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# Cu Nanoparticles absorbed on chitosan hydrogels positively alter morphological, production, and quality characteristics of tomato

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## Summary

Use of nanoparticles as nano copper (nCu) may be useful in agriculture. The objective of the present study was to evaluate responses in plant growth and antioxidants in tomato fruits upon application of nCu absorbed on chitosan hydrogels. The study was performed in two stages. The first stage, with tomato seedlings, was done to determine the most appropriate nCu concentration. nCu was absorbed on a chitosan hydrogel at 100 mg nCu $kg^{-1}$ of hydrogel, and five different treatments of hydrogel were applied to the substrate prior to transplantation: 0.3, 0.15, 0.06, 0.03 and 0.015 g $L^{-1}$, plus a control. The second stage evaluated the best treatment results from the previous stage, a chitosan treatment without nCu and a control. Effects of the treatments on antioxidants in the leaves and fruit were evaluated, along with fruit quality. The results from the first stage demonstrated that 0.06 g $L^{-1}$ nCu-chitosan hydrogel treatments had better results. Outcomes from the second stage demonstrated that treatment with nCu had the best results for most of the plant growth variables, with differences in catalase activity in the leaves and lycopene concentration in the fruit. Application of chitosan hydrogels with nCu was favorable to tomato growth and quality.

## Introduction

The use of nanoparticles (NPs) on a scale from 1 to 100 nm is increasingly common for a variety of clinical and commercial purposes (Somasundaran et al., 2010). NPs are also used in agriculture and the food industry. Compared to bulk materials, NPs exert effects on organisms at very low concentration thresholds, interact with membrane transport proteins and, because of their small size, are able to enter the cell cytoplasm directly through the membrane (Adhikari et al., 2013). Despite these potentially beneficial features, the use of nanotechnology in agriculture is still limited due to the lack of information on nanomaterial or nanoparticle toxicity (Narayanan et al., 2013). Plants need metals like Fe, Zn, Mn, and Cu, to develop biochemical redox reactions for the activation of enzymes and proteins and their cofactors and gas transport, such as $CO_2$ and $O_2$ (Epstein and Bloom, 2005). Because the availability of transition metals in soils is highly variable, plants have developed different mechanisms for their capture, transport and assimilation. The uptake of these metals usually occurs in the roots, where they are then transported to the rest of the plant. Some of these elements can be found stored in ionic form in the cell vacuole but are usually complexed with organic acids, in non-charged metallic nanostructured particles or in specialized proteins for storage, such as ferritin (Lobréaux et al., 1992). The concentrations of transition metals at sites of absorption, transport and storage are under very fine control, as even in small concentrations, these metal ions cause oxidative damage through Fenton reactions in membranes, proteins, and nucleic acids (Pastori and Foyer, 2002).

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Many studies have been reported where toxic effects of NPs of different elements (Musante and White, 2012; Song et al., 2013) are verified. However it is known that the toxic effect depends on the concentration; the NPs in low concentration exert stimulatory effects of plant growth (Iavicoli et al., 2014). $SiO_2$ NPs promote the activities of superoxide dismutase and indoleacetic acid in cotton plants, being present in the root and transported to the rest of the plant by the xylem (Nhan et al., 2014). In rice, the Si and Mo NPs performed best at concentrations of 40 and 5 mg $L^{-1}$, respectively (Adhikari et al., 2013); in soybeans and chickpeas, it was observed that the optimum concentration of nCu to promote growth was between 60 and 100 mg $L^{-1}$ (Adhikari et al., 2012). However, growth was inhibited beyond these concentrations, attributed to the excessive accumulation of NPs in the roots. Recently, several studies have demonstrated that Cu NPs (nCu) are absorbed by plants and accumulate in the cells of roots, leaves and other plant tissues (Shi et al., 2014). During this process, the activity of some enzymes can increase, such as catalase, or decrease, such as ascorbate peroxidase (Trujillo-Reyes et al., 2014). It is believed that the stimulatory effects of Cu Nps relate to the induction of antioxidant activity (Fu et al., 2014). Thus, it would be interesting verify the effect of nCu in the tomato fruit which is consumed by its nutraceutical value (Shalaby and El-Banna, 2013). However, this requires a system that controls the nCu concentration in the medium.

Hydrogel is a gel-type polymer network that is water-insoluble due to its extensive cