were placed in a thermostatic Dubnoff bath (M428-BD, tecnovetro S.r.l., Monza, Italy) at 99 °C for 5 min then, 1.5 mL of distilled water was added. The readings were taken using a spectrophotometer (Evolution 300 UV–Vis Thermo Scientific, UK) at 530 nm [23]. Results were expressed as mg g^{-1} of FW. Analysis was conducted in biological quadruplicate.

2.4.6. Sucrose

The sample preparation was the same as described for total sugar determination. A 30 % HCl solution (125 mL final volume) was prepared and added to a mixture of 8.75 mg of resorcinol, 22.5 mg of thiourea, 6.25 mL of glacial acetic acid, and 2.5 mL of deionized water. The

concentration of sucrose in the samples was calculated by preparing a calibration curve (sucrose 0–2.0 mM, y = 0.3014x + 0.0253, R^2 = 0.992) using a sucrose standard solution. To a test tube, 100 mL of leaf extract and 100 mL of 2N NaOH were added. The test tubes were placed in a thermostatic Dubnoff bath (M428-BD, tecnovetro S.r.l., Monza, Italy) at 100 °C for 10 min then, 750 mL of resorcinol was added, and the tubes were placed in a thermostatic Dubnoff bath (M428-BD, tecnovetro S.r.l., Monza, Italy) at 80 °C for 10 min. Then the tubes were cooled to RT and the extracts were read using a spectrophotometer (Evolution 300 UV–Vis Thermo Scientific, UK) at 500 nm [24]. Results were expressed as mg g^{-1} of FW. Analysis was conducted in biological quadruplicate.

2.4.7. Nitrate

The sample preparation was the same as described for total sugar determination. To determine the nitrate concentration the salicylsulfuric acid method was employed [25]. For each sample, 80 μL of a 5 % salicylic acid in sulfuric acid solution were added to 20 μL of the extract, then 3 mL of 1.5 N NaOH were added in each tube. The samples were allowed to cool to room temperature, and readings were taken at 410 nm, using a spectrophotometer (Evolution 300 UV–Vis Thermo Scientific, UK). The nitrate content was determined by preparing a calibration curve (KNO_3 0–10 mM, y = 0.08x + 0.0026, R^2 = 0.992) using a standard solution of potassium nitrate (KNO_3). Results were expressed as mg of NO^{-3} on kg of FW. Analysis was conducted in biological quadruplicate.

2.4.8. Proline

Proline concentration was assessed by the ninhydrin-based colorimetric assay [26]. Approximately 1 g of leaf tissue randomly selected from the 4 pots was homogenized with 10 mL of 3 % sulfosalicylic acid. Samples were centrifuged (PK 130 Centrifuge, ALC International SRL, Milano, Italy) at 4000 rpm for 5 min at RT. A reaction mixture was prepared with 3 % sulfosalicylic acid (100 μL), glacial acetic acid (200 μL) and acidic ninhydrin (200 μL) and posed in a tube. Afterward, 100 μL of the extract was added to the mixture. The tubes were stirred and incubated at 96 °C for 60 min in a thermostatic Dubnoff bath (M428-BD, tecnovetro S.r.l., Monza, Italy). The reaction was stopped placing the tubes on ice. Finally, 1 mL of toluene was added to the mixture. The tubes were vortexed (Zx3 Vortex, Velp Scientific, Francesco Sassone, Milano Italy) and then let them rest for 5 min to allow the separation between the organic and water phases. The chromophore phase was read at 520 nm using a spectrophotometer (Evolution 300 UV–Vis Thermo Scientific, UK), utilizing toluene as reference. The proline concentration was determined by preparing a calibration curve (proline 0–150 μM, y = 0.0014x + 0.0079, R^2 = 0.992) using a standard solution of proline. Results were expressed as μg g^{-1} of FW. Analysis was conducted in biological quadruplicate.

2.4.9. Thiobarbituric reactive substances (TBARS) assay

Lipid peroxidation was determined using the TBARS method [27]. For each treatment, 1 g of fresh leaves were randomly selected from the 4 pots and was homogenized in 5 mL trichloroacetic acid (TCA, 0.1 % w/v) and centrifuged at 4500 g for 10 min at RT (PK 130 Centrifuge, ALC International SRL, Milano, Italy).

One mL of the supernatant was mixed with 4 mL 20 % TCA containing 0.5 % thiobarbituric acid, vortexed, and incubated in a thermostatic Dubnoff bath (M428-BD, tecnovetro S.r.l., Monza, Italy) at 95 °C for 30 min. The reaction was stopped by placing the tube in ice. Absorbance readings were recorded at 600 and 532 nm using a spectrophotometer (Evolution 300 UV–Vis Thermo Scientific, UK). The concentration of TBARS was expressed as malondialdehyde (MDA) equivalents, calculated using the Lambert-Beer law with an extinction coefficient of ε = 155 mM^{-1} cm^{-1} (MDA equivalent (nmol/mL) = [(Abs_{532} -Abs_{600})/155000] 10^6) [28]. Analysis was conducted in biological quadruplicate.

2.5. Statistical analysis

Data were reported as mean ± standard error (SE) of the mean. One-way ANOVA followed by Tukey's multiple comparisons test ($p < 0.05$) was performed using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, Boston, Massachusetts USA, www.graphpad.com. Results of normality test and of equality of variances are shown in Supplementary Table S2.

3. Results

3.1. In vivo chlorophyll and chlorophyll a fluorescence determination

The chlorophyll level in control plants is stable until 7 days, while it significantly increases after 10 days (Fig. 3). The application of salicylic acid induced a significant ($p < 0.05$) but temporary decrease of chlorophyll values at 5 days, however, at the end of the experiment (day 10), the level was similar to those observed after 1 day. A significant ($p < 0.05$) decline in chlorophyll content happened in Swiss chard leaves, in response to water shortage until 5 days, then an opposite trend occurred, and chlorophyll values increased. Chlorophyll content did not change in response to the combination of salicylic acid and water shortage until 7 days, whereas it significantly ($p < 0.05$) increased after 10 days. The combination of salicylic acid and water shortage induced a significant ($p < 0.05$) increase of chlorophyll in Swiss chard leaves starting from 5 days. The value was higher than those measured in control plants and plants treated with salicylic acid. The difference persisted until the end of the experiment (10 days). A similar but delayed and less intense effect occurred in response to water shortage starting from 7 days.

Overall, the variation of several chlorophyll *a* fluorescence parameters (Fig. 4) measured at the end of the growing cycle, were observed in plants grown under water shortage and treated with salicylic acid, as shown by the greater distance between the yellow line (S + WS) and the control grey line (C). In particular, the biggest differences have been observed for performance index (PI), the number of active reaction centres per cross section (RC/CSm), the electron transport flux per cross section (ET_0/CS), the number of active reaction centres per chlorophyll unit (RC/ABS), and the time to reach the maximum level of fluorescence (TFm). None of the tested conditions induced a variation in the quantum maximum efficiency of PSII (Fv/Fm).

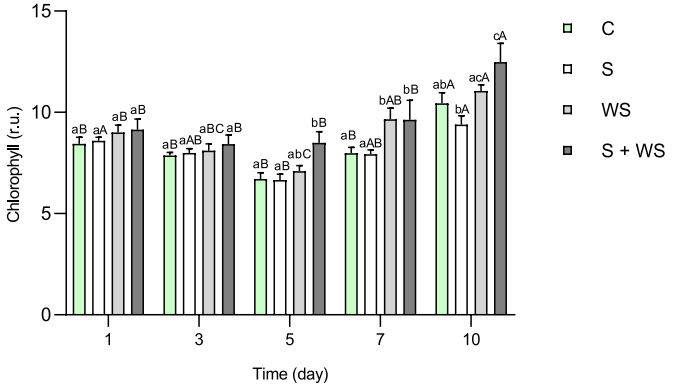

Fig. 3. In vivo chlorophyll level (r.u) in Swiss chard leaves under control and stress treatments applied for 10 days. Treatments: control (C), 0.01 mM salicylic acid (S), water shortage (WS), and combination of 0.01 mM salicylic acid and water shortage (S + WS). Different uppercase letters indicate significant ($p < 0.05$) differences along time and lowercase letters among treatments (two ways ANOVA and Tukey's multiple comparison test). Values are means ± SE (n = 10).

