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Foliar Application of Copper Nanoparticles Increases the Fruit Quality and the Content of Bioactive Compounds in Tomatoes

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Abstract: Nanotechnology is a potential and emerging field with multiple applications in different areas of study. The beneficial effects of the use of nanoparticles in agriculture have already been proven. The objective of this research was to determine if the foliar application of Cu nanoparticles (NPs) could increase the content of the bioactive compounds in tomato fruits. Our study considered four treatments with different concentrations of Cu nanoparticles (50, 125, 250, 500 mg L⁻¹, diameter 50 nm) applied twice during the development of the culture. The effects on the fruit quality and the contents of the antioxidant compounds were determined. The application of the Cu nanoparticles induced the production of fruits with greater firmness. Vitamin C, lycopene, and the ABTS antioxidant capacity increased compared to the Control. In addition, a decrease in the ascorbate peroxidase (APX) and glutathione peroxidase (GPX) enzymatic activity was observed, while the superoxide dismutase (SOD) and catalase (CAT) enzymes showed a significant increase. The application of Cu NPs induced a greater accumulation of bioactive compounds in tomato fruits.

Keywords: nanotechnology; Cu nanoparticles; antioxidant capacity; vitamin C; lycopene

1. Introduction

There is currently a strong interest in human food quality—specifically in foods' bioactive compound contents. These compounds may help to prevent chronic and degenerative diseases and generate positive effects to promote and restore the physiological functions of the human organism. Therefore, it is important to generate healthier and better food quality. The tomato fruit (*Solanum lycopersicum* L.) has been identified as a functional and nutraceutical food [1]. This fruit is an important source of bioactive compounds such as vitamins, carotenoids, and phenolic compounds, which produce a greater antioxidant activity that represents a direct benefit to human health [2]. Besides its importance to the human diet, it is the second most commercially consumed vegetable after the potato [3].

The currently available technology affords several approaches to increasing crop production and crop nutraceutical quality. One of these is the application of nanotechnology to improve

productivity [4,5]. In recent years, nanotechnology has been used by means of the application of nanomaterials such as nanofertilizers, nanoparticles, or nanopesticides for nutrient management, genetic improvement, plant disease treatment, and plant growth promotion [4]. Nanoparticles (NPs) and nanomaterials (NMs) are materials with at least one dimension less than 100 nm. This small size gives rise to properties different from those exhibited by the bulk of the material of the same composition. Indeed, for metallic elements such as Cu, Fe, Ce, Ti, and Ag, the cellular responses are very different when induced by ionic forms compared to the nanometric forms [6]. These new properties provide the material with an added value that has multiple applications in the automotive, energy, pharmaceutical, medical, and agricultural industries, among others [7]. These nanomaterials can induce a faster germination, or help plants tolerate biotic and abiotic stresses, favoring a more efficient management of nutrients and increasing plant growth while reducing the environmental impact in comparison with traditional methods [8]. Recently, the use of nanoparticles such as insecticides, fungicides, and nanofertilizers has been investigated [9]. It has been shown that the impact of NPs on plants depends on many factors, such as the composition, concentration, size, the physical and chemical properties, and even the plant species under study [10]. A concentration of NPs above the optimal ranges of Zn, Cu, Ag, Ce, and Ti, among others, produces stress and/or toxicity, generating reactive oxygen species (ROS) and resulting in the disruption of cellular metabolism. Under these conditions, plants produce antioxidant enzymes and non-enzymatic components that protect the cellular and subcellular system from ROS cytotoxic effects [11]. Cu nanoparticles (Cu NPs) have properties linked to their small size and high specific surface area which provides them with a physical resistance, chemical reactivity, electrical conductivity, magnetism, and optical effects [12]. Low doses of Cu NPs (5–20 mg Cu per plant) generate metabolic effects due to the Cu accumulation and ROS generation [13]. CuO NPs applied in *Arabidopsis thaliana* (L.) Heynh. increased the flavonoid content at concentrations of 5 mg L⁻¹. A significant induction of genes related to the responses to oxidative stress, sulfur assimilation, glutathione, and proline biosynthesis has also been shown under CuO NPs stress [14]. While the CuO NPs (0–200 mg L⁻¹) applied to the leaves in cucumber plants significantly reduced the firmness of the fruit [15], it has also been shown that when applied to the substrate, Cu NPs (0.006 mg L⁻¹) increased the total phenols and modified the concentration of the enzymatic and non-enzymatic compounds in tomato fruits [16]. Similar results were obtained in jalapeno peppers with Cu NPs + Chitosan-polyvinyl alcohol (Cs-PVA) (0–10 mg L⁻¹) applied on the substrate [17]. Through the application of Cu NPs, the production of antioxidant compounds (glutathione (GSH), vitamin C, and carotenoids) including antioxidant enzymes (ascorbate peroxidase (APX), superoxide dismutase (SOD), and catalase (CAT)) are activated in plants to reduce oxidative stress caused by ROS [18].

Considering the beneficial effects reported on the increase of bioactive compounds by the application of copper nanoparticles in other crops, the objective of this work was to determine the effect of the foliar application of Cu NPs in the accumulation of bioactive compounds in tomato fruits.

