Effect of salt stress in the regulation of anthocyanins and color of *Hibiscus* flowers by digital image analysis

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ABSTRACT

The effect of salinity stress (200mM NaCl for 28 days) on physiological characteristics of *Hibiscus rosa-sinensis* such as abscisic acid content, electrolyte leakage, and photochemical efficiency in leaves; and its influence on the biomass production, anthocyanin composition and color expression of flowers was evaluated. Salinity significantly increased electrolyte leakage and ABA content in leaves, and reduced the flower fresh weight. Chlorophyll fluorescence parameters were lower in salt stress condition, compared to control. Moreover, salt stress negatively affected the content of anthocyanins (mainly cyanidin-3-sophoroside), which resulted in a visually perceptible loss of color. The detailed anthocyanin composition monitored by HPLC-DAD-MS and the color variations by digital image analysis due to saline stress showed that the effect was more noticeable at the basal portion of petals. A Forward Stepwise Multiple Regression was performed for predicting the content of anthocyanins from appearance characteristics obtained by Image Analysis, reaching R−square values up to 0.90.

Keywords: *Hibiscus rosa-sinensis*, salt stress, anthocyanins, color, image analysis.

INTRODUCTION

Hibiscus genus (Malvaceae) is one of the most popular ornamental plants cultivated in tropical and subtropical regions since it presents several species with varying attractive flower colors[1]. Species of the genus *Hibiscus*, as *H. rosa-sinensis,* are particularly appreciated for the color of the flowers with the base of the corolla forming a deep-colored heart and a bright red limb[2]. The main compounds responsible for the characteristic heterogeneous color pattern of *Hibiscus* flowers in red cultivars are anthocyanins, being preferentially accumulate on calyces[3]. More specifically, the major anthocyanins described in *Hibiscus* spp. are delphinidin and cyanidin derivatives glycosylated with various sugar moieties and occasionally acylated with organic acids and other phenolic compounds also present in the flower organs[4].

Anthocyanins are the largest group of water-soluble flavonoids pigments widely spread in vascular plants where imparts the red, purple, and blue colors. As prominent members of the secondary metabolite of plants, anthocyanins have multi-biological functions including visual signal for pollinating insects, protection against different biotic and abiotic stresses, hormone-like activity, etc. Consequently, they are considered very efficient bioactive compounds and one of the most important natural and innocuous pigments in the plant-derived food. Thus, despite its undeniable importance as coloring agents, anthocyanins are responsible for the commercial and medicinal value of *Hibiscus* spp. because of their role as antioxidants and the health benefits that have been associated with their presence in the diet[5,6]. Indeed, based on of their high content on anthocyanin pigments (2.5 g/100 g DW), *Hibiscus* calyces continue attracting much attention for the pharmaceutical and food industry as a functional food or a good source of nutraceutical constituents. In this sense, they are worldwide utilized in the preparation of beverages and color extracts[1,3].

Previous investigations have reported that the content and composition of pigments in plants is conditioned by genetic and agronomic factors associated with biosynthetic changes[7]. Among them, salt stress is one of the most important problems for many species, especially in the Mediterranean areas where water resource is the main limiting factor[8,9]. Under salt stress conditions, plants response with several physiological disorders affecting the osmotic potential, photosynthetic rate and hormone synthesis (among others), which can determine changes on phenological characteristics such as the root/shoot ratio, size and turn-over of flowers or the pigment accumulation in plant tissues[7,10]. These eco-physiological responses are usually visible through biomass reduction, leaf necrosis, chlorophyll loss or flower discoloration, which are quality attributes markedly relevant from an ornamental and phytochemical perspective.

Surprisingly, few studies have been carried out on ornamental pot plants and little have been published regarding the response of these plants to saline fertirrigation, probably because they have been generally irrigated with high-quality water. Those studies reported that the saline fertirrigation had a negative effect on yield and decreased the flower quality[11,12]. However, the positive or negative effects of saline stress will depend on the degree of tolerance of plants and stress saline conditions; so the mechanisms of salt stress action are not yet completely clear, especially the impact on the pigment composition and color expression of the flower organ.