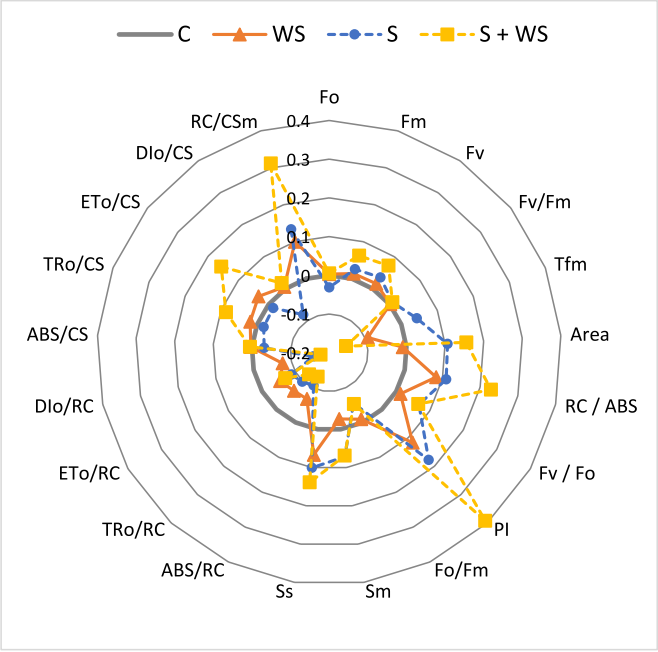

Fig. 4. Chlorophyll *a* fluorescence parameters of Swiss chard plants at harvest after 10 days of control and stress treatments: control (C), 0.01 mM salicylic acid (S), water shortage (WS), and combination of 0.01 mM salicylic acid and water shortage (S + WS). (Ft-Fc)/Fc formula was used to normalize the data, where Ft and Fc represent treated and control plants, respectively. Fc values were normalized to 0 (grey circle). Values are means ± SE (n = 6).

3.2. Biomass production and morphological characteristics

All the treatments influenced the biomass production (g), and morphology of Swiss chard leaves measured at harvest by digital imaging (Table 1). Salicylic acid application and water shortage led to leaves having shorter length and width than control ones, and consequently, lower average area and perimeter sizes. However, this unfavourably effect was only significant ($p < 0.05$) when water shortage was applied reducing the leave area size by 26 % respect to control plants. This trend was even more notable when water shortage was applied in combination with salicylic acid, resulting in an average leave area reduction of 37.5 %.

Table 1
Biomass production and morphological parameters of Swiss chard leaves at harvest. Values are means ± SE (n = 20).

	Treatment[a]			
	C	S	WS	S + WS
Biomass production				
Fresh weight (g)	1.73 ± 0.061^{a}	1.60 ± 0.086 ^{ab}	1.32 ± 0.055 ^{bc}	1.04 ± 0.084^{c}
Morphological parameters by digital imaging				
Area (cm^2)	42.66 ± 1.282^{a}	37.52 ± 1.752 ^{ab}	31.57 ± 1.966 ^{bc}	26.30 ± 1.435^{c}
Perimeter (cm)	25.97 ± 0.717^{a}	24.24 ± 0.498 ^{ab}	22.17 ± 0.667 ^{bc}	20.43 ± 0.549^{c}
Length (cm)	9.92 ± 0.253^{a}	9.27 ± 0.214 ^{ab}	8.68 ± 0.266 ^{bc}	7.95 ± 0.197^{c}
Width (cm)	5.66 ± 0.192^{a}	5.52 ± 0.181 ^{bc}	4.96 ± 0.187 ^{ab}	4.53 ± 0.145^{c}

Different letters in the same row indicate significant ($p<0.05$) differences according to Tukey test (ANOVA).

[a] C: control; S: 0.01 mM salicylic acid; WS: water shortage; S + WS: salicylic acid + water shortage.

Similarly, the same treatments caused a significant decrease in leaves fresh mass (g), which was about 24 % and 40 % lower than control samples after 10 days of WS and S + WS treatments. In the case of salicylic acid treatment (S), the observed biomass reduction in leaves was not significant.

3.3. Colour and visual appearance

Fig. 5 shows the images taken of one replicate of Swiss chard leaves of each treatment and the location of the colour matrixes obtained in the CIELAB (a*b*)-plane by pairs of samples (C versus S, WS, S + WS, respectively in Fig. 5A, B, and 5C). In this study, the segmentation criterion applied recognized every leave, automatically removing the red areas (petiole and veins), and so did not compute for CIELAB colour matrixes data. Independently of the treatment, all samples were located between hue (h_{ab}) values of 100° and 120°, which correspond to the green-yellow region of the CIELAB colour space. Leaves treated with salicylic acid (S) and water shortage (WS) treatments had the highest values of lightness (L* = 53–65) indicating in both cases lighter colourations (Fig. 5A and B). As well, the colour matrix of S leave was slightly shift toward 95°–110° of hue, and hence, showed more yellowish green colouration. Analogously, S + WS treated leave having the lowest values of lightness (L* = 45–50) was characterised by a darker green colouration (Fig. 5C).

From each colour matrix, the mean CIELAB parameters and heterogeneity characteristics were calculated (Table 2) in order to statistically compare the impact of treatments at harvest relative to the global average values of visual appearance and colour (n = 20 leaves/treatment). When the whole set of samples were considered altogether in each treatment, it was observed that the isolated application of salicylic acid or water shortage (S and WS) did not significantly ($p < 0.05$) impact the colour characteristics of Swiss chard leaves, neither the % of red area nor colour heterogeneity, although their leaves tend to be lighter. However, when applied in combination (S + WS treatment), leaves show a significant ($p < 0.05$) darker and less saturated green colouration (lower mean values of L* and C^*_{ab}), a lower percentage of red colouration, and consequently, more homogeneous colour (lower values of MCDM).In order to establish whether the observed mean changes in colour were visually relevant, the CIELAB colour difference (ΔE^*_{ab}) was assessed comparing treatments by pairs. According to Martínez et al. (2001) [29], ΔE^*_{ab} around 3–4 units indicates, approximately, colour differences appreciable to the human eyes (as an average observer). Based on this premise, results showed that the application of S + WS treatment led to colour effects in Swiss chard leaves than can be slightly discernible from the other treatments (ΔE^*_{ab} = 3.2, 3.5 and 3.9 u respect to C, S, and WS respectively).

3.4. Betalains

A large extent number of betalains (7 betacyanins and 4 betaxanthins) were identified in Swiss chard cv. Jupiter F1. The chromatographic analysis showed that all plants (control and treated) presented the same betalain profile (Fig. S1). Considering the betacyanin family (Fig. S1A), the major pigments in Swiss chard leaves corresponded to betanin followed by isobetanin, which accounted for nearly 85 % of the total betacyanin content (78 % and 10–13 %, respectively). Regarding betaxanthins (Fig. S1B), it was the valine moiety derivative the major compound, which represents around 60 % of total betaxanthins.