2. Materials and Methods

2.1. Crop Development

A “Huno F1” indeterminate growth tomato variety (Harris Moran, Davis, CA, USA) of the saladette type was used for this experiment. The transplant took place 36 days after sowing in black 10 L capacity polyethylene bags. The crop was established under greenhouse conditions, with temperatures of 35 °C/20 °C for day and night, relative humidity of 60%, and photosynthetically active radiation of 1400 μmol m⁻² s⁻¹. The crop was grown from a single stem and developed for 100 days from the transplant to obtain fruits for the evaluations. A substrate composed of a mixture of perlite–peat moss (1–1) was used with a directed irrigation system. The Steiner nutrient solution was used for crop nutrition [19] using the following micronutrients in chelated form using EDTA (2,2',2'',2'''-[Ethane-1,2-diyl dinitrilo] tetraacetic acid)/Fe EDTA = 3.75 mg L⁻¹; Mn EDTA =

1.85 mg L⁻¹; B = 0.35 mg L⁻¹; Zn EDTA = 0.30 mg L⁻¹; Cu EDTA = 0.15 mg L⁻¹; Mo = 0.10 mg L⁻¹, and the solution pH was adjusted to 6.5 with sulfuric acid each time it was prepared.

2.2. Application of Treatments

The treatments consisted of foliar applications of four different Cu NPs concentrations: 500, 250, 125, and 50 mg L⁻¹. Distilled water was applied to a Control. Two Cu NPs applications were carried out during the development of the crop. The first application was made 26 days after transplant (DAT) (flowering) and the second one at 46 DAT (fruit setting). In total, 30 mL of solution was applied per plant corresponding to 15, 7.5, 3.75, and 1.5 mg Cu NPs per plant in each treatment. The copper nanoparticles were synthesized in the Research Center for Applied Chemistry, located in Saltillo, Coahuila, México, following the methodology described by Ortega-Ortiz et al. [20]. The average size of the spherical Cu NPs was 50 nm.

2.3. Mineral Content in Fruit

The mineral content in tomato fruits was determined using a plasma emission spectrophotometer (ICP, Thermo Jarrel Ash Irish Advantage 14034000). The following elements were determined: K, Na, and Cu. For this process, one gram of each sample was digested with HNO₃ at 400 °C following the method of Hernández-Hernández et al. [21].

2.4. Fruit Quality

Uniformly sized fruits were collected at stage 6 (light red) of maturity according to the color visual scale of the USDA [22]. Six fruits per treatment were selected, each one from a different plant, and it was verified that they were not physically damaged and that they were uniform. These fruits were washed and used whole to perform the fruit quality analyses immediately. The hydrogen potential (pH) and electrical conductivity (EC) were determined using an HI 98130 digital potentiometer (Hanna Instruments). The total soluble solids (TSS) were measured in 10 mL of fruit pulps using a digital refractometer (ATAGO, MASTER-100H model, Bellevue, WA, USA). The fruit firmness was determined with a manual penetrometer (WAGNER INSTRUMENTS, FDK 20 model, Greenwich, CT, USA). For this, measurements were taken in three different points of the fruit and the average was obtained. The oxidation–reduction potential (ORP) was measured with a pH/oxidation–reduction potential potentiometer (Hanna Instruments, HI2211 model, Woonsocket, RI, USA). The titratable acidity (TA) was determined by the colorimetric technique according to the AOAC methodology [23] using 10 mL of fruit pulp with two drops of phenolphthalein (1%) added. It was titrated with NaOH (0.1 N) and the data were expressed as a percentage of citric acid.

2.5. Bioactive Compounds

For this, uniformly sized fruits were collected at stage 6 (light red) of maturity according to the color visual scale of USDA [22]. The fruits were selected, two per plant, verifying that they were not physically damaged and that they were uniform. These fruits were washed. Six fruits were used whole to perform the determinations in fresh tissue immediately, and six fruits were frozen at –80 °C and then lyophilized for 72 h at –84 °C and 0.060 mbar in a freeze dryer (Labconco, FreeZone 2.5 L model, Kansas City, MO, USA). These samples were ground to a fine powder to perform the determinations in lyophilized tissue.

The vitamin C content was determined by the titration method with 2,6-dichlorophenolindophenol, using 1 g of fresh tissue and HCl (2%) [24]. The lycopene content was quantified according to the methodology of Fish et al. [25] using 1 g of fresh tissue and hexane:acetone (3:2).

The total phenols were determined according to the methodology of Singleton et al. [26]. The sample (0.2 g) was extracted with 1 mL of a water:acetone solution (1:1). The mixture was vortexed for 30 s. The tubes were centrifuged (Thermo Scientific Mod. ST 16R centrifuge, Langensfeld, Germany) at 17,500 × g for 10 min at 4 °C. In a test tube, 50 µL of the supernatant, 200 µL of the

Folin–Ciocalteu reagent, 500 μL of 20% sodium carbonate (Na_2CO_3), and 5 mL of distilled water were added and then vortexed for 30 s. The samples were placed in a water bath at 45 °C for 30 min. Finally, the reading was taken at an absorbance of 750 nm using a plastic cell in a UV-Vis spectrophotometer (Thermo Fisher Scientific, G10S model, Waltham, MA, USA). The results were expressed in equivalent milligrams of gallic acid per 100 g dry weight ($\text{mg EGA } 100 \text{ g}^{-1} \text{ DW}$).

The flavonoids quantification was carried out using Dowd's method adapted by Arvouet-Grand et al. [27]. For the extraction, 100 mg of lyophilized tissue was placed in a test tube, where 10 mL of reagent grade methanol was added and shaken for 30 s until the mixture was homogenized. The mixture was filtered using No. 1 Whatman paper. For the quantification, 2 mL of the extract and 2 mL of methanolic solution of aluminum trichloride (AlCl_3) 2% were added in a test tube and left to rest for 20 min in the dark. The reading was then taken in a UV-Vis spectrophotometer (Thermo Fisher Scientific, G10S model, MA, USA) at a wavelength of 415 nm using a quartz cell. The flavonoid content was expressed in equivalent milligrams of quercetin per 100 g of dry weight ($\text{mg EQ } 100 \text{ g}^{-1} \text{ DW}$).

The same extract was used to determine the total proteins [28], glutathione (GSH) [29], ascorbate peroxidase (APX) [30], glutathione peroxidase (GPX) [31], superoxide dismutase (SOD) (SOD Cayman 706002[®] kit [16]), catalase (CAT) [32], and the antioxidant capacity by 2,2'-azino-bis(3-ethylbenzthiazolin-6-sulfonic acid) (ABTS) [33]. For this, 200 mg of lyophilized tissue was placed in a 2 mL Eppendorf tube. Then, 20 mg of polyvinylpyrrolidone and 1.5 mL of phosphate buffer were added.

The quantification of total proteins was determined using Bradford's colorimetric technique, [28]. In a microplate, 5 μL of the extract and 250 μL of Bradford reagent were placed in each well. They were incubated for 10 min at room temperature (26 °C) and then read at a wavelength of 630 nm on a microplate reader (BioTek, ELx808 model, Winooski, VT, USA). The total proteins were expressed in mg g^{-1} of DW.

Glutathione quantification was performed using the spectrophotometric technique by Xue et al. [29], by means of a 5,5-dithio-bis-2 nitrobenzoic acid (DTNB) reaction. A mix of 0.480 mL of the extract, 2.2 mL of sodium dibasic phosphate (Na_2HPO_4 at 0.32 M), and 0.32 mL of the DTNB dye (1 mM) were placed in a test tube. Then, the mix was vortexed and read on a UV-Vis spectrophotometer (Thermo Fisher Scientific, G10S model, MA, USA) at 412 nm using a quartz cell. The results were expressed in mM equivalent of GSH $\text{mL}^{-1} \text{ min}^{-1} \text{ total protein}^{-1}$.

The measurement of the enzymatic activity of ascorbate peroxidase was carried out according to what was established by Nakano and Asada [30]. The measurement was made in two moments (at time 0 (T0) and at time 1 (T1)). At T0, a mix of 100 μL of extract, 500 μL of ascorbate (10 mg L^{-1}), 400 μL of H_2SO_4 (5%), and 1 mL of H_2O_2 (100 mM) were placed in a test tube and then vortexed for 30 s. The absorbance was measured in a UV-Vis spectrophotometer (Thermo Fisher Scientific, G10S model, MA, USA) at 266 nm with a quartz cell. At T1, 100 μL of extract, 500 μL of ascorbate (10 mg L^{-1}), and 1 mL of H_2O_2 (100 mM) were added to the previous mixture and vortexed for 1 min at a temperature of 26 °C. To stop the reaction, 400 μL of H_2SO_4 (5%) was added, and the absorbance was measured. Ascorbate peroxidase determination is based on the quantification of the ascorbate oxidation rate by means of the absorbance difference (T0 - T1). The enzymatic activity was expressed as U per total proteins (mg g^{-1}), where U is equal to $\mu\text{mol QE}$ of oxidized ascorbate per milliliter per minute.

The glutathione peroxidase (QE 1.11.1.9) enzyme was determined with the method modified by Flohé and Günzler [31] and adapted by Xue et al. [29] using H_2O_2 as the substrate. A mix of 200 μL of extract, 400 μL of GSH (0.1 mM), and 200 μL of Na_2HPO_4 (0.067 M) were placed in a test tube. The mixture was preheated in a water bath at 25 °C for 5 min, then 200 μL of H_2O_2 (1.3 mM) was added to start the catalytic reaction for 10 min at a temperature of 26 °C. The reaction was stopped by the addition of 1 mL of 1% trichloroacetic acid. The mixture was placed in an ice bath for 30 min and then centrifuged at $1008 \times g$ for 10 min at 4 °C. To assess the glutathione peroxidase, 480 μL of the supernatant, 2.2 mL of Na_2HPO_4 (0.32 M), and 320 μL of 5,5-dithio-bis-2-nitrobenzoic acid dye (DTNB) of 1 mM were placed in a test tube. The absorbance was measured by a UV-Vis spectrophotometer

(Thermo Fisher Scientific, G10S model, MA, USA) at 412 nm with a quartz cell. The results are expressed in U per total proteins (mg g^{-1}), where U is equal to mM equivalent of GSH per milliliter per minute.

The determination of superoxide dismutase (QE 1.15.1.1) enzymatic activity was carried out using the SOD Cayman 706002[®] kit. A mix of 20 μL of extract, 200 μL of the radical detector (tetrazolium salt), and 20 μL of xanthine oxidase solution were placed in a microplate. The microplate was covered with a transparent cover (kit), stirred for 10 s, and then incubated at 26 °C for 30 min. The absorbance was then measured at a length of 450 nm using a plate reader (BioTek, ELx808 model, VT, USA). The principle of the test is based on the use of a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical. The results are expressed in U mL^{-1} by the total proteins (mg g^{-1}).

The catalase (QE 1.11.1.6) enzymatic activity was quantified by the spectrophotometric method used by Dhindsa et al. [32]. The measurement was carried out in two steps (at time 0 (T0) and at time 1 (T1)). At T0, 100 μL of extract, 400 μL of H_2SO_4 (5%), and 1 mL of H_2O_2 (100 mM) were added to an Eppendorf tube and vortexed for 30 s. The absorbance was then measured on a UV-Vis spectrophotometer (Thermo Fisher Scientific, G10S model, MA, USA) with a quartz cell at 270 nm. At T1, 100 μL of extract and 1 mL of H_2O_2 (100 μL) were added and stirred for 1 min in a vortex at 26 °C. Then, 400 μL of H_2SO_4 (5%) was added to stop the reaction and the absorbance was measured by a UV-Vis spectrophotometer (Thermo Scientific Model G10S) with a quartz cell at 270 nm. The determination of catalase is based on the quantification of the oxidation rate of H_2O_2 by absorbance difference (T0–T1). The values were expressed in U per total proteins (mg g^{-1}), where U is equal to mM equivalent of H_2O_2 consumed per milliliter per minute.

The antioxidant activity by ABTS (2,2'-azino-bis(3-ethylbenzthiazolin-6-sulfonic acid)) was determined by the spectrophotometric method of Re et al. [33], which is based on the discoloration of the ABTS radical cation. This radical was obtained from the reaction of ABTS at 7 mM with potassium persulfate at 2.45 mM (1:1) in the dark at 26 °C during 16 h and then diluted with 20% ethanol to obtain an absorbance of 0.7 ± 0.01 at 750 nm. Afterwards, 5 μL of enzyme extract and 245 μL of the ABTS radical dilution (7 mM) were placed in a microplate and stirred for 5 s and then allowed to stand for 7 min in darkness. The absorbance was measured by a plate reader (BioTek, ELx808 model, VT, USA) at a wavelength of 750 nm. The blank was prepared with 250 μL of phosphate buffer (pH 7.0–7.2, 0.1 M). Two calibration curves were prepared, one with ascorbate (0–0.2 mg mL^{-1}) and the other with Trolox (0–1 mM). The results were expressed as mM equivalent of ascorbic acid 100 g^{-1} DW and mg equivalent of Trolox 100 g^{-1} DW, respectively.

2.6. Statistical Analysis

Six replicates per treatment were considered for each of the evaluated variables in a completely random design. Each replicate was obtained from one different plant. The analysis of variance and Fisher Least Significant Difference (LSD) mean test ($p \leq 0.05$) were performed using the software Infostat 2016 (<http://www.infostat.com.ar>).

3. Results and Discussion

3.1. Mineral Content in Fruit

Significant differences were observed in the K and Na contents (Table 1). There were no statistically significant differences in the Cu content between treatments. This indicates that the application of Cu NPs did not necessarily generate an accumulation of Cu in the fruits. This may be due to the fact that Cu NPs can penetrate tissues and move through the phloem to other organs [34]. In this study where the application was foliar, it is most likely that the NPs were accumulated in the leaves. Considering

this, the application of NPs did not necessarily produce an accumulation of these in the tomato fruits, and thus there is no risk when consuming the fruits.

Table 1. The mineral content in tomato fruits with copper nanoparticles (Cu NPs) application.

Treatment	K (g kg ⁻¹ DW)	Na (g kg ⁻¹ DW)	Cu (mg kg ⁻¹ DW)
500 mg L ⁻¹ Cu NPs	21.21 a	1.22 b	26.59 a
250 mg L ⁻¹ Cu NPs	20.88 a	0.88 b	35.17 a
125 mg L ⁻¹ Cu NPs	21.05 a	0.98 b	12.97 a
50 mg L ⁻¹ Cu NPs	20.27 ab	1.08 b	11.70 a
Control	18.31 b	2.16 a	48.78 a
CV (%)	6.13	17.17	144.69

Each unit of data is the average of six replicates. DW: dry weight. CV: coefficient of variation. Different letters per column indicate the significant differences between treatments according to the LSD Fisher test ($p \leq 0.05$).

Regarding potassium, an accumulation was observed in all treatments with the application of Cu NPs. This is a positive result because K is fundamental in the quality of the fruits, improving characteristics such as firmness, sugar content, vitamin C content, and other antioxidant contents [35]. Therefore, a greater accumulation of K in fruits can potentially result in higher-quality fruits. The sodium content was lower in all of the treatments in which Cu NPs were applied. This may be due to a greater accumulation of K. In any case, it is a positive result, since a higher K/Na ratio is beneficial for the plant [36].

3.2. Fruit Quality

The results of tomato fruit quality (firmness, pH, electrical conductivity (CE), Total soluble solids (TSS), Titratable Acidity (TA), and ORP) showed statistical differences in all the evaluated variables (Table 2). The fruit firmness increased with all treatments of Cu NPs (500, 250, 125, and 50 mg L⁻¹) with respect to the Control. The treatment with 250 mg L⁻¹ of Cu NPs increased the firmness by 28.90%, and the treatment with 125 mg L⁻¹ Cu NPs produced a 23.62% increase in the firmness with respect to the Control. The other two treatments (500 and 50 mg L⁻¹) showed an increase of 20.10% and 18.85%, respectively. Juárez-Maldonado et al. [16] reported that the application of Cu NPs + chitosan increased the firmness of tomato fruits by 9%. This is consistent with the results shown in this study. It has been shown that cell wall lignification occurs in plants with copper stress [37]. Additionally, there is a relationship between the accumulation of copper in the cell wall and the responses of lignifying enzymes such as phenylalanine ammonia lyase (PAL). This response catalyzes the transformation of L-phenylalanine to cinnamic acid, which is the first step of the phenylpropanoid pathway leading to the synthesis of lignin [37,38]. Nair and Chung [14] observed root lignification when CuO NPs were applied, and mentioned that NPs could have translocated through the vascular tissues, and their subsequent dissolution to Cu ions could have given rise to the observed lignification. Thus, the increase in the firmness of tomato fruits may be the result of an increase in the pericarp cell walls' lignification due to Cu NPs application.

The fruit pH in the treatments of 125 and 50 mg L⁻¹ Cu NPs, were 2.50 and 2.94% higher than that of the Control (Table 2). This agreed with Juárez-Maldonado et al. [16], who registered a pH increase of 2.79% in tomato fruits with Cu NPs + chitosan application. However, this differs from Pinedo-Guerrero et al. [17], who reported a pH decrease with the application of Cu NPs + Cs-PVA in jalapeño pepper. The pH range in tomatoes is normally found between 4.0 and 4.5. Fruit quality is highly influenced by pH, and less-acidic fruits are more appreciated by consumers for their better flavor feature [39,40]. The increase in fruit pH is due to the transformation of the organic acids in the vacuoles of the vegetables into simple sugars that are later used in cellular respiration to obtain energy. This causes a decrease in the acidity, and therefore an increase in pH [41]. The results seem to

indicate that the application of Cu NPs induced the transformation of organic acids to simple sugars in tomato fruits.

Table 2. The effect of the application of Cu NPs on tomato fruit quality.

Treatment	Firmness (kg cm ⁻²)	pH	EC (mS cm ⁻¹)	TSS (°Brix)	TA (% citric acid)	ORP (mV)
500 mg L ⁻¹ Cu NPs	4.78 a	4.37 ab	3.60 ab	5.10 b	0.53 a	165.20 a
250 mg L ⁻¹ Cu NPs	5.13 a	4.35 ab	3.78 a	5.25 ab	0.51 a	157.33 b
125 mg L ⁻¹ Cu NPs	4.92 a	4.41 a	3.42 bc	5.30 ab	0.41 b	165.00 a
50 mg L ⁻¹ Cu NPs	4.73 a	4.43 a	3.59 ab	5.22 ab	0.50 a	164.17 a
Control	3.98 b	4.30 b	3.22 c	5.40 a	0.49 a	166.83 a
CV (%)	12.83	1.81	6.69	3.45	8.43	2.27

Each unit of data is the average of six replicates. CV: coefficient of variation. Different letters per column indicate significant differences between treatments according to the LSD Fisher test ($p \leq 0.05$). EC: electrical conductivity. TSS: total soluble solids. TA: titratable acidity. ORP: oxidation–reduction potential.

The application of Cu NPs increased the EC of tomato fruits, indicating a higher salt content (Table 2). The lowest values for EC were observed in the Control fruits, while fruits in a treatment with 250 mg L⁻¹ Cu NPs showed increases in the EC by 17.39% (i.e., 3.78 mS cm⁻¹). This can be attributed to the increase in K, since this element is one of the most important inorganic solutes in plants [36].

The TSS content of the tomato fruits also showed significant differences between treatments (Table 2). The Control presented the highest value with 5.4° Brix, surpassing the 500 mg L⁻¹ of Cu NPs treatment, which presented a decrease of 5.55%. Juárez-Maldonado et al. [16] reported that the application of Cu NPs + chitosan did not generate differences in the TSS in tomato fruits. The decrease in TSS can be caused by the use of simple sugars in cellular respiration [41].

A decrease in the titratable acidity (TA) was observed in the 125 mg L⁻¹ Cu NPs treatment. With respect to the Control, it decreased by 16.33% (Table 2). Juárez-Maldonado et al. [16] reported an increase in the TA in tomato fruits with the application of Cu NPs + chitosan. Pinedo-Guerrero et al. [17] reported that the application of 0.2 and 2.0 mg of Cu NPs increased the TA in jalapeño peppers by 25 and 35.42%, respectively. Oms-Oliu et al. [42] mentioned that the accumulation of citric acid is due to the decrease of malic and fumaric acids derived from changes in glycolysis and the Krebs cycle since they are the predominant carbon fluxes during the fruit ripening process. An increase in respiration during maturation, characteristic of the climacteric fruit, will determine the increase in the content of some intermediate products of the Krebs cycle. Therefore, it is likely that the application of Cu NPs influenced the respiration of the fruit, which translates into a delay in the maturation and, as a consequence, a decrease in the titratable acidity. However, this effect depends on the dose, as well as on the species under study.

With respect to the oxidation–reduction potential (ORP), the 250 mg L⁻¹ of Cu NPs treatment was 5.7% lower than that of the Control. Juárez-Maldonado et al. [16] reported a decrease in ORP with the application of Cu NPs + chitosan. An ORP with low values indicates better fruit quality, which can be translated into greater antioxidant potential [16]. This indicates a beneficial effect of the Cu NPs application (250 mg L⁻¹) on fruit quality.

3.3. Bioactive Compounds

Statistically significant differences were found for all non-enzymatic antioxidant compounds evaluated in tomato fruits, observing that at least one treatment with Cu NPs surpassed the Control (Table 3). The content of total proteins was positively affected by the application of Cu NPs. An increase of 99.74% was observed with the application of 50 mg L⁻¹ of Cu NPs compared to the Control. However, as the dose increased, a decrease in protein content was consistently observed, and no effects were observed at a Cu NPs concentration of 500 mg L⁻¹. This may be related to the plant's

response to different types of stress, including the alteration of the protein gene expression. The stress caused by nanoparticles can produce ROS, in turn modifying the activity of antioxidant enzymes, some transcription factors, and other proteins involved in the response to stress [43,44]. ROS have a double role that depends on duration, site, and concentration. A low concentration acts as a signal and generates a stress response in plants, while a higher concentration interrupts cellular homeostasis which produces oxidative damage to proteins, DNA, and lipids [45]. This explains the observed results, where the total proteins increased when Cu NPs were applied in concentrations of 50, 125, and 250 mg L⁻¹, in opposition to the concentration of 500 mg L⁻¹.

Table 3. The non-enzymatic antioxidant compounds in tomato fruits with the application of Cu NPs.

Treatment	T Prot	Vit C	Lyc	GSH	T Phen	Flav	ABTS (t)	ABTS (aa)
500 mg L ⁻¹ Cu NPs	8.66 c	6.34 c	3.17 a	2.68 a	306.24 ab	64.08 e	7.44 ab	1.46 ab
250 mg L ⁻¹ Cu NPs	13.88 b	10.41 a	3.51 a	1.55 b	326.19 a	84.71 b	7.17 bc	1.41 bc
125 mg L ⁻¹ Cu NPs	13.65 b	8.80 b	3.01 a	1.91 b	264.68 c	80.77 c	7.55 a	1.48 a
50 mg L ⁻¹ Cu NPs	15.34 a	6.45 c	3.96 a	1.87 b	276.41 bc	99.67 a	7.67 a	1.50 a
Control	7.68 c	4.69 d	1.92 b	1.97 b	309.37 b	73.21 d	7.09 c	1.40 c
CV (%)	9.17	12.49	25.30	22.15	9.03	3.70	3.37	3.07

Each unit of data is the average of six replicates. CV: coefficient of variation. Different letters per column indicate significant differences between treatments according to the LSD Fisher test ($p \leq 0.05$). T Prot: total proteins, expressed as mg g⁻¹ of dry weight. Vit C: vitamin C as mg 100 g⁻¹ Fresh Weight (FW). Lyc: lycopene as µg 100 g⁻¹ FW. GSH: glutathione as mM equivalent of GSH mL⁻¹ min⁻¹ total protein⁻¹. T Phen: total phenols as the mg equivalent of gallic acid 100 g⁻¹ of dry weight. Flav: flavonoids as mg equivalent quercetin 100 g⁻¹ of dry weight. ABTS: 2,2'-azinobis(3-ethylbenzthiazolin-6-sulfonic acid). t: mg equivalent Trolox 100 g⁻¹ dry weight. aa: mM equivalent ascorbic acid 100 g⁻¹ dry weight.

The vitamin C content in tomato fruits significantly increased in all treatments. The highest value of vitamin C was found in fruits from plants treated with 250 mg L⁻¹ of Cu NPs, resulting in a 121.97% increase (Table 3). The doses of 125, 50, and 500 mg L⁻¹ of Cu NPs showed increases of 87.64, 37.53, and 35.18%, respectively, compared to the Control. The results obtained showed that the application of Cu NPs significantly increased the vitamin C content, which in turn increased the nutraceutical quality of tomato fruits. This can be attributed to the induction of antioxidant compounds, reported as one of the functions of Cu NPs [17]. Vitamin C is essential in the human diet, and must be ingested from fruits rich in its content, since the human body cannot produce it [24]. In plants, vitamin C has many cellular functions, mostly linked to its ability to donate electrons [46]. Specifically, in tomato fruits, vitamin C is one of the most important compounds [3] which may act directly as an antioxidant by trapping reactive oxygen species (ROS), preventing or minimizing oxidative damage [47]. In addition, it can also regenerate the glycoprotein and tocopherol radicals, and act as a cofactor for many enzymes (e.g., APX) [2]. It plays an important role in photosynthesis as an enzymatic cofactor (including the synthesis of ethylene, gibberellins, flavonoids, and anthocyanins) [48]. Therefore, the greater accumulation of vitamin C will provide a plant with better characteristics and better fruit quality.