Thus, the aim of this study was to assess the effect of salinity stress on various physiological characteristics (flowers biomass production, ABA content, ion leakage and leaf photochemical efficiency) of *Hibiscus rosa-sinensis*, which has been described as moderately salt tolerant specie[13]. More deeply, the anthocyanin composition and content of *Hibiscus* petals was also studied to evaluate the influence of stress saline conditions on the color expression of the flower organ, objectively measure by digital image analysis.

Recent advances in image acquisition technology offer the possibility of using technically sophisticated apparatus available at relatively low cost, and the ability to evaluate areas in terms of millions of pixels. In this way, more detailed evaluations of objects with heterogeneous color as plant tissues is possible, since every different color present in the image can be accounted for one or more pixels. Thus, Image Analysis arises as a good option for satisfying the need of measuring color in this kind of samples. In addition, possible color-anthocyanin composition relationships were studied by applying multivariate statistical analysis.

MATERIAL AND METHODS

Chemicals and standards

Folin Ciocalteau reagent, gallic acid and quercetin standards were purchased from Sigma (St. Louis, Mo., U.S.A). Cyanidin-*3*-glucoside was purchased from Extrasynthese (Genay, France). The solvents used in chromatography were HPLC-grade and purchased from Merck (Darmstadt, Germany). All other chemicals were analytical grade and supplied by Panreac Química (Barcelona, Spain).

Plant material, growing technique and treatments

Hibiscus rosa-sinensis L. (cv. Porto) plants were grown in a greenhouse located in Pisa (latitude 43°43'N; longitude 10°23'E; Italy), under natural environmental conditions (22-26 °C). The plants were grown in pots containing soil:pumice mixture (3:1, v/v) and were irrigated twice a day with a drip irrigation. Drip irrigation was carried out using a standard nutrient solution (120 g per plant) with an electrical conductivity (EC) of 2.6 dS m^{-1} and pH 6.5. The composition of the standard nutrient solution was as follows (concentrations are expressed in mol m^{-3}): 11 N-NO_3 h, 1.3 P-PO, 6.2 K^+, 3.1 Ca^{2+}, 1.2 Mg^{2+}, 9 Na^+, and 1.7 S-SO_4^{2-}. Micronutrients were added at Hoagland's concentration (in mmol m^{-3}: B 25, 88 Fe, 1 Cu, 5 Zn, and 10 Mn).

When experiment started, 40 plants were subjected to salt-stress fertirrigation with NaCl at 200 mM, and the others 40 to standard fertirrigation (as control plants). This saline condition was selected to achieve a clearly marked discoloration of the petals to be related with the qualitative and quantitative modification of flower pigments. Plants were daily irrigated during 28 days. Fresh leaves and flowers samples with no apparent physical, insect or microbial damage were collected from control and treated *Hibiscus* plants, during or at the end of the experiment, and carefully transported to the laboratory for the downstream analysis.

Flowers fresh weight

Ten fresh flowers per treatment were collected to determine the fresh weight biomass production, expressed in g flower^{-1}. Flowers were sampled from different *Hibiscus* plants just at the end of the experiment (after 28 days) and directly analyzed.

Abscisic acid content determination, electrolyte leakage and chlorophyll a fluorescence measurements

Leaves, petals, style-stigma plus stamens, and ovary samples (100 mg fresh weight, FW) were collected, weighed, frozen in liquid nitrogen, and then stored at –80 °C until analysis. ABA was measured after extraction in distilled water (water:tissue ratio=10:1 v/w) overnight at 4 °C. Then ABA was determined by an indirect enzyme linked immunosorbent assay (ELISA) based on the use of DBPA1 monoclonal antibody, raised against S(+)-ABA[14] as described previously[15].

For the electrolyte leakage, the leaves were sampled every 7 days until the end of the experiment. Ten leaves discs were incubated by shaking in 5 mL of distilled water for 4 h at room temperature. After the incubation, the conductivity in the solution was determined by HI8733 conductivity meter (Hanna Instruments). Then, the samples were autoclaved, cooled and the conductivity was read again in the solutions. Ion leakage was expressed as a