Betanin, isobetanin, betanidin, lampranthin II, and betaxanthin derived from valine were the betalains more affected by the studied treatments (Table 3). Compared to the control plants (C), both the application of salicylic acid (S) and of water shortage (WS) positively and significantly ($p < 0.05$) impacted the content of betanidin, lampranthin II, and valine moiety betaxanthin. In particular, S treatment exhibited a 1.6-fold higher betanidin content, whereas WS treatment led to an amount 1.26 and 2.2-fold higher of lampranthin II and valine-

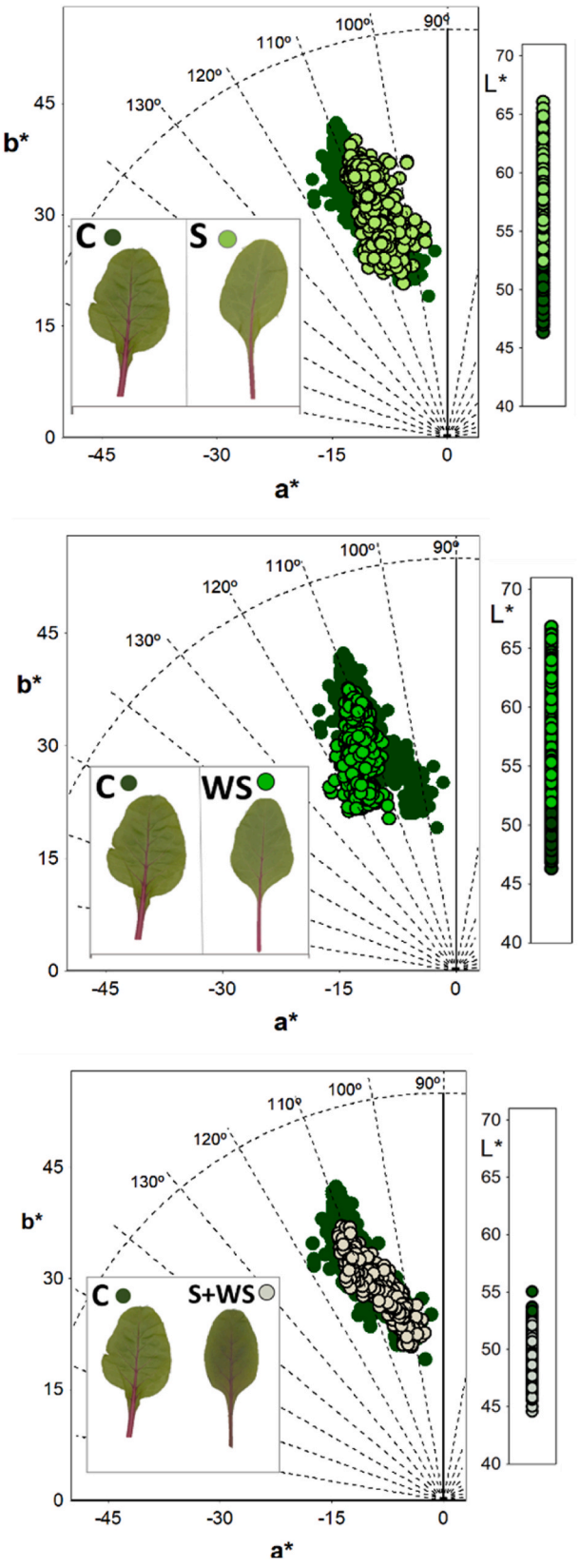

Fig. 5. Location of the colour matrixes (500 colour pixel/image) of leave samples in the CIELAB a*b* diagram, together to the lightness (L*) values. A) C versus S treatment, B) C versus WS treatment, D) C versus S + WS treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Visual appearance features and CIELAB colour parameters of Swiss chard leaves at harvest. Values are means ± SE (n = 20).

	Treatment[a]			
	C	S	WS	S + WS
L*	52.61 ± 0.367[a]	53.06 ± 0.534[a]	53.95 ± 0.536[a]	50.04 ± 0.731 [b]
a*	−10.42 ± 0.144[a]	−10.62 ± 0.202[a]	−10.01 ± 0.154 [ab]	−9.54 ± 0.226 [b]
b*	32.82 ± 0.284[a]	32.54 ± 0.510[a]	31.93 ± 0.357[a]	31.31 ± 0.423[a]
C^*_{ab}	36.67 ± 0.240	36.44 ± 0.524[a]	35.98 ± 0.330[a]	34.57 ± 0.331 [b]
h_{ab}	104.23 ± 0.254[a]	104.75 ± 0.261[a]	103.49 ± 0.322[a]	103.99 ± 0.487[a]
% Red area	8.41 ± 0.282 [ab]	7.80 ± 0.323[a]	8.76 ± 0.240 [ab]	7.81 ± 0.418 [b]
MCDM	9.28 ± 0.175[a]	9.15 ± 0.424 [ab]	9.47 ± 0.290 [ab]	8.03 ± 0.396 [b]

Different letters in the same row indicate significant ($p < 0.05$) differences according to Tukey test (ANOVA).

[a] C: control; S: 0.01 mM salicylic acid; WS: water shortage; S + WS: salicylic acid + water shortage.

Table 3
Content (μg g^{-1} DW) of individual betalains identified in Swiss chard leaves at harvest, together to the Total betacyanins (525 nm), Total betaxanthin (482 nm), and Total Betalains. Values are means ± SE (n = 4).

	Treatment[a]			
	C	S	WS	S + WS
Betanin	310.43 ± 16.498[a]	277.48 ± 7.645 [ab]	276.10 ± 19.191 [ab]	208.21 ± 21.911 [b]
Isobetanin	52.03 ± 0.293[a]	36.92 ± 3.939 [b]	31.51 ± 2.263 [b]	28.57 ± 5.599 [b]
Betanidin	5.11 ± 0.472[a]	8.21 ± 0.595 [b]	7.70 ± 0.557 [ab]	10.39 ± 1.376 [b]
Isobetanidin	1.84 ± 0.079[a]	1.94 ± 0.024[a]	1.81 ± 0.497[a]	1.86 ± 0.196[a]
17-Decarboxy-neobetanin	2.33 ± 0.280[a]	2.51 ± 0.211[a]	3.46 ± 0.995[a]	3.77 ± 0.049[a]
Neobetanin	3.49 ± 0.278[a]	3.82 ± 0.274[a]	5.41 ± 0.985[a]	4.25 ± 0.505[a]
Lampranthin II	20.79 ± 1.264[a]	23.57 ± 0.408 [ab]	26.40 ± 0.844 [b]	20.45 ± 0.677[a]
Total betacyanins	396.02 ± 17.265[a]	354.45 ± 12.640 [ab]	352.39 ± 21.238 [ab]	277.51 ± 29.396 [b]
Valine-btx	10.46 ± 2.244[a]	11.38 ± 1.624 [ab]	23.43 ± 4.428 [b]	15.06 ± 2.388 [ab]
Tyrosine-btx	1.59 ± 0.127[a]	1.52 ± 0.087[a]	1.84 ± 0.214[a]	1.35 ± 0.123[a]
Isoleucine-btx	2.57 ± 0.201[a]	2.92 ± 0.179[a]	3.30 ± 0.245[a]	3.12 ± 0.506[a]
Leucine-btx	2.76 ± 0.379[a]	2.69 ± 0.473[a]	3.29 ± 0.474[a]	3.36 ± 0.465[a]
Total betaxanthins	17.37 ± 2.616[a]	18.51 ± 1.973[a]	31.86 ± 5.349[a]	22.88 ± 2.967[a]
Total betalains	413.39 ± 18.327[a]	372.96 ± 14.556 [ab]	384.26 ± 26.186 [ab]	300.39 ± 29.739 [b]

Different letters in the same row indicate significant ($p < 0.05$) differences according to Tukey test (ANOVA).

[a] C: control; S: 0.01 mM salicylic acid; WS: water shortage; S + WS: salicylic acid + water shortage.

betaxanthin, respectively. On contrast, the separate application of both treatments exerted a negative impact on isobetanin content, with a significant ($p < 0.05$) decrease among 30–40 %. The combined application of both treatments (S + WS) did not produce any additional advantage to the isolated application of salicylic acid (S), and even significantly ($p < 0.05$) decreased the betanin content, and, thus, the

total betacyanins and betalains on Swiss chard leaves.

3.5. Phenolic compounds

The chromatographic analysis showed that all plants (control and treated) presented the same phenolic profile (Fig. S2). However, the levels of the most individual phenolic compounds identified in Swiss chard leaves (Table 4) were significantly ($p < 0.05$) affected by the treatments applied (gallic acid, ferulic and sinapic acid dvs., and flavonoids dvs.) which was reflected in the total content of the major phenolic families (benzoic acids and flavonoids). As expected, the application of salicylic acid (S) was the treatment most influenced the phenolic compounds increasing the total content of benzoic acids and flavonoids about 2.5 and 1.7-fold higher respect to control plants, respectively. This positive effect, although to a lesser extent, was also observed for the total content of benzoic acids when water shortage (WS) was applied (1.6-fold higher than in control plants). On contrast, the accumulation of total flavonoids slightly decreased in WS plants respect to control ones, but the differences were not significant. On the other hand, the combination of salicylic acid treatment and water shortage (S + WS) seems to slightly increase both the content of benzoic acids and flavonoids in Swiss chard plants respect to the only application of water stress (WS), but not significantly. As a consequence, the isolated application of salicylic acid (S) led to Swiss chard plants having a significant ($p < 0.05$) higher accumulation of Total phenolics in leaves (4979 μg g^{-1} DW) than any other treatment. In comparison, the application of S + WS treatment led to plants with significant higher Total phenolic content (3184 μg g^{-1} DW) than control plants (2897.31 μg g^{-1} DW) but not than WS plants (2943.82 μg g^{-1} DW).

Table 4
Content (μg g^{-1} DW) of individual phenolic compounds identified in Swiss chard leaves at harvest, together to the Total benzoic acids, Total cinnamic acids, Total flavonoids and Total phenolics. Values are means ± SE (n = 4).

	Treatment[a]			
	C	S	WS	S + WS
Gallic acid	330.31 ± 19.784[a]	1024.35 ± 25.999 [b]	581.14 ± 51.173[c]	693.92 ± 38.314[c]
Ferulic acid dv.1	72.74 ± 6.834[a]	107.80 ± 6.845 [b]	62.41 ± 4.038[a]	74.60 ± 3.791[a]
Gallic acid dv.	129.82 ± 6.131[a]	139.47 ± 8.708[a]	133.41 ± 9.688[a]	105.89 ± 10.131[a]
Caffeic acid dv.	127.77 ± 2.548[a]	119.53 ± 6.092[a]	128.76 ± 9.754[a]	112.10 ± 11.235[a]
Sinapic acid	105.94 ± 3.784[a]	111.33 ± 3.439[a]	110.01 ± 3.619[a]	112.63 ± 1.948[a]
Sinapic acid dv.	102.79 ± 0.872[a]	115.85 ± 1.861 [ab]	123.44 ± 3.800 [b]	109.74 ± 7.322 [ab]
Flavonoid dv.1	834.41 ± 53.590[a]	794.36 ± 43.647[a]	479.13 ± 136.854 [b]	453.28 ± 46.052 [b]
Flavonoid dv.2	335.33 ± 9.865[a]	503.69 ± 29.599 [b]	335.19 ± 14.881[a]	345.81 ± 53.646[a]
Flavonoid dv.3	646.12 ± 11.299[a]	1795.10 ± 36.417 [b]	748.86 ± 84.457[a]	992.63 ± 36.117[c]
Flavonoid dv.4	101.63 ± 2.768[a]	127.78 ± 3.049 [b]	99.89 ± 3.425[a]	101.38 ± 2.744[a]
Ferulic dv. 2	106.69 ± 2.695[a]	140.30 ± 8.361 [b]	108.16 ± 5.575[a]	100.50 ± 3.634[a]
Total benzoic acids	402.89 ± 16.662[a]	1132.19 ± 26.178 [b]	643.50 ± 47.172[c]	768.50 ± 37.301[c]
Total cinnamic acids	572.91 ± 14.335[a]	626.49 ± 11.074[a]	603.78 ± 21.529[a]	540.93 ± 32.091[a]
Total flavonoids	1921.40 ± 52.177[a]	3220.89 ± 36.739 [b]	1633.12 ± 123.294[a]	1875.12 ± 66.948[a]
Total phenolics	2897.31 ± 44.701[a]	4979.58 ± 15.855 [b]	2943.82 ± 93.343 [ac]	3184.62 ± 83.183[c]

Different letters in the same row indicate significant ($p<0.05$) differences according to Tukey test (ANOVA).

[a] C, control; S, salicylic acid at 0.01 mM; WS, water shortage; S + WS, salicylic acid water shortage.

3.6. Photosynthetic pigments, and carbohydrates

The concentration of total chlorophyll and carotenoids in Swiss chard leaves was not significantly affected by the water shortage and by the application of salicylic acid (Table 5). However, the values were generally lower in control than in treated plants.

The combination of salicylic acid and water shortage induced a significant ($p < 0.05$) increase in total sugars, reducing sugars, and sucrose (Table 5). In particular, the concentration of total sugars and reducing sugars in S + WS samples was almost four times higher than the value of control plants. The concentration of total sugars was also significantly ($p < 0.05$) affected by the water shortage, whereas the levels of reducing sugars and sucrose were generally higher than control but not significant. Similarly, the application of salicylic acid solution did not significantly affect the concentration of sugars.

3.7. Nitrate, proline, and TBARS

The concentration of nitrate was significantly ($p < 0.05$) affected by the amount of water supplied to the plants, regardless the application of the salicylic acid solution (Fig. 6 A). In particular, control plants had a mean value of 1792 mg kg^{-1}, whereas the plants grown under water shortage condition showed a mean value of 4402 mg kg^{-1}.

At the same time, the concentration of proline significantly ($p < 0.05$) decreased (−74 %) in plant grown under with a low amount of water (Fig. 6 B). The application of the salicylic acid solution in plants grown under the same water management condition induced a concentration of proline similar to the control.

The level of MDA in Swiss chard leaves was not significantly affected by any of the tested conditions and the average value observed in samples was 4.6 nmol g^{-1} (Fig. 6C).

4. Discussion

Baby leaf typically refers to leafy vegetables harvested after the seedling stage, once true leaves have emerged but before reaching full maturity, usually before developing eight true leaves. Consumer demand for ready-to-eat products increased over time due to the convenience of consuming nutrient-rich vegetables [30]. Among different compounds, chlorophylls are particularly important in leafy vegetables

Table 5
Total chlorophyll, carotenoids, total sugars, reducing sugars, and sucrose in Swiss chard leaves at harvest. Values are means ± SE (n = 3 for pigments, n = 4 for carbohydrates).

	Treatment[a]			
	C	S	WS	S + WS
Pigments				
Chlorophyll *a*+b (μg mg^{-1})	0.54 ± 0.12[a]	0.76 ± 0.085[a]	0.65 ± 0.041[a]	0.76 ± 0.104[a]
Carotenoid (μg mg^{-1})	0.08 ± 0.005[a]	0.11 ± 0.008[a]	0.12 ± 0.011[a]	0.11 ± 0.017[a]
Carbohydrates				
Total sugars (mg g^{-1})	0.79 ± 0.085[a]	2.53 ± 0.140 [ab]	3.76 ± 0.490 [b]	3.71 ± 0.854 [b]
Reducing sugars (mg g^{-1})	0.68 ± 0.075[a]	1.20 ± 0.116[a]	1.63 ± 0.170 [ab]	2.50 ± 0.490 [b]
Sucrose (mg g^{-1})	0.27 ± 0.024[a]	0.29 ± 0.058 [ab]	0.61 ± 0.069 [ab]	0.71 ± 0.185 [b]

Different letters in the same row indicate significant ($p < 0.05$) differences according to Tukey test (ANOVA).

[a] C: control; S: 0.01 mM salicylic acid; WS: water shortage; S + WS: salicylic acid + water shortage.