The fruit lycopene content increased significantly with the application of Cu NPs (Table 3). The application of 50 mg L⁻¹ of Cu NPs increased it by 105.26%, 250 mg L⁻¹ of Cu NPs increased it by 61.24%, 500 mg L⁻¹ of Cu NPs increased it by 65.1%, and 125 mg L⁻¹ of Cu NPs increased it by 56.77% compared to the Control. Similar results were obtained in tomato fruits when applying Cu NPs + chitosan, where an increase of 12% was reported compared to the Control [16]. Likewise, the foliar application of NPs of TiO₂ and ZnO (0–1000 mg kg⁻¹) increased the lycopene content (80–113%) in tomato fruits [49]. As in the case of vitamin C, the increase in lycopene is directly related to the induction of antioxidant compounds by the application of Cu NPs [17]. In plants, lycopene synthesis is usually derived from the mevalonic acid (MVA) and methylerythritol phosphate (MEP) pathways. These pathways synthesize isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), which function as precursors to the synthesis of carotenoids [50]. Lycopene is one of the main carotenoids in tomato fruits, and it is also one of the main antioxidants [3,41]. Moreover, the antioxidant actions of carotenoids are based on their ability to quench singlet oxygen and to trap

peroxyl radicals [51–53]. Specifically, lycopene is one of the most efficient singlet oxygen neutralizers among the natural carotenoids [52]. The results indicate that the overproduction of these carotenoids is a response to oxidative stress caused by Cu NPs [54]. One of the functions of lycopene in plants is related to photo- and chemo-protection. As an antioxidant, lycopene counteracts the ROS [51]. The production of foods rich in lycopene is of utmost importance; therefore, the application of Cu NPs represents a viable option.

The GSH increased by 36.04% with the application of 500 mg L⁻¹, while the rest of the treatments equaled the Control (Table 3). This response can be derived from the stress caused by the applied nanoparticles [43,44]. This indicates that the highest dose of Cu NPs generated a certain degree of toxicity in tomato plants. This result is consistent with that observed in the protein content.

Regarding the total phenols content of fruits, treatment with the application of 250 mg L⁻¹ of Cu NPs generated an increase of 5.43% in relation to the Control, while treatment with 125 mg L⁻¹ of Cu NPs showed a decrease of 14.44%. The rest of the treatments were similar to the Control (Table 3). Jalapeno pepper fruits showed an increase in the content of the total phenols (5.9%) when 2.0 mg of Cu NPs + Chitosan-PVA were applied [17]. Additionally, foliar application of nano-fertilizers of Zn and B increased the phenols content in pomegranate fruits [55]. Phenols are antioxidant compounds that trigger the synthesis of a series of secondary metabolites from the shikimic acid pathway or through phenylpropanoids under conditions of abiotic stress [56,57]. In tomato fruits, the main flavonoid compounds are rutin, naringenin, chalcone naringenin, quercetin, hydroxycinnamic, chlorogenic, and caffeic acids [3]. Their function as antioxidants in fruits is due to the reduction of free radical levels in cells [41,58]. Therefore, the observed response may be related to the ROS formation due to Cu NPs. In addition, an accumulation of this type of compound is beneficial for fruit quality.

The flavonoid content in fruits also showed statistically significant differences between treatments (Table 3). The highest content of flavonoids in the tomato fruits was obtained with the application of 50 mg L⁻¹ of Cu NPs, exceeding the Control by 36.14%. Additionally, fruits of treatments with 250 and 125 mg L⁻¹ of Cu NPs presented a flavonoid content that surpassed the Control by 15.71% and 10.33%, respectively. However, there was a clear decreasing tendency in the flavonoid content as the Cu NPs dose increased. When 500 mg L⁻¹ of Cu NPs was applied, it was observed that the flavonoid content in fruits decreased by 12.48% compared to the Control. These results agree with those observed in jalapeño pepper fruits, where the flavonoid content increased 13% with Cu NPs + Chitosan-PVA [17]. In addition, in *A. Thaliana*, the concentration of anthocyanins increased when CuO nanoparticles were applied [59]. These metabolites act as antioxidants, protecting plants from oxidative stress by the elimination of H₂O₂ and singlet oxygen generated under biotic or abiotic stress [60,61]. Therefore, the observed increase in flavonoids content is probably related to oxidative stress caused by Cu NPs [62].

The results obtained clearly indicate that the application of Cu NPs induced a higher content of non-enzymatic antioxidant compounds in tomato fruits. This is consistent with the increase in the antioxidant capacity observed in fruits with treatments containing 50 and 125 mg L⁻¹ of Cu NPs (Table 3)—increases of 8.18% and 6.49%, respectively, in the ABTS antioxidant capacity, were observed compared to the Control. García et al. [63] reported that the application of ZnO NPs to the soil (3, 20, and 225 mg kg⁻¹) in bean and tomato crops stimulated the antioxidant capacity considerably. Moreover, the results indicated that Cu NPs application can generate certain stress in tomato plants. This produced an increase in ROS, which activates a defense mechanism [54,62], increasing the production of antioxidant compounds such as ascorbate, glutathione, carotenoids, and flavonoids, among others [64]. The same response has been observed under conditions of mild stress caused by various NPs. However, the response changes when high doses are applied due to an overproduction of ROS, resulting in a decrease of antioxidants [62]. In the present study, the results indicated that the foliar application of 500 mg L⁻¹ of Cu NPs had a slightly adverse effect. On the other hand, lower doses generally showed positive effects in the content of the different non-enzymatic antioxidant compounds (Table 3).