123 percentage of the total conductivity after autoclaving[16]. Chlorophyll a fluorescence
124 transients were determined on dark-adapted leaves kept for 30 min at room temperature,
125 using a portable Handy PEA (Hansatech, UK). Leaf fluorescence detection was measured by
126 fast-response PIN photodiode with RG9 longpass filter and an excitation light intensity
127 (ultrabright red LEDs with a peak at 650 nm) of 3000 μmol m^{-2} s^{-1} (600W m^{-2}) (Hansatech,
128 technical manual). The parameters measured were Fo, Fm, and Fv/Fm. JIP analysis was
129 performed to determine the performance index (PI).
130 The chlorophyll fluorescence in plants adapted under ambient light conditions was measured
131 using FMS2 portable chlorophyll fluorometer (Hansatech, UK). The light was measured
132 using a PAR sensor and the parameters measured were the efficiency of the photosystem II
133 (FPSII), maximum fluorescence under light conditions (Fm'), electron transport rate (ETR).
134 **Anthocyanin extracts from *Hibiscus* petals**
135 Anthocyanin extracts were obtained from red petals of *Hibiscus* flowers. Nine petals per
136 treatment were carefully taken from fresh flowers just at the end of the experiment (after 28
137 days) and samples were frozen at -20 °C until the analysis
138 The extraction of anthocyanin compounds was carried out as follows. Petals were manually
139 divided in two pieces: the base (narrow basal portion) and the limb (cup-shaped upper
140 portion). Each part was individually analyzed (petal bases n=18; petal limbs n=18). Samples
141 were freeze-dried for 24 h (lyophilizer CRYODOS -80, Telstar® Varian DS 102). The total
142 amount of each homogeneous lyophilized powder obtained was separately extracted with
143 methanol containing 5% of concentrated hydrochloric acid, for 12 h, with occasional
144 agitation and sonication, in darkness at room temperature (18-20 °C). Then, supernatants
145 were centrifuged (4190 g, 10 min) and all the methanolic extracts were evaporated to
146 dryness in a rotary evaporator at temperature below 30 °C. The residues were dissolved in
147 500-1000 μL of distilled water and centrifuged at 4190 g for 3 min.

When experiment started, 40 plants were subjected to salt-stress fertirrigation with NaCl at 200 mM, and the others 40 to standard fertirrigation (as control plants). This saline condition to 9 replicates per 2 treatments and 2 petal zones, as follows: 9 petal bases from control plants; 9 petal limbs from control plants; 9 petal bases from salt-stress plants, and 9 petal limbs from salt-stress plants. Moreover, each sample was analyzed in triplicate.

Total anthocyanin, flavonols and phenolic content

The spectrophotometric determination of total anthocyanin, flavonols and phenolic content were performed with a Hewlett–Packard UV–vis HP8452 spectrophotometer (Palo Alto, CA, USA), using 10 mm path length glass cells and distilled water as reference.

Total anthocyanin content was determined by the pH differential method[17]. Samples were diluted with aqueous pH 1.0 and 4.5 buffers (Potassium chloride solution, 0.025 M, pH 1; sodium acetate buffer, 0.4 M, pH 4.5) and left standing for 10 min, after which the absorbance measurements were taken at 520 and 700 nm. The method assumes that anthocyanin pigments show zero absorbance at pH 4.5. The molar extinction coefficient of cyanidin-3-glucoside was 26 900 and its molecular weight was 449.2 Da. Results were expressed in mg of anthocyanins (as cyanidin-*3*-glucoside equivalent) per 100 g petal dry weight (DW).

Total flavonols content was determined using a modification of the Glories method[18,19], originally for wine phenols. Samples were diluted 1:10 with 10% ethanol. The method consisted of placing 0.25 mL of sample or standard in a test tube and adding 0.25 mL of 0.1% HCl in 95% ethanol and 4.55 mL of 2% HCl. The solution was mixed and allowed to sit for approximately 15 min before reading the absorbance at 360 nm. Standard used was quercetin in 95% ethanol and the results were expressed as mg of flavonols (expressed as quercetin equivalents) per 100 g petal dry weight (DW).

Total phenolic content (TPC) was determined using a modification of the Folin-Ciocalteau method[20]. Absorbance was measured at 765 nm and the results were expressed as mg of polyphenol (expressed as gallic acid equivalents) per 100 g petal dry weight (DW).

