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Effect of foliar copper application on yield and anthocyanin concentration in *Hibiscus sabdariffa* calyces

Aplicación foliar de cobre sobre el rendimiento y concentración de antocianinas en cálices de *Hibiscus sabdariffa*

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Abstract

Frequent anthocyanin consumption improves health and prevents diseases due to its antioxidant activity. Exposure of some plants to high concentrations of heavy metals may increase anthocyanin concentration and thus improve their food quality. This study determined the effect of Cu spraying on hibiscus (*Hibiscus sabdariffa* L.) leaves at various dosages and number of doses on anthocyanin content, physical and chemical characteristics, and calyces yield. For this purpose, hibiscus genotype Reina Roja was grown under rainfed conditions. During the vegetative stage, Cu was sprayed two, four or six times with 150, 300 and 450 mg L^{-1}. The results indicate that four and six sprayings with 150, 300 and 450 mg Cu L^{-1} reduced dry calyces yield. Two sprayings at either Cu dosage did not modify calyces yield. Added Cu increased significantly anthocyanin content and titratable acidity and decreased ascorbic acid content in the calyces. Anthocyanin content increased the most (57 and 44%) when Cu was sprayed six times at 300 and 450 mg L^{-1}. The data suggests that two sprayings with 150 mg Cu L^{-1} could improve nutritional quality of hibiscus extracts without affecting dry calyces yield.

Keywords
Hibiscus sabdariffa • anthocyanins • ascorbic acid • titratable acidity

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Resumen

El consumo frecuente de antocianinas mejora la salud y previene enfermedades, debido a su actividad antioxidante. La exposición de algunas plantas a concentraciones altas de metales pesados puede incrementar la concentración de antocianinas y mejorar su calidad funcional. Este estudio determinó el efecto de la aplicación foliar de Cu en varias dosis y número de aplicaciones sobre el contenido de antocianinas, características físico-químicas y rendimiento de cálices de jamaica (*Hibiscus sabdariffa* L.). Para este propósito se cultivó el genotipo Reina Roja que durante la etapa vegetativa, se asperjó al follaje con Cu en dos, cuatro y seis ocasiones con 150, 300 y 450 mg L^{-1}. Los resultados indican que cuatro y seis aplicaciones de cobre redujeron el rendimiento de cálices secos para todas las dosis. Dos aplicaciones no modificaron el rendimiento de cálices. El Cu incrementó significativamente el contenido de antocianinas, acidez titulable y disminuyó el contenido de ácido ascórbico en los cálices. Los aumentos mayores de antocianinas (57 y 44%) fueron con seis aplicaciones de 300 y 450 mg de Cu L^{-1}. Los datos sugieren que dos aplicaciones de 150 mg de Cu L^{-1} pueden mejorar la calidad nutracéutica de los extractos de jamaica sin afectar el rendimiento de cálices secos.

Palabras clave

Hibiscus sabdariffa • antocianinas • ácido ascórbico • acidez titulable.

Introduction

The demand of plant products as functional food staple is increasing due to the direct relationship between diet and health (11, 30). The properties of some plant substances and their possible use in prevention and mitigation of chronic diseases caused mainly by free radicals have been identified (10, 15, 20). Some of the phytochemical compounds with nutraceutical activity are carotenoids, phytosterols, flavones, isoflavones, flavonols, flavonones, stilbenes, phenolic acids, lignans and anthocyanins (15, 18).

Anthocyanins are polyphenols that color leaves, flowers and fruits of red, purple and blue. The main plant sources for anthocyanins are berries like cranberries (*Vaccinium sp.*), blackberries (*Rubus spp.*), raspberries (*Rubus idaeus*) and strawberries (*Fragaria x ananassa* D.), as well as grapes (*Vitis vinifera* L.) and hibiscus calyces (*Hibiscus sabdariffa* L.) (23, 29). Anthocyanins function as antioxidants, diuretic, antimicrobial, antiobesity, inmune modulators, antiurolithiatic, hepatoprotective and hypocholesterolcemic (20). Additionally, they reduce cholesterol levels and decrease the risk of acquiring diabetes, cancer, and other diseases (19, 20).