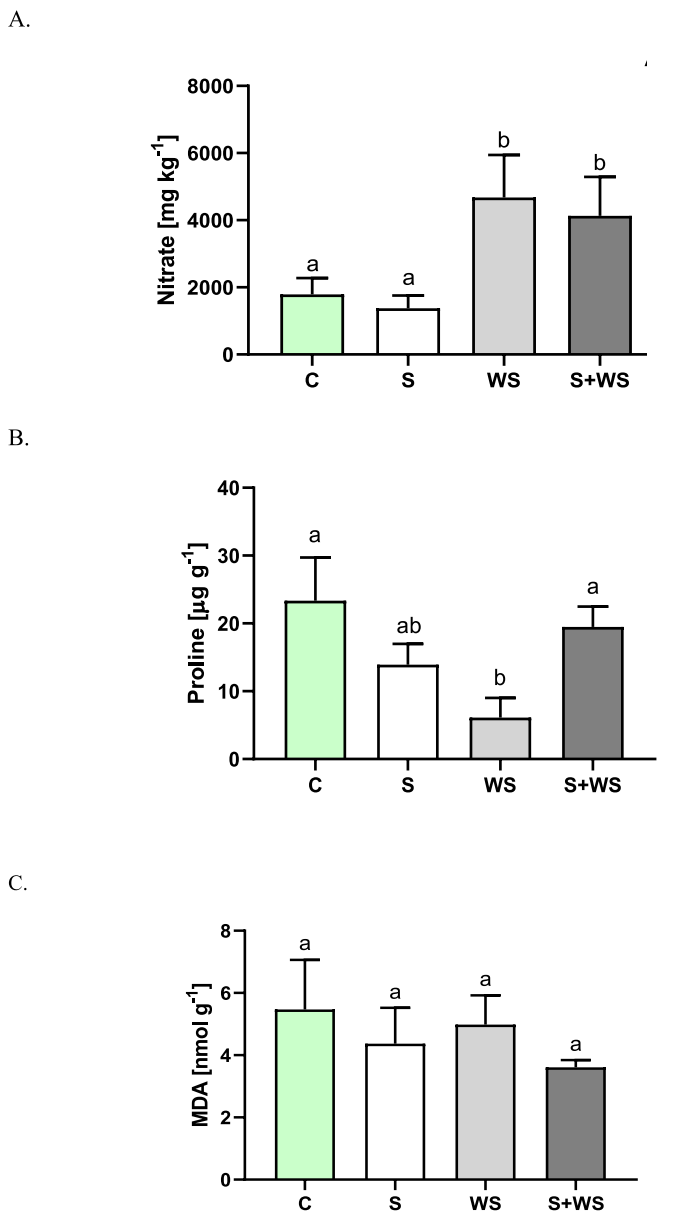

Fig. 6. Concentration of nitrate (A), proline (B), and MDA (C) in Swiss chard leaves at harvest. Treatments: control (C), 0.01 mM salicylic acid (S), water shortage (WS), and combination of 0.01 mM salicylic acid and water shortage (S + WS). Different letters indicate significant ($p < 0.05$) differences among treatments (Tukey test, ANOVA). Values are means ± SE (n = 4).

for their role as photosynthetic pigments and since they are responsible for the green colour of leaves. Food colour is a central criterion in consumer choice and for leafy vegetables a bright green colour is visually appealing conveying the idea of a fresh and healthy product [31]. As expected, the leaves chlorophyll concentration (estimated non-destructively) increased over time in accordance with leaf age [32]. Moreover, various studies show that chlorophyll concentration can increase when plants are exposed to mild stress but declines under severe stress conditions [33]. It is possible to hypothesize that in our experiment water shortage induced only a transient decrease in chlorophyll content, and the values at harvest were similar to those of control plants. In addition, the analytical determination of chlorophyll levels confirmed the behaviour observed with non-destructive measurements.

The application of salicylic acid has been reported to affect chlorophyll accumulation and different responses can be observed according to the concentration, and the combination with different environmental conditions, and genotype [34]. Interestingly, the combination of salicylic acid and water stress induced a significant increase in chlorophyll content (measured *in vivo*) after 3 days, compared to control plants. Similarly, an increase in total chlorophyll has been observed in peppermint plants grown under drought stress and treated with salicylic acid [35].

The molecular and biochemical mechanisms through which SA affects chlorophyll accumulation are complex and involve several regulatory pathways including the modulation of antioxidant enzyme activity. Salicylic acid boosts antioxidant enzymes like CAT, and APX, which reduce oxidative stress by neutralizing ROS, thereby protecting chlorophyll from damage and potentially promoting its accumulation [36]. Also, salicylic acid could have been effective by boosting chlorophyll biosynthesis by and promoting the synthesis of intermediate molecules, or by limiting the stress-induced chlorophyll degradation. Finally, SA treatment could have interacted with phytohormones like cytokinin, helping maintain chlorophyll levels and delaying senescence [37–39].

Foliar application of salicylic acid slightly affected the chlorophyll *a* fluorescence-related parameters, especially when applied in combination with water deprivation. For example, it has been observed a tendency to increase the performance index (PI), the electron transport flux (ET_0/CS), the density of active reaction centres per excited cross section (RC/CSm), and the amount of photosynthetic reaction centres per absorption (RC/ABS) resulted in Swiss chard plants. Different studies have reported how the exogenous application of salicylic acid affects chlorophyll *a* fluorescence parameters in various ways, according to the plant species, the environmental condition, and the concentration applied. Seed priming with SA increased chlorophyll *a* fluorescence in basil plant grown under dodder infestation most likely by increasing the probability of energy trapping by PSII centres and reducing the number of inactive PSII centres [40]. Positive effects have been reported also in saffron plants grown under salt stress in response to the foliar application of SA, showing the ability to affect the water content, increase the energy available for reaction centre, and enhance the electron fluidity to the PSII [41]. In barley plants, instead, it has been observed a dose effect and a low concentration of salicylic acid (0.5 mM) boosted PSII activity, while a high concentration (5 mM) caused PSII damage and reduced chlorophyll levels [42]. The protective effect deriving by SA application, in combination with other abiotic stress factors such as high temperature and intense light has been linked to the electron transport rate, the quantum yield of PSII (Fv/Fm), and the relative amounts of D1 protein and Deg1 protease which were influenced by exogenous SA under the combined stress of high temperature and intense light [43].In addition to chlorophylls, the betalain profile and content affects the overall Swiss chard quality since is one of the compounds responsible of its sensorial (red colour) and nutraceutical value (antioxidant capacity). In Jupiter variety, betalains are accumulated both in the petiole and in the veins of the leaves contributing to the attractive visual appearance of such as vegetables characterised by bright green rounded leaf and contrasting deep red veins and petioles. However, very scarce reports of betalains have been previously found in Swiss chard, and only Kugler et al. [44, 45] carried out the identification of individual betalains in Swiss chard petioles. The present study is a step forward in that regard, being the first attempt to carry out a deep identification and quantification of the individual betalains in Swiss chard leaves and deepen their behaviour against different stress-inducing agents. Similar results respect to the major betalains identified in Jupiter variety were reported in the petioles of Swiss chard [44]. Among the scarce literature reported on the effect of salicylic acid addition to plants on betalain content, a dosage dependence is established depending on the plant. Pari et al. [46] recently established that concentrations (25–50 µM) of salicylic acid stimulate the betalain synthesis of *Gomphrena globose* L. callus, while red pitaya needed 100–200 µM for a similar purpose [11] but the use of a higher dosage was counterproductive [47]. Based on the results, it

would be necessary to optimize the applied dose of salicylic acid to more efficiently activate the betalain synthesis in the studied Swiss chard leaves since most of the identifies compounds were not significantly affected by the treatment, except for two betacyanins (isobetanin and betanidin). Regarding water deprivation, and similarly to the obtained results, Ceja-López et al. [47] stated that total betalains content was downregulated when osmotic stress was induced. However, in our experiment Lampranthin II and Valine-btx significantly increased in response to water shortage. On the other hand, the impact of the exogenous supply of salicylic acid on the accumulation of phenolics in plants submitted to different abiotic stresses is well documented in different species [14]. However, as for betalains, the isolated effect of salicylic acid on the antioxidant levels in Swiss chard plants has been scarcely reported [13]. Some studies have assessed the interactive effect of growth regulators and water stress on other components of sugar beet (*Beta vulgaris* L.) but not in phenolics [16]. In our study, in agreement with previous reports [14,48], the application of salicylic acid (both isolated and under water stress) significantly increased the global levels of phenolics in Swiss chard leaves, indicating that it represents a useful strategy to increase its nutritional value. Phenolic acids and flavonoids, which were the phenolic compounds most positively affected in leaves, are considered important dietary phytonutrients that contribute to the antioxidant defence system of the organism against oxidative stress.