HPLC-DAD-ESI/MS analysis

HPLC-DAD analysis was performed in a Hewlett-Packard 1200 series liquid chromatograph (Palo Alto, CA), equipped with a quaternary pump, column heater, an UV-Vis diode-array detector, an automatic injector, and the ChemStation software. A Zorbax SB C18 column (4.6 mm x 250 mm, 4.6 μm particle size) thermostated at 35 °C was used. All analyses were made in triplicate by direct injection of the samples, previously filtered through a 0.45 μm Nylon filter (E0034, ANALISIS VINICOS, Spain).

Chromatographic analysis was carried out following a modification of the method described in Quijada-Morin et al.[21]. The solvents used were: (A) an aqueous solution (0.1%) of trifluoroacetic acid (TFA), and (B) 100% HPLC-grade acetonitrile.

The elution profile was as follows: isocratic 10% B for 3.25 min, from 10 to 15% B for 12.37 min, isocratic 15% B for 5.21 min, from 15 to 18% B for 5.21 min, from 18 to 30% B for 20.84 min and from 30 to 35% B for 5.20 min. The flow-rate was 0.8 mL min^{-1} and the injection volume was 50 μL. The UV-vis spectra were recorded from 220 to 600 nm with a bandwidth of 2.0 nm. Detection was carried out at 520 nm as the preferred wavelength.

For the anthocyanin identification, the mass spectrometer was connected to HPLC system via the DAD cell outlet. MS detection was performed in a API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source and a triple quadrupole-ion trap mass analyzer that was controlled by Analyst 5.1 software. Zero grade air served as nebulizer gas (GS1) and turbo gas (GS2) for solvent drying. Nitrogen served as curtain (CUR) and collision gas (CAD). MS analysis was carried out in positive mode (ESI+). Setting used was optimized by direct infusion of a malvidin 3-O-glucoside solution:

198 declustering potential (DP) 41 V, entrance potential (EP) 7.5V, ion spray voltage (IS)
199 5000V, GS1 40 psi, GS2 50 psi (600 ºC), CUR 20 psi, and CAD was set as "High". Both
200 quadrupoles were set at unit resolution. Mass method consisted of three mass experiments:
201 full mass analysis (EMS mode, collision energy (CE) 10V), MS2 analysis (EPI mode, CE
202 25V) and MS3 analysis (CE 30V, excitation energy (AF2) 50V). Spectra were recorded
203 between m/z 150 and 1100.
204 Anthocyanin compounds were identified by comparison of their retention time, UV-vis
205 spectra, and mass spectra features with data reported in literature. Quantification of
206 anthocyanins was calculated from the peak areas recorded at 520 nm using a calibration
207 curve purchase standard of cyanidin-3-glucoside. Results were expressed as mg/100 g of
208 petal dry weight (DW).
209 **Color analysis of *Hibiscus* petals by Digital Image Analysis**
210 Previous to the frozen treatment, the same fresh petals considered for the anthocyanin
211 determination (n=9 per treatment) were used to analyze the color of *Hibiscus* flowers by
212 digital images analysis.
213 The image acquisition was performed with a DigiEye imaging system[22] consisting of a
214 calibrated digital camera connected to a controlled illumination cabinet and a computer with
215 appropriate software (DigiPix). The digital camera used for image acquisition was a 10.2-
216 megapixel Nikon® D80 with Nikkor® 35 mm f/2D objective. It was connected via USB to a
217 computer with Pentium IV processor at 3.00 GHz. The cabinet (VeriVide DigiEye®,
218 Leicester, UK) was equipped with two fluorescent tubes that emulate the standard illuminant
219 D65 and offer stable lighting conditions. Lamps were switched on at least ten minutes before
220 being used, according to manufacturer indications, to stabilize them. The camera was
221 calibrated with the Gretag Macbeth Colour Checker DC Chart in order to characterize its
222 response by relating the RGB signals to CIE specifications under the fixed lightning

conditions in the cabinet. For the color measurements, fresh petal samples were appropriately placed into the cabinet using a white background that ensures an adequate segmentation of sample image.