Hibiscus sabdariffa L. is a malvaceae grown mainly in tropical and subtropical regions at Sudan, Taiwan, Thailand and Mexico. In Mexico it is cultivated in approximately 20,055 ha with a production of 7,528 t (25). This crop is made by producers of scarce resources without mechanization, which favors occupation (5). Dry flower calyces are used to prepare tea, wine, cocktails, sauce, jelly, candies and cookie flour; however, its main use is preparation of refreshing beverages from extracts (20). Its consumption has increased mainly due to promotion efforts that classify it as a functional food (10, 20). Efforts to increase secondary metabolite content, like anthocyanins, in hibiscus calyces could increment health benefits and improve prices for producers. Environmental stresses like high and low temperatures, drought, salinity, UV stress or heavy metals could alter their metabolism and thus the physical and chemical characteristics of the plants (2, 31).

Exposure to heavy metals increases free radical production. Anthocyanins have greater capacity than vitamins C and E to protect the plant against oxidative stress by donation of hydrogen atoms that stabilize unpaired electrons from free radicals. It is then possible to induce secondary metabolite synthesis by stress conditions (27).

Application of As on *Lemna gibba* with 0,25, 0,5, 1,0 and 1,5 mg L^{-1} increased anthocyanin content; the highest content was found at the highest dosage and produced 45 mg 100 g^{-1} of fresh biomass (FB) (17).

In *Arabidopsis thaliana* under *in vitro* conditions, exposure to Mn, Cu, Zn, Pb and Hg increased anthocyanin content: Cu with 250 µM increased anthocyanins more efficiently from 2 to 32,5 µg g^{-1} of fresh biomass (FB). Zn increased the highest (14 µg g^{-1} FB) with 1000 µM (7). In both studies, the variables measured included chlorophyll content, dry biomass weight, root length and shoot length (7, 17).

According to Leão *et al*. (2014), plants respond differently to stress by heavy metals, and the response depends on the metal, the concentration and the duration of the stress. However, in *Atriplex hortensis* var Purpurea and *A. rosea* exposure to different concentrations of Cu, Ni, Pb and Zn did not modify anthocyanin content (22).

Hibiscus calyces respond to cobalt and nickel applications, either separate o combined, by increasing content of anthocyanins and other flavonoids. The highest values (31.7 mg anthocyanins g^{-1} dry biomass) occurred with 20 mg of Co + 25 mg Ni kg^{-1}: a 96% increment compared to the control. Plant height, number of fruits and calix yield also improved (6). Leaf spraying of heavy metals could improve this response. Cu is sold as leaf fertilizer, is easily purchased, contributes to plant nutrition, and improves crop yield. There are limited number of hibiscus-related studies that explore changes in anthocyanin production and improvements on physical and chemical characteristics of extracts. This study determined the effect of Cu leaf spraying at different dosages and number of doses on anthocyanin content, physical and chemical characteristics of calyces, and calyces yield.

Materials and methods

The experiments were conducted under rainfed conditions in Cajones, Municipality of Gabriel Zamora, Michoacán, México at 19°10'49" N; 101°58'13" and 503 a m. s. l. The climate is warm sub-humid, with summer rains (AW_0) (13).

On July 2016, Reina Roja hibiscus seeds were handed out to local producers. Two seeds per bush were planted at 0.5 m between each bush and 0.8 m between rows. Population density was 50.000 plants per hectare.

Previous tests determined that Cu leaf spraying should start 15 days after sowing at 150, 300 and 450 mg L^{-1} of water per spraying (D150, D300, D450). Plants were dosed two, four and six sprayings (A2, A4 and A6) spaced every seven days.

Nine treatment combinations were tested (D150-A2, D150-A4, D150-A6, D300-A2, D300-A4, D300-A6, D450-A2, D450-A4, D450-A6), as well as a control (D0-A0), which were distributed in the field under a randomized complete block experimental design in a split-plot arrangement. The large plot was the dosage and the small plot the number of applications. Each treatment was repeated four times for a total of 40 experimental units. Each unit included three meter long rows with 36 plants.

The Cu source was pentahydrated cupric sulfate applied with adherent agent Inex (1 mL L^{-1}). Leaves were sprayed in the mornings until dripping. The sprayer was 15 L in capacity and included an empty cone tip (hollow).

Crop fertilization included 80 and 40 kg ha^{-1} of N and P_2O_5 to the soil as urea (46-0-0) and triple calcium superphosphate (0-46-0). Half of the N dosage and all the P_2O_5 dosage were applied mixed 15 days after sowing (das); the rest of the N dosage was applied at 45 das.