Digital imaging allowed assessing in an objective manner the effects of the treatments on the global visual appearance and heterogeneous colour of Swiss chard leaves by means of the shifts on the location and orientation of colour matrixes respect to the control sample, which cannot be easily performed by conventional colorimetric instruments such as spectroradiometers or colourimeters. From mean colour an appearance data, the effects mainly affected the colour saturation and lightness of leaves, which in turn is related to the content of pigments (chlorophylls, carotenoids and betalains) strongly influenced by the treatments at harvests. The application of S + WS in combination led to leaves with significantly higher content of chlorophylls (quantified both *in vivo* and in vitro analyses) but lower of total betalains, which is consistent with the visually darker, less saturated, and more homogenous green colouration where the typical contrasting red colouration became less notable. From the morphological analyses, it can be concluded that leaves of Swiss chard plant grown under WS condition were smaller than control leaves. This effect was even enhanced when WS was applied in combination to salicylic acid treatment (S + WS), which was also reflected on the reduction of fresh biomass production. Our results are in accordance with data reported for other species such as *Thymus vulgaris* L., *Salvia officinalis*, or *Hypericum perforatum* in which water deficit negatively affected number and size of leaves and flowers, height and fresh and dry weight [48]. In the case of *Thymus vulgaris* L. plants, the application of salicylic acid reversed the negative impact of water drought below an upper limit concentration (2 mM). Other factors such as the duration of the treatment, plant species, age, plant organ, mode of application, or the state of the plant also influence the salicylic acid effects, where induced or inhibited plant functions are both possible [14].

Plant responses to water stress involves several mechanisms, among them sugars may act as osmotic agents in turgor maintenance or as metabolic signals. In the present experiment, the water shortage significantly affected the accumulation of total sugars in Swiss chard leaves. At the same time, a tendency to increase the concentration of sugars has been observed also in plants treated with the salicylic acid solution. The effect of exogenous application of salicylic acid on sugars has been reported also by Gorni et al. [14], in red beetroot juice and explained as a reduction of the polysaccharide's degradation. The combination of S + WS emphasised these effects inducing an increase in both total and reducing sugars, and sucrose in Swiss chard leaves. A link between salicylic acid and sugar metabolism has been reported in other species as cucumber seedling growing under salt stress conditions [49]. Sugars are an important source of carbon and energy in plant and their increased concentration in leaves is expected to positively impact the post-harvest life, by counteraction sugar starvation reaction that stimulate senescence [50]. Moreover, high level of sucrose is correlated with an extended shelf life in different salad species [51]. In addition to the accumulation of sugars in response to water shortage, it has been also observed a significant increase in nitrate concentration both in WS and S + WS Swiss chard plants. Nitrates play a crucial role in plant responses to water stress, as they are stored in vacuoles and can reduce osmotic potential, helping plants absorb water under changing stress conditions [52]. This finding suggests that the application of exogenous salicylic acid at 0.01 mM does not affect the nitrate accumulation in Swiss chard leaves and the nitrate metabolism is apparently more dependent on other factors, including the water shortage. The effect of salicylic acid on nitrate accumulation has been reported to be dose dependent in other species. In particular, it has been observed that low concentration (10^{-5} M) induces an increase in nitrate reductase activity, whereas higher concentration (10^{-4} M and 10^{-3} M) decrease its activity [53].

It is well known that proline accumulation in plants is induced by drought conditions [54]. However, an opposite trend resulted in our experiment, with a decreased amount of proline in plants grown under water shortage condition. At the same time, Swiss chard plants subjected to a period of water deprivation showed an increase in sugars and nitrate concentrations, suggesting that, in the conditions tested, a major involvement of these molecules, rather than proline. The concentration of proline was restored to control levels in plants subjected to S + WS. Its decrease in plants grown under water deprivation may suggests that the level of water reduction applied in our experiment did not induce a severe stressful condition for Swiss chard, given the plant's resilient nature [55].

5. Conclusions

Morphological attributes, appearance and colour characteristics, pigment levels (chlorophylls, carotenoids and betalains), phenolic composition, and carbohydrates/nitrates metabolisms have been compared in Swiss chard plants subjected to salicylic elicitation under water stress conditions, both isolated and in combination. Stressing treatments show differences for most of the physiological and qualitative parameters evaluated compared to control plants, which importantly influenced the sensorial and nutritional properties of Swiss chard leaves that could impact the consumer's preferences on the market. The results demonstrated that both favourable and unfavourably effects can be modulated depending on the type (salicylic acid foliar application or water shortage) and interaction (isolated or in combined application) of the stress-inducing treatments. The foliar supply of salicylic acid has proven to be a good strategy to increase important bioactive compounds (some betalains and most of phenolics) and, hence, the nutritional value of Swiss chard edible leaves cultivated under non-stressing water conditions. According to the hypothesis, a slightly positive effect of salicylic acid application was found for the content of some phenolic compounds in plants submitted to water stress, suggesting a positive role for the biosynthesis of these antioxidant molecules. However, the concentration or timing of application should be revised to better support the hypothesis of the potential efficacy of salicylic acid in counteracting the Swiss chard response to water stress in the case of betalains biosynthesis and accumulation in leaves.

CRediT authorship contribution statement

Giulia Franzoni: Writing – original draft, Methodology, Investigation, Data curation. **Davide Guffanti:** Methodology, Investigation, Data curation. **Antonio Ferrante:** Resources, Project administration, Conceptualization. **María Jesús Cejudo-Bastante:** Writing – original draft, Validation, Methodology, Data curation. **Francisco J. Rodríguez-Pulido:** Writing – original draft, Methodology, Data curation. **Belén Gordillo:** Writing – review & editing, Validation, Methodology, Data

curation. **Giacomo Cocetta:** Writing – review & editing, Resources, Project administration, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2024.101524.

Data availability

Data will be made available on request.

References

[1] M. Gamba, P.F. Raguindin, E. Asllanaj, F. Merlo, M. Glisic, B. Minder, W. Bussler, B. Metzger, H. Kern, T. Muka, Bioactive compounds and nutritional composition of Swiss chard (*beta vulgaris* L. Var. *Cicla* and *flavescens*): a systematic review, Crit. Rev. Food Sci. Nutr. 61 (2021) 3465–3480, https://doi.org/10.1080/10408398.2020.1799326.

[2] Z. Mzoughi, H. Chahdoura, Y. Chakroun, M. Cámara, V. Fernández-Ruiz, P. Morales, H. Mosbah, G. Flamini, M. Snoussi, H. Majdoub, Wild edible Swiss chard leaves (Beta vulgaris L. var. cicla): nutritional, phytochemical composition and biological activities, Food Res. Int. 119 (2019) 612–621, https://doi.org/10.1016/j.foodres.2018.10.039.

[3] D. Strack, T. Vogt, W. Schliemann, Recent advances in betalain research, Phytochemistry 62 (2003) 247–269, https://doi.org/10.1016/S0031-9422(02)00564-2.

[4] M. M Giusti, B. Gordillo, L. González-Miret, Color analysis, in: P. Ismail, S. Nielsen (Eds.), Nielsen's Food Analysis, sixth ed., Springer Nature, Switzerland, 2024, pp. 509–521.