For each image, a 625×625 pixel fixed area was taken and CIELAB color parameters data (L*, a*, b*, C^*_{ab}, and h_{ab}) were estimated according to Rodríguez-Pulido et al.[23], with the software Matlab®[24]. The color data obtained were averages of three measurements. In addition, the Euclidean distance between two points in the three-dimensional space define by L*, a*, and b* were used to calculate the color difference between control and saline samples as follows: $\Delta E^*_{ab} = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2.}$

Statistical analysis

All statistical analyses were performed using software Statistica v.8.0[25]. Univariate analysis of variance (ANOVA) was applied, using the general linear model procedure (GLM), to establish whether mean values of the phenological and physicochemical data obtained differed significantly as a function of the salinity treatment applied. The means values were compared by *t*-test, Bonferroni test, or Tukey test at a significance level of $p<0.05$. Correlations between color parameters and anthocyanin content were studied by Forward Stepwise Multiple Regression at a significance level of $p<0.05$.

RESULTS AND DISCUSSION

Effect of salinity stress on abscisic acid content, ion leakage and photochemical efficiency

The application of salinity stress for a prolonged period influenced the morphology and the physiology of *H. rosa-sinensis* plants.

In our conditions, the saline irrigation caused a significant decrease in flower fresh mass, which was about 60% after 28 days of treatment. The biomass reduction due to salt stress may either be determined by the decreased availability of water or to the NaCl toxicity[26].

248 Previous research on other ornamental flowers showed that quality parameter like flower
249 weight was unfavorably affected by salinity[27]. In fact, plants subjected to saline irrigation
250 require additional energy on plant cells. This extra metabolic energy is likely to be
251 consumed in processes related to osmotic adjustment within the cells and hence less carbon
252 is available for growth[28]. This is particularly relevant for plant visual quality, because the
253 most decorative elements for ornamental plants are usually the flowers.
254 As reviewed by Ashraf and Harris[29], the salt sensitive species, showed an increase in
255 electrolyte leakage in the leaves under saline conditions. Electrolyte leakage is an indicator
256 for the extent of membrane damage under salinity stress and was determined in the fully
257 expanded leaves of salt-stressed *H. rosa-sinensis*. Low values of electrolyte leakage were
258 recorded in the controls (0 mM). The presence of NaCl in the rooting medium induced a
259 significant increase in electrolyte leakage in the leaves (Figure 1) and was greater as
260 exposure time to saline treatment increased reaching the maximum values after 28 days.
261 The hormone ABA is well known as a mediator in plant responses for both biotic and
262 abiotic stresses. Endogenous ABA concentrations fluctuate in response to environmental
263 conditions[30], allowing plants to survive under adverse conditions, such as salt stress. In *H.*
264 *rosa-sinensis* plants, salinity increased ABA content in leaves (Figure 2). This is consistent
265 with other plant species that increase ABA production in response to salinity[31]. Moreover,
266 ABA levels varied among the flower tissues examined, and as showed in Figure 2, its
267 content enhanced in the style stigma plus stamens whereas in petals and ovary tissues
268 decreased. A possible relationship among the differential tissue-rate of growth under salinity
269 could explain the divergent trend of ABA levels in floral organs and needs to be further
270 explored.
271 To better characterize the physiology of *Hibiscus*, the chlorophyll a fluorescence was
272 measured. In dark adapted leaves the PI declined drastically after 28 days of saline

273 treatment, being 8.4 units in the control leaves whereas in the treated plants was 3.9 units. PI
274 is an overall index that combines different fluorescence parameters and has been frequently
275 employed to understand the responses of the photosynthetic apparatus to different
276 physiological and environmental conditions[10,32]. Our results confirmed PI as a sensitive
277 indicator of salt stress. Also the chlorophyll a fluorescence measured from the light adapted
278 leaves subjected to saline irrigation, revealed a statistically relevant reduction in the relative
279 quantum yield of PSII (0.7 in the control plants versus 0.4 under saline treatment) and these
280 results are in agreement with the literature[33].
281 To summarize, salinity promoted several physiological and biochemical changes in *Hibiscus*
282 plant tissues including reduction of flower biomass and parameters associated with the
283 functioning of the photosynthetic apparatus as well as fluctuation of ABA levels, and
284 increase electrolyte leakage.
285 **Effect of salinity stress on the anthocyanin composition and color**
286 Considering the heterogeneous color pattern of *H. rosa-sinensis* flowers with two red zones
287 visually discernible, the base and the limb of petals were analyzed as separate groups to
288 determinate possible differences regarding their anthocyanin composition and content; as
289 well as to evaluate the influence of saline stress in each zone.
290 Results showed that the base and limb of flowers presented the same anthocyanin profile.
291 The chromatographic analysis of the extracts, registered at 520 nm, shows the presence of
292 five anthocyanins (Figure 3.A), all of them identified as cyanidin derivatives according to
293 their chromatographic and spectrometric features obtained in the HPLC-DAD-MS analysis
294 (Table 1).
295 The major anthocyanin found in *Hibiscus rosa-sinensis* flowers was identified as cyanidin-
296 3-sophoroside (peak 1), which accounted for 80% of total peak area. The mass spectrum of
297 peak 1 (Figure 3.B) obtained in the ESI positive mode exhibited an ion at m/z 611. MS/MS