At plant physiological maturity (147 das) when the fruits started with brown coloration and the seeds get a black coloration the harvest of fruits with calyces was carried with the hands, from the middle row from each experimental unit. Afterwards, the fruit was separated from the calyces with a punch. Subsequently, the number of calyces per plant were counted, and their diameter and length recorded. Calyces were stored in paper bags and dried in a forced circulation oven at 50°C until constant weight was achieved. After drying, the weight of 10 calyces was recorded, as well as dry calyces yield.

From each experimental unit, 15 g of dry calyces were ground in an IKA mill model MF 10 equipped with a 0.5 mm screen. From each sample, duplicate water extracts were prepared with 2,5 g of powder each. The extract was transferred to 250 mL Schott Duran glass vials, and 200 mL of distilled water were added. The mixture was left untouched for 24 h at ambient temperature in the dark. The following day, the extracts were placed in a water bath at 40°C for 15 minutes.

The following variables were measured in the extracts: pH with a Beckman pH Meter (method 981.12); total soluble solids with an ATAGO digital refractometer (0 to 32% range) (method 932.12); titratable acidity as percentage of citric acid (method 942.15); and ascorbic acid content with 2, 6 dichlorophenol indophenol (method 967.21). The determinations were made according to the methodology of the Association of Official Analysis Chemistry (4).

Anthocyanin content was determined with the Abdel-Aal and Hucl method (1999). This method required 100 mg of powder from each ground sample. This sample was added to centrifuge tubes and 10 mL of extraction solution [acidified methanol (methanol: HCl 1,5N in an 85:15 v/v ratio)].

The mixture was kept in the darkness for 24 h at 4°C. After this period, the tubes were centrifuged at 5000 rpm for 20 minutes in a Heraeus Biofuge Primo R. Samples were then diluted to a 1:10 ratio (100 µL of concentrated extract + 900 µL of acidified methanol) and their concentration determined in a Genesys 10 UV (Termo Spectronic) spectrophotometer at 533 nm with extraction solution as blank.

Anthocyanin content was estimated as equivalents of cyanidine-3-glucoside using the equation:

$$A = (Ab/\varepsilon)(Ve/1000)MW(1/Ph)(10^6)$$

where:
A = anthocyanin content
Ab=the absorbance for the sample,
sigma= molar extinction coefficient for cyanidine-3-glucoside (25965 cm-1 mol^{-1})
Ve = total extract volume
MW = the molecular weight for cyanidine-3-glucoside (449)
Ph = sample weight

Total anthocyanin content was recorded in mg of anthocyanins per g of dry calyces biomass.

Data was analyzed using ANOVA and mean comparison among treatments (Tukey ≤ 0.05) in SAS version 9.0 (24).

During the development of the crop the maximum temperature, minimum temperature and rainfall were recorded. On average, the maximum and minimum temperatures were 33.5°C and 17.7°C, respectively with accumulated precipitation of 367.9 mm (figure 1).

Results and discussion

Calyces yield and yield components

Supplied Cu in two applications at either dosage did not modify statistically calyces yield: yields for 150, 300 and 450 mg L^{-1} were similar to yields produced by untreated plants (1272 kg ha^{-1}).

The 300 mg L^{-1} dosage produced heavy calyces, 12.8% heavier than the control, but it did not improve yield because the number of calyces per plant decreased (table 1, page 70).

Cu spraying reduced significantly ($P \leq 0.01$) calyces yield when applied four and six times in all dosages; it significantly decreased calyces length, calyces diameter, number of calyces per plant and calyces weight (table 1 and 2, page 70).

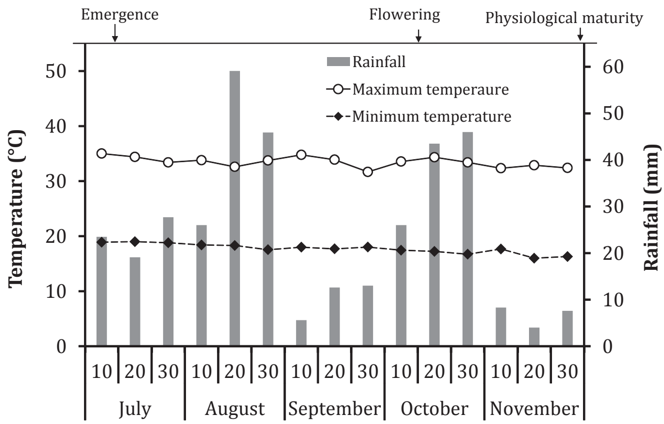

Figure 1. Rainfall distribution (ten days sum), maximum and minimum temperature (ten days average) during crop growth.