Regarding the enzymatic antioxidant compounds in tomato fruits, statistically significant differences were observed in all evaluated enzymes (Table 4). The fruits from the Control showed the highest values of APX and GPX enzymes. Regarding the SOD and CAT enzymes, an increase was observed with at least one Cu NPs treatment compared to the Control. The APX enzyme showed a similar effect, since the 250 mg L⁻¹ of Cu NPs treatment generated the lowest value, 54.55% lower than the Control. APX is an enzyme that removes H₂O₂ using ascorbate as an electron donor to reduce H₂O₂ to water, and it is extremely sensitive to ascorbate concentrations [65]. A decrease in the concentration of APX leads to a higher ascorbate content [66], as observed in Tables 3 and 4. APX plays a central role in the ascorbate–glutathione cycle; this is why the induction of APX is a response to oxidative stress caused in plants by biotic or abiotic stress [67]. The concentration of GPX also decreased with the application of Cu NPs in all concentrations applied. Juárez-Maldonado et al. [16] reported that the application of Cu NPs + chitosan in tomato plants showed no differences between the treatments in fruit GPX content, which was attributed to low concentrations used in that study. A decrease in enzymatic antioxidant activity in fruits could be due to the inactivation of more enzymes due to the toxic effects of ROS [68]. This is possible because Cu NPs induce oxidative stress [62]. In addition, due to its redox activity at concentrations higher than the physiological level, Cu can catalyze the overproduction of ROS, causing an oxidative stress [69]. The previous results suggest that the foliar application of Cu NPs in the concentrations shown in this study had a negative effect on APX and GPX enzymes in the tomato fruits, since they decreased their activity in all cases.

Table 4. The enzymatic antioxidant compounds in tomato fruits with the application of Cu NPs.

Treatment	APX	GPX	SOD	CAT
500 mg L ⁻¹ Cu NPs	625.58 b	2.08 b	1.67 d	1927.5 a
250 mg L ⁻¹ Cu NPs	329.69 e	1.28 c	8.44 b	1735.1 a
125 mg L ⁻¹ Cu NPs	427.23 c	1.28 c	16.31 a	1575.7 a
50 mg L ⁻¹ Cu NPs	369.25 d	1.36 c	4.52 c	808.46 b
Control	725.28 a	2.54 a	4.53 c	618.00 b
CV (%)	5.93	17.21	9.71	21.42

Each unit of data is the average of six replicates. CV: coefficient of variation. Different letters per column indicate significant differences between treatments according to the LSD Fisher test ($p \leq 0.05$). APX: ascorbate peroxidase (U total proteins⁻¹ (mg g⁻¹)), GPX: glutathione peroxidase (U total proteins⁻¹ (mg g⁻¹)), SOD: superoxide dismutase (U total proteins⁻¹ (mg g⁻¹)), CAT: enzymatic activity of catalase (U total proteins⁻¹ (mg g⁻¹)).

Conversely, SOD activity increased significantly in tomato fruits (Table 4). The treatments with the application of Cu NPs in 125 and 250 mg L⁻¹ doses presented an increase of 260% and 86%, respectively, in comparison to the Control. This is because SOD acts as the first line of defense against ROS [45,70] generated by stress caused by Cu NPs. In addition, the SOD activity is also induced by metal ions such as Cu, or by the increase in singlet oxygen levels [71]. However, the dose of 500 mg L⁻¹ of Cu NPs presented a decrease of 63.14%. This may be due to the stress caused by the application of Cu NPs that exceeded the response capacity of the plant, causing the observed effect [62]. The CAT enzymatic activity also increased significantly with the application of Cu NPs. The application of 500, 250, and 125 mg L⁻¹ generated an increase in CAT activity by 211%, 180%, and 155%, respectively, compared to the Control. These results indicate an increasing tendency in the CAT activity as the concentration of Cu NPs in foliar application increased. CAT is one of the enzymes that protects cells against ROS since it converts H₂O₂ into H₂O and O₂ [72,73]. Therefore, its activity is directly related to the stress caused by the application of Cu NPs [62]. An increase in CAT activity is dependent on the concentration of H₂O₂ [70]. Thus, there is a correlation between the components of the ROS elimination systems (e.g., CAT and SOD) [74]. In tomato fruits, it has been shown that under conditions of high-temperature stress, the CAT enzymatic activity increases [73]. Similar results were obtained in tomato leaves [75]. Therefore, the increase in CAT activity has a positive response in fruits.

4. Conclusions

The application of Cu NPs increased the firmness of tomato fruits, and so the shelf life of the tomato fruits could potentially be increased. Therefore, the quality of the tomato fruits could be maintained for a longer time.

The application of Cu NPs induces the accumulation of bioactive compounds such as vitamin C, lycopene, total phenols, and flavonoids in tomato fruits. All treatments with foliar application of Cu NPs increased the content of Vitamin C and lycopene in tomato fruits and induced greater ABTS antioxidant capacity.

Likewise, it increased the activity of the main enzymes (CAT and SOD) responsible for eliminating ROS. Therefore, with the application of Cu NPs, better-quality tomato fruits are produced for the human diet due to the accumulation of bioactive compounds.

Considering the results obtained in Vitamin C and the total phenols, a treatment of 250 mg L⁻¹ NPs Cu is the best. However, the application of 500 mg L⁻¹ NPs Cu is not recommended, since in some cases it induced negative effects in the content of bioactive compounds or simply did not have an effect.

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