298 fragmentation of m/z 611 produced a daughter ion at m/z 287 which was indicative of the
299 cyanidin moiety, as previously found in literature[34].
300 Peak 2, was identified as cyanidin-3-sambubidoside based on its characteristic molecular ion
301 at m/z 581 and a major fragment in MS^2 at m/z 287, which has been described as one of the
302 most common anthocyanin in diverse *Hisbiscus* spp[1]. However, in our *H. rosa-sinensis*
303 extracts, it was only detected in trace levels.
304 Peak 3 represented the second predominant anthocyanin in the analyzed extracts accounting
305 for nearly 12% of total anthocyanins. Its fragmentation pattern showed a molecular ion at
306 m/z 639 which produced two MS^2 daughter ions at m/z 449 (M+-190) which corresponds to
307 cyanidin-hexose moiety; and m/z ratio at 287 (M+-190-162) which corresponds to cyanidin-
308 aglycone moiety were found (Figure 3.C). Despite MS^2 fragmentation pattern could not be
309 confirmed with reported data, it indicated the sequential losses of an unidentified substituent
310 (190 amu) and one hexose residue (162 amu, i.e. glucose as abundant sugar in plants) from
311 aglycone. According to the literature, the glycoside moieties of anthocyanins are usually
312 esterified with different organic or phenolic acids. Among them, the hibiscus acid has been
313 described as a common compound of Hibiscus species showing a characteristic loss of 190
314 amu[35], when it appears as an esterification substituent. Moreover, the acylated nature of this
315 compound is consistent with their relative retention time in the HPLC analysis, eluting
316 before the corresponding 3-glycosides derivatives. Thus, peak 3 would tentatively
317 correspond to a cyanidin-3-glucoside esterified by an hibiscus acid moiety.
318 In addition to the abovementioned compounds, other two minor pigments were also detected
319 (peaks 4 and 5) although their absorption or mass spectra were insufficient to allow
320 speculation about their identity. Their structures still need to be elucidated by comparison of
321 MS data with reference standards or using other spectroscopic techniques such as NMR.

322 The concentration of the different anthocyanins detected by HPLC-DAD-MS together with
323 the total content of anthocyanins, flavonols and polyphenols assessed by rapid
324 spectrophometric methods are shown in Table 2, where the effect of saline fertirrigation can
325 be observed.
326 Although there were five anthocyanins detected, quantitative differences for all the
327 individual compounds were observed among the two distinct areas of the petals. Generally,
328 the petal limb contained more anthocyanins and other phenolics than the base, which
329 support the heterogeneous color pattern exhibited by *H. rosa-sinensis* flowers. For the
330 control sample, the average values of Total anthocyanins, flavonols and polyphenolic
331 content in the petal limb were found to be 821.5±37.3, 23.0±0.9 and 2252.4±129.4 mg/100g
332 DW, whereas the global levels in the petal base were 228.0±48.9, 12.9±1.2 and 1077.9±98.5
333 mg/100g DW, respectively.
334 As can be observed, the saline treatment significant ($p<0.05$) affected the anthocyanin
335 composition of *Hibiscus* flowers reducing its content (Figure 3.A), but the magnitude of the
336 effect was different in each zone of the petal (Table 2). The increase in the salinity of the
337 nutrient solution was accompanied by a stronger decrease of the Total anthocyanin content
338 in the base than in the limb (30% versus 20%, approximately), which is mainly due to the
339 cyanidin-3-sophoroside reduction in both cases. In particular, some of the individual
340 anthocyanin compounds present in the limb were not affected by the saline treatment, which
341 could explain the lower loss of pigments in this zone of the flower. As reported by other
342 authors[3], differences between the glycosyl units and acyl groups attached to the aglycone,
343 and the site of their bonding, have a significant influence on the stability and reactivity of
344 the anthocyanin molecule.
345 Furthermore, the saline fertirrigation caused a negative impact in the accumulation of other
346 phenolic compounds since their total content was lower in flowers from plants grown with