Figura 1. Distribución de la precipitación pluvial (suma decenal), temperatura máxima y mínima (promedio decenal) durante el crecimiento del cultivo.

Table 1. Dry calyces yield (DCY), calyces diameter (CD), calyces length (CL), number of calyces per plant (NC), weight of 10 calyces (W10C) of *Hibiscus sabdariffa* in response to dosage and number of foliar Cu applications. Tukey means comparisons.

Tabla 1. Rendimiento de cálices secos (DCY), diámetro de cálices (CD), longitud de cálices (CL), número de cálices por planta (NC) y peso de 10 cálices (W10C) de *Hibiscus sabdariffa* en función de la dosis y número de aplicaciones foliares de Cu. Comparación de medias de T.

Cu dosage (mg L^{-1})	Number of doses	DCY (kg ha^{-1})	CD (cm)	CL (cm)	NC	W10C (g)
0	0	1272.1 a	3.22 a¶	4.32 a	33.8 ab	7.65 b
150	2	1299.2 a	3.17 ab	4.16 abc	36.3 a	7.2 bc
	4	1061.1 c	3.07 ab	3.91 c	24.3 c	7.68 b
	6	1045.9 c	3.16 ab	4.15 abc	32.8 ab	6.48 d
300	2	1245.4 ab	3.14 ab	4.18 ab	29.0 bc	8.63 a
	4	1067.5 c	2.81 c	3.57 d	29.3 bc	7.35 bc
	6	1069.2 c	3.19 ab	4.27 a	30.8 ab	7.03 bcd
450	2	1244.2 ab	3.09 ab	3.95 bc	35.5 a	7.03 bcd
	4	928.4 c	3.13 ab	4.14 abc	29.3 bc	6.98 cd
	6	1087.5 bc	3.01 b	4.16 abc	29.0 bc	7.53 bc
General Mean		1132.0	3.1	4.08	30.1	7.45
$MSD_{0.05}$		168.0	0.18	0.25	6.16	0.65
CV		6.10	6.32	8.50	7.9	9.40

¶ Means with the same later within each column are not statistically different (Tukey, P ≤ 0.05). $MSD_{0.05}$ = minimum significant difference at 5% error probability. CV = coefficient of variation.

¶ Medias con la misma letra dentro de cada columna no son diferentes estadísticamente (Tukey, P ≤ 0,05). $MSD_{0,05}$ = diferencia mínima significativa al 5% de probabilidad de error. CV = coeficiente de variación.

Table 2. Level of significance for dry calyces yield (DCY), calyces diameter (CD), calyces length (CL), number of calyces per plant (NCP) and weight of 10 calyces (W10C) in *Hibiscus sabdariffa* in response to Cu dosage, number of leaf sprayings and the interaction dosage x doses.

Tabla 2. Nivel de significancia para rendimiento de cálices secos (DCY), diámetro de cálices (CD), longitud de cálices (CL), número de cálices por planta (NCP) y peso de 10 cálices (W10C) en *Hibiscus sabdariffa* en función de la dosis de Cu, número de aplicaciones foliares y la interacción dosis x número de aplicaciones.

Factor	DCY	CD	CL	NCP	W10C
Dosage (D)	ns	ns	**	*	ns
Doses (A)	**	**	**	**	**
D x A	**	**	**	**	**

*, ** P ≤ 0.01 y 0.05, respectively. ns = not significant.

*, ** P ≤ 0,01 y 0,05, respectivamente. ns = no significativo.

Plants sprayed four times with 300 mg L^{-1} per application produced the smallest calyces: 12.7% smaller diameter than the control treatment. The 150 mg L^{-1} treatment applied four times registered the lowest number of calyces per plant: 28.1% lower number and 9.5% shorter calyces.

Results from the statistical analysis showed that dry calyces yield and yield components were most affected by the number of Cu applications rather than dosage (table 2, page 70). According to the interaction dosage (D) * number of doses (N) it was found that the increase in the number of copper applications at any dose reduced the yield of dry calyces and the values of the yield components. It should be noted that this effect was not proportional in all doses of Cu, to be less the dejection with 300 mg L^{-1} (table 1 and 2, page 70).

Reduction of calyces yield by Cu sprayings could be attributed to application of higher than recommended concentrations for crops according to Fageria *et al*. (2009). Plant exposure to high heavy metal dosages could cause toxicity, but it is also possible that plants develop mechanisms to tolerate stress.