[5] S. Kumar, Abiotic stresses and their effects on plant growth, yield and nutritional quality of agricultural produce, Int. J. Food Sci. Agric. 4 (2020) 367–378, https://doi.org/10.26855/ijfsa.2020.12.002.

[6] R. Singh, P. Parihar, S. Singh, R.K. Mishra, P. Singh, S.M. V Prasad, Reactive oxygen species signaling and stomatal movement: current updates and future perspectives, Redox Biol. 11 (2017) 213–218, https://doi.org/10.1016/j.redox.2016.11.006.

[7] G. Franzoni, G. Cocetta, A. Ferrante, Effect of glutamic acid foliar applications on lettuce under water stress, Physiol. Mol. Biol. Plants 27 (2021) 1059–1072, https://doi.org/10.1007/s12298-021-00984-6.

[8] M.K. Nyathi, G.E. Van Halsema, Y.G. Beletse, J.G. Annandale, P.C. Struik, Nutritional water productivity of selected leafy vegetables, Agric. Water Manag. 209 (2018) 111–122, https://doi.org/10.1016/j.agwat.2018.07.02.

[9] M. Dujmović, N. Opačić, S. Radman, S. Fabek Uher, S. Voća, J. Šic Žlabur, Accumulation of stinging nettle bioactive compounds as a response to controlled drought stress, Agriculture 13 (2023) 1358, https://doi.org/10.3390/agriculture13071358.

[10] K. Li, R. Xing, S. Liu, P. Li, Chitin and chitosan fragments responsible for plant elicitor and growth stimulator, J. Agric. Food Chem. 68 (2020) 12203–12211, https://doi.org/10.1021/acs.jafc.0c05316.

[11] C.Y. Wee, R. Sekeli, N.H.C. Asari, S.F. Yahya, C. Machap, Enhancement of bioactive compounds in hylocereus polyrhzus callus mediated by plant growth regulators and elicitors, J. Trop. Plant Physiol. 10 (2018) 1–10.

[12] P.H. Gorni, L. da Silva Gonçalves, K.D. Spera, A.C. Pacheco, A. de Marcos Lapaz, Exogenous salicylic acid increases productivity and elicits betalains and other bioactive compounds in red beetroot, Trop. Plant. Biol. 16 (2023) 41–52, https://doi.org/10.1007/s12042-023-09329-x.

[13] H. Ramírez, J.H. Rancaño, A. Benavides, R. Mendoza, V. Robledo, J. Hernandez, Stress signalling substances influence in vegetables and their antioxidant relationship: a preliminary study, Acta Hortic. 774 (2008) 127–132, https://doi.org/10.17660/ActaHortic.2008.774.15.

[14] M.I.R. Khan, M. Fatma, T.S. Per, N.A. Anjum, N.A. Khan, Salicylic acid-induced abiotic stress tolerance and underlying mechanisms in plants, Front. Plant Sci. 6 (2015) 1–17, https://doi.org/10.3389/fpls.2015.00462.

[15] D.A. Dempsey, A.C. Vlot, M.C. Wildermuth, D.F. Klessig, Salicylic acid biosynthesis and metabolism, Arabidopsis Book 9 e0156 (2011), https://doi.org/10.1199/tab.0156.

[16] S. Khodadadi, M.A. Chegini, A. Soltani, H.A. Norouzi, S.S. Hemayati, Influence of foliar-applied humic acid and some key growth regulators on sugar beet (beta vulgaris L.) under drought stress: antioxidant defense system, photosynthetic characteristics and sugar yield, Sugar Tech 22 (2020) 765–772, https://doi.org/10.1007/s12355-020-00839-6.

[17] F.J. Rodríguez-Pulido, B. Gordillo, M.L. González-Miret, F.J. Heredia, Analysis of food appearance properties by computer vision applying ellipsoids to colour data, Comput. Electron. Agric. 99 (2013) 108–115, https://doi.org/10.1016/j.compag.2013.08.027.

[18] M.J. Cejudo-Bastante, M. Chaalal, H. Louaileche, J. Parrado, F.J. Heredia, Betalain profile, phenolic content, and color characterization of different parts and varieties of *opuntia ficus-indica*, J. Agric. Food Chem. 62 (2014) 8491–8499, https://doi.org/10.1021/jf502465g.

[19] M.J. Cejudo-Bastante, N. Hurtado, P. Muñoz-Burguillos, F.J. Heredia, *Stenocereus griseus* (haw) pitaya as source of natural colourant: technological stability of colour and individual betalains, Int. J. Food Sci. Technol. 54 (2019) 3024–3031, https://doi.org/10.1111/ijfs.14215.

[20] B. Gordillo, M.J. Cejudo-Bastante, F.J. Rodríguez-Pulido, M.L. González-Miret, F. J. Heredia, Application of the differential colorimetry and polyphenolic profile to the evaluation of the chromatic quality of tempranillo red wines elaborated in warm climate. Influence of the presence of oak wood chips during fermentation, Food Chem. 14 (2013) 2184–2190, https://doi.org/10.1016/j.foodchem.2013.05.014.

[21] H.K. Lichtenthaler, Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, Methods Enzimol 148 (1987) 350–382, https://doi.org/10.1016/0076-6879(87)48036-1.

[22] E.W. Yemm, A.J. Willis, The estimation of carbohydrates in plant extracts by anthrone, Biochem. J. 57 (1954) 508–514, https://doi.org/10.1042/bj0570508.

[23] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 31 (1959) 426–428, https://doi.org/10.1021/ac60147a030.

[24] E.S. Rorem, H.G. Walker, R.M. McCready, Biosynthesis of sucrose and sucrose-phosphate by sugar beet leaf extracts, Plant Physiol. 35 (1960) 269–272, https://doi.org/10.1104/pp.35.2.269.

[25] D.A. Cataldo, M. Maroon, L.E. Schrader, V.L. Youngs, Rapid colorimetric determination of nitrate in plant tissue by nitration of salicylic acid, Commun. Soil Sci. Plant Anal. 6 (1975) 71–80, https://doi.org/10.1080/00103627509366547.

[26] L.S. Bates, R.P. Waldren, I.D. Teare, Rapid determination of free proline for water-stress studies, Plant Soil 39 (1973) 205–207, https://doi.org/10.1007/BF00018060.

[27] R.L. Heath, L. Packer, Photoperoxidation in isolated chloroplasts, Arch. Biochem. Biophys. 125 (1968) 189–198, https://doi.org/10.1016/0003-9861(68)90654-1.

[28] Z. Du, W.J. Bramlage, Modified thiobarbituric acid assay for measuring lipid oxidation in sugar-rich plant tissue extracts, J. Agric. Food Chem. 40 (1992) 1566–1570, https://doi.org/10.1021/jf00021a018.

[29] J.A. Martínez, M. Melgosa, M.M. Pérez, E. Hita, A.I. Negueruela, Visual and instrumental color evaluation in red wines, Food Sci. Technol. Int. 7 (2001) 439–444, https://doi.org/10.1106/VFAT-5REN-1WK2-5JGQ.

[30] F. Di Gioia, M. Renna, P. Santamaria, Sprouts, microgreens and "baby leaf" vegetables, in: F. Yildiz, R. Wiley (Eds.), Minimally Processed Refrigerated Fruits and Vegetables. Food Engineering Series, Springer, Boston, MA, 2017, pp. 403–432, https://doi.org/10.1007/978-1-4939-7018-6_1.

[31] C. Spence, Gastrophysics: nudging consumers toward eating more leafy (salad) greens, Food Qual. Prefer. 80 (2020) 103800, https://doi.org/10.1016/j.foodqual.2019.103800.

[32] M. Lefsrud, D. Kopsell, A. Wenzel, J. Sheehan, Changes in kale (Brassica oleracea L. Var. Acephala) carotenoid and chlorophyll pigment concentrations during leaf ontogeny, Sci. Hortic. 112 (2007) 136–141, https://doi.org/10.1016/j.scienta.2006.12.026.