salinity stress conditions (25% and 15% lower for Total flavonols, and 29% and 10% lower for Total polyphenols; in the base and limb respectively). There is considerable controversy in the literature concerning the positive and negative effects of salt stress on the phenolic composition of many plant species. Some authors have shown that the application of moderate salt stress stimulates the anthocyanin synthesis[7]. However, our results are in agreement with those reported by Li et al.[36], which observed that the anthocyanin content in grapes was negatively affected by high saline conditions (100-150 mM NaCl).

Regarding the color characteristics of *H. rosa-sinensis* flowers, Figure 4 shows the three-dimensional colorimetric representation of a petal sample (control plant) obtained by Digital Image analysis. It presents the L*, C^*_{ab}, and h_{ab} CIELAB values assigned to each pixel of the petal image grouped regarding the petal zone (base and limb; n=100 pixel/petal zone), which provide a full characterization of the color variability of samples[37]. As can be seen, the color of the base could be easily distinguishable from the limb. Color points corresponding to the limb area appeared more disperse than those of the base, and showed higher values of lightness (L* ranging from 23 to 42 versus 26 to 37 CIELAB u., respectively), chroma (C^*_{ab} from 31 to 47 versus 29 to 35 CIELAB u.) and hue (h_{ab} from 35 to 53 ° versus 30 to 41°). This meant that the petal limb exhibits more vivid color with red-orange hues, which is consistent with the higher accumulation of red-orange forms of anthocyanins (cyanidin derivatives) in this flower zone[38]. On the contrary, the base of the petal corresponds to a more homogeneous redness area with darker and less saturated color, due to their lower concentration in cyanidin derivative pigments. Apart from the different amounts in pigments, the differences observed for the total content of flavonols and polyphenols in each petal zone (Table 2) may also account for the natural color variability of *Hibiscus* flowers since colorless phenolics are usually implied in the color expression of anthocyanins by means of copigmentation reactions contributing with color changes[39].

The concentration of the different anthocyanins detected by HPLC-DAD-MS together with the fertirrigation treatment are summarized in Table 2, where *p*-values for comparison among control and saline samples are shown. Results indicate that the color of *Hibiscus* flowers was significantly ($p<0.05$) affected by salt treatment, which was in accordance with the effect induced in the pigment composition. The addition of 200 mM of NaCl into nutrient solution resulted in quantitative (L* and C^*_{ab}) and qualitative (h_{ab}) color variations both in the base and the limb of *Hibiscus* petals (Figures 5.A and 5.B). Specifically, salt stress resulted in a general loss of color, as can be seen in Table 2. In each zone, L* and h_{ab} values increased while C^*_{ab} values decreased (L* by 21% and 11%; h_{ab} by 20% and 5%, C^*_{ab} by 12% and 3%, in the base and the limb respectively), so *Hibiscus* flowers exhibited clearer and less vivid red color when they were fertirrigated under saline stress conditions, being the effect more marked in the base of the petals.

These results were also confirmed through the quantitative mean color difference (ΔE^*_{ab}) calculated among control and saline samples in each petal zone, which were 4.1 and 9.6 CIELAB u. for the petal limb and base, respectively. Taking into account that ΔE^*_{ab} of up to three CIELAB units indicates color differences appreciable to the human eyes[40], it was confirmed that more important and hence more easily perceptible color loss was caused in the base of the *Hibiscus* petal, which is consistent with the higher reduction of the pigment concentration in this flower zone.

Apart from the assessment of color itself, the objective measurement of color can be harnessed for the rapid determination of pigments. In this regard, color measurements offer a series of advantages for this purpose, like rapidity, non destructiveness and simplicity, among others. A Forward Stepwise Multiple Regression was performed for predicting the Total Anthocyanin Content (mg/100g DW) from the colorimetric data obtained by Image Analysis. In this respect, not only conventional colorimetric coordinates (CIELAB) were

Furthermore, the saline fertirrigation caused a negative impact in the accumulation of other phenolic compounds since their total content was lower in flowers from plants grown with