The mechanisms that stand out are metal exclusion, metal accumulation and metal union to cysteine rich proteins. Plants can also promote the antioxidant system to fight oxidative lesion induced by heavy metals (3, 9). This behavior might explain how calyces yield did not change with two Cu sprayings at 150, 300 and 450 mg L^{-1} dosages.

Calyces yield reduction in hibiscus with four and six Cu sprayings for all dosages tested might respond to repeated and prolonged stress to the plant with these treatments. Previous research mentions this type of stress catalyzes the production of oxygen reactive species like superoxide, hydrogen peroxide and hydroxyl radicals that damage cellular components like DNA, proteins and lipids and affect crop growth and yield (28).

Similar response was found with a single application of $ZnSO_4$ at 2000 μM and $CdCl_2$ at 250 μM on genotypes Drakkar, Cossair and Pactol of *Brassica napus* L. then reduced significantly dry biomass production (14). Likewise, in rice (*Oryza sativa* L.) 100 μM of Cu caused significant reductions in growth and dry biomass accumulation (28).

A positive effect was found in soil-grown hibiscus treated with Co (20 and 40 mg Kg^{-1}) and Ni (25 and 50 mg Kg^{-1}), alone or in combination. These elements improved up to 137% fresh and dry calyces yield by significantly increasing the number of calyces per plant (6). This response, which is opposite to the response found in this study, might depend on the differential response of plants to heavy metals: their response depends on type of metal, application mode, concentration, and length of stress (8, 17).

Physical and chemical characteristics of hibiscus extracts

Cu spraying for all the treatments reduced significantly ($P \leq 0.01$) ascorbic acid content in all calyces. The highest reduction (41.5%) occurred with two sprayings at 300 mg Cu L^{-1}. Four sprayings at 450 mg Cu L^{-1} caused the lowest reduction (19.4%) (table 3 and 4, page 72).

Regarding D * N, it was found that the reduction of ascorbic acid with the increase in the number of applications was not proportional in all the doses of Cu evaluated, when there was greater abatement with 150 mg Cu L^{-1}, followed by 300 mg L^{-1} (table 3 and 4, page 72).

Table 3. Ascorbic acid (AA), titratable acidity (TA), pH, soluble solids (SS) and anthocyanin content in *Hibiscus sabdariffa* calyces in response to dosage and number of Cu leaf doses. Tukey means comparisons.

Tabla 3. Ácido ascórbico (AA), acidez titulable (TA), pH, sólidos solubles (SS) y contenido de antocianinas en cálices de *Hibiscus sabdariffa* en función de la dosis y número de aplicaciones de Cu. Comparación de medias de Tukey.

Cu dosage (mg L^{-1})	Number of doses	AA (mg 100 g^{-1})	AT (%)	pH	SS (°Brix)	Anthocyanin (mg g^{-1})
0	0	85.5 a¶	10.5 b	2.94 a	4.32 a	18.9 d
150	2	57.6 d	12.2 a	2.85 a	4.18 a	24.8 bc
	4	56.1 de	10.5 b	2.94 a	4.31 a	24.3 bc
	6	59 d	12.2 a	2.84 a	4.17 a	23.4 c
300	2	50 e	11.7 ab	2.91 a	4.27 a	24.7 bc
	4	61.8 cd	10.8 ab	2.96 a	4.34 a	24.3 bc
	6	58 d	12 ab	2.92 a	4.29 a	26.9 a
450	2	57.6 d	10.8 ab	2.91 a	4.27 a	24.7 bc
	4	67.5 bc	12 ab	2.87 a	4.22 a	25.7 ab
	6	68.9 b	12.2 a	2.86 a	4.20 a	27.3 a
General Mean		62.2	11.5	2.90	4.26	24.5
$MSD_{0.05}$		6.5	1.7	0.17	0.25	1.97
CV		4.7	4.2	2.4	2.4	3.30

¶ Means with the same later within each column are not statistically different (Tukey, P ≤ 0.05). $MSD_{0.05}$ = minimum significant difference at 5% error probability. CV = coefficient of variation.

¶ Medias con la misma letra dentro de cada columna no son diferentes estadísticamente (Tukey, P ≤ 0,05). $MSD_{0,05\ =}$ diferencia minima significativa al 5% de probabilidad de error. CV = coeficiente de variación.

Table 4. Level of significance for ascorbic acid (AA), titratable acidity (TA), pH, soluble solids (SS) and anthocyanins (ANT) in *Hibiscus sabdariffa* calyces in response to Cu dosage, number of leaf doses and the interaction dosage x doses.