[33] E. Agathokleous, Z. Feng, J. Peñuelas, Chlorophyll hormesis: are chlorophylls major components of stress Biology in higher plants? Sci. Total Environ. 726 (2020) 138637 https://doi.org/10.1016/j.scitotenv.2020.138637.

[34] M. Moustakas, I. Sperdouli, I.D.S. Adamakis, J. Moustaka, S. İşgören, B. Şaş, Harnessing the role of foliar applied salicylic acid in decreasing chlorophyll content to reassess photosystem II photoprotection in crop plants, Int. J. Mol. Sci. 23 (2022) 7038, https://doi.org/10.3390/ijms23137038.

[35] F. Jahani, H.R. Tohidi-Moghadam, H.R. Larijani, F. Ghooshchi, M. Oveysi, Influence of zinc and salicylic acid foliar application on total chlorophyll, phenolic components, yield and essential oil composition of peppermint (*mentha piperita* L.) under drought stress condition, Arabian J. Geosci. 14 (2021) 691, https://doi.org/10.1007/s12517-021-07024-3.

[36] M. Azeem, R. Sultana, A. Mahmood, M. Qasim, Z.S. Siddiqui, S. Mumtaz, T. Javed, M. Umar, M.Y. Adnan, M.H. Siddiqui, Ascorbic and salicylic acids vitalized growth, biochemical responses, antioxidant enzymes, photosynthetic efficiency, and ionic regulation to alleviate salinity stress in sorghum bicolor, J. Plant Growth Regul. 42 (2023) 5266–5279, https://doi.org/10.1007/s00344-023-10907-2.

[37] S.T. Moharekar, S. D Lokhande, T. Hara, R. Tanaka, A. Tanaka, P.D. Chavan, Effect of salicylic acid on chlorophyll and carotenoid contents of wheat and moong

seedlings, Photosynthetica 41 (2003) 315–317, https://doi.org/10.1023/B:PHOT.0000011970.62172.15.
[38] D. Singh, V.K. Dhiman, H. Pandey, V.K. Dhiman, D. Pandey, Crosstalk between salicylic acid and auxins, cytokinins and gibberellins under biotic stress, in: Tariq Aftab (Ed.), Auxins, Cytokinins and Gibberellins Signaling in Plants, Springer Nature, Switzerland, 2022, pp. 249–262, https://doi.org/10.1007/978-3-031-05427-3_11.
[39] H. Zhang, Y. Cun, J. Wang, M. Wu, X. Li, Q. Liang, C. Wang, L. Zhao, J. Deng, Acetylsalicylic acid and salicylic acid alleviate postharvest leaf senescence in Chinese flowering cabbage (Brassica rapa var. parachinensis), Postharvest Biol. Technol. 194 (2022) 112070, https://doi.org/10.1016/j.postharvbio.2022.112070.
[40] E. Abbasvand, S. Hassannejad, S. Zehtab Salmasi, S. Alizadeh Salteh, Effects of seed priming with salicylic acid on chlorophyll a fluorescence parameters of basil (ocimum basilicum L.) infested by field dodder (cuscuta campestris yunk.), J. Plant Physiol. Breed. 9 (2019) 11–18, https://doi.org/10.22034/jppb.2019.10440.
[41] K. Ghassemi-Golezani, A. Hosseinzadeh-Mahootchi, S. Farhangi-Abriz, Chlorophyll a fluorescence of safflower affected by salt stress and hormonal treatments, SN Appl. Sci. 2 (2020) 1306, https://doi.org/10.1007/s42452-020-3133-1.
[42] G. Habibi, A. Vaziri, High salicylic acid concentration alters the electron flow associated with photosystem II in barley, Acta Agric. Slov. 109 (2017) 165–173, https://doi.org/10.14720/aas.2017.109.2.22.
[43] Y.E. Chen, H.T. Mao, N. Wu, A. Mohi Ud Din, A. Khan, H.Y. Zhang, S. Yuan, Salicylic acid protects photosystem II by alleviating photoinhibition in Arabidopsis thaliana under high light, Int. J. Mol. Sci. 21 (2020) 1229, https://doi.org/10.3390/ijms21041229.
[44] F. Kugler, F.C. Stintzing, R. Carle, Identification of betalains from petioles of differently colored Swiss chard (*beta vulgaris* L. Ssp. *cicla* [L.] alef. Cv. Bright lights) by high-performance liquid chromatography-electrospray ionization mass spectrometry, J. Agric. Food Chem. 52 (2004) 2975–2981, https://doi.org/10.1021/jf035491w.
[45] F. Kugler, S. Graneis, F.C. Stintzing, R. Carle, Studies on betaxanthin profiles of vegetables and fruits from the chenopodiaceae and cactaceae, Z. Naturforsch. C Biosci. 62 (2007) 311–318, https://doi.org/10.1515/znc-2007-5-601.
[46] M. Pari, W.Q. Lee, C.K.F. Wong, C.Y. Teh, Induction of callus culture through plant growth regulators supplementation and the effect of elicitors on enhancement of betalain synthesis using Gomphrena globosa, P.C.T.O.C 156 (2024) 19, https://doi.org/10.1007/s11240-023-02628-x.
[47] J.A. Ceja-López, J. Morales-Morales, J. Araujo-Sánchez, W.G. Kantún, A. Ku, M. L. Miranda-Ham, L.C. Rodriguez-Zapata, E. Castaño, Evaluation of natural pigments production in response to various stress signals in cell lines of stenocereus queretaroensis, Plants 11 (2022) 2948, https://doi.org/10.3390/plants11212948.
[48] N. Khalil, M. Fekry, M. Bishr, S. El-Zalabani, O. Salama, Foliar spraying of salicylic acid induced accumulation of phenolics, increased radical scavenging activity and modified the composition of the essential oil of water stressed Thymus vulgaris L, Plant Physiol. Biochem. (Issy les Moulineaux, Fr.) 123 (2018) 65–74, https://doi.org/10.1016/j.plaphy.2017.12.007.
[49] C.J. Dong, X.L. Wang, Q.M. Shang, Salicylic acid regulates sugar metabolism that confers tolerance to salinity stress in cucumber seedlings, Sci. Hortic. 129 (2011) 629–636, https://doi.org/10.1016/j.scienta.2011.05.005.
[50] E.J. Woltering, I.M. Witkowska, Effects of pre- and postharvest lighting on quality and shelf life of fresh-cut lettuce, ISHS Acta Hortic (2016) 357–366, https://doi.org/10.17660/ActaHortic.2016.1134.47.
[51] G.J.J. Clarkson, S.D. Rothwell, G. Taylor, End of day harvest extends shelf life, Hortscience 40 (2005) 1431–1435, https://doi.org/10.21273/hortsci.40.5.1431.
[52] R. He, Y. Liu, C. Song, G. Feng, J. Song, Osmotic regulation beyond nitrate nutrients in plant resistance to stress: a review, Plant Growth Regul. 103 (2024) 1–8, https://doi.org/10.1007/s10725-023-01093-y.
[53] Q. Fariduddin, S. Hayat, A. Ahmad, Salicylic acid influences net photosynthetic rate, carboxylation efficiency, nitrate reductase activity, and seed yield in *Brassica juncea*, Photosynthetica 41 (2003) 281–284, https://doi.org/10.1023/B:PHOT.0000011962.05991.6c.
[54] S. Hayat, Q. Hayat, M.N. Alyemeni, A.S. Wani, J. Pichtel, A. Ahmad, Role of proline under changing environments, Plant Signal. Behav. 7 (2012) 1456–1466, https://doi.org/10.4161/psb.21949.
[55] V. Shrivastava, N. Edayilam, B. Singla Just, O. Castaño-Sanchez, L. Díaz-Guerra, E. Meers, Evaluation of agronomic efficiency and stress resistance on Swiss chard via use of biostimulants, Sci. Hortic. 330 (2024) 113053, https://doi.org/10.1016/j.scienta.2024.113053.