Tabla 4. Nivel de significancia para ácido ascórbico (AA), acidez titulable (TA), pH, sólidos solubles (SS) y antocianinas (ANT), en cálices de *Hibiscus sabdariffa* en función de la dosis de Cu, número de aplicaciones y la interacción dosis x número de aplicaciones.

Factor	AA	TA	pH	SS	ANT
Dosages (D)	**	**	ns	ns	**
Doses (A)	**	**	ns	ns	**
D x A	**	**	ns	ns	**

*, ** P ≤ 0.01 y 0.05, respectively. ns = not signficant.

*, ** P ≤ 0,01 y 0,05, respectivamente. ns = no signficativo.

The percentage of titratable acidity increases with most treatments: the highest values were registered with two and six sprayings at 150 mg Cu L^{-1}, and six sprayings at 450 mg Cu L^{-1}. Both treatments were statistically similar, and acidity was 16% higher.

The statistical analysis indicated that number of sprayings was the determinant factor in increasing titratable acidity since in most dosages six sprayings produced the highest increments (table 3 and 4, page 72). Interaction was found between D * N, which indicates that the increase in the number of applications of Cu caused higher rates of increase in the titratable acidity of the calyces of Jamaica with the dose of 450 mg Cu L^{-1}, followed by 3-00 mg Cu L^{-1}.

The increase in titratable acidity percentage is important because it carries a bactericide effect, favors assimilation of metallic ions in the body, acts against formation of low solubility salts, and promotes the freshness sensation when ingesting hibiscus-based drinks (21).

Starting at two Cu sprayings, any of the tested dosages increased significantly ($P \leq 0.01$) anthocyanin content in hibiscus calyces. Two 150 mg L^{-1} sprayings increased anthocyanin content by 31%. This response was stronger with six sprayings at 300 and 450 mg Cu L^{-1}: mg of anthocyanins g^{-1} of dry biomass increased by 42 and 44 %, respectively (26,9 and 27.3). According to the interaction D * A the increase in the number of applications of Cu caused greater increases in the content of anthocyanins with 450 mg L^{-1} followed by 300 mg Cu L^{-1} (table 3 and 4, page 72).

Anthocyanins are secondary metabolites that protect plants against oxidative stress caused by diverse factors, including exposure to high concentrations of heavy metals. Under these conditions, plants might increase anthocyanin content (6), as the results in this study indicate from two sprayings at 150 mg L^{-1}. Increments in anthocyanin content due to exposure to high concentrations of heavy metals and semi-metals has been documented in other crops: in gibbous duckweed (*Lemna gibba* L.) by exposure to arsenic (17); in *Arabidopsis thaliana* under *in vitro* conditions by addition to the growth media of Cu, Zn, Mn, Pb and Hg (7); in *Capsicum annum* L. by leaf spraying of Cu and Zn to young leaves (26); and in greenhouse-grown Hibiscus supplied at the soil with Co (20 and 40 mg Kg^{-1}), Ni (25 and 50 mg Kg^{-1}), and their combinations (6).

The study shows that foliar Cu applications improves anthocyanin content as well as titratable acidity percentage, but decreased ascorbic acid content. These results are like those reported by Salinas-Moreno *et al*. (2012): they observed that ascorbic acid content in hibiscus calyces is inversely proportional to anthocyanin concentration. Even though ascorbic acid has antioxidant activity, it has been shown that its activity is lower than that of anthocyanins (16, 27).

All Cu treatments improved anthocyanin content in hibiscus calyces. This polyphenol is highly important because of its beneficial properties on health when consumed (19, 20). Four and six sprayings at 150, 300 and 450 mg L^{-1} increased anthocyanin content the highest, but dry calyces yield decreased. Thus, two sprayings at any of the highest dosages could improve anthocyanin content without reducing calyces yield.

Conclusions

Twice Cu application with 150, 300 and 450 mg L^{-1} did not modify caly yield, but four and six applications reduced yield by decreasing calyx size, weight and number per plant. Added Cu increased anthocyanin content and percentage of titratable acidity and decreased ascorbic acid concentration in hibiscus calyces.

The response was higher with four and six Cu sprayings in the three dosages applied.

pH and soluble solids in Hibiscus calyces did not change in response to applied Cu. The treatments that increased anthocyanins content without affecting yield were two Cu applications with 150, 300, 450 mg L^{-1}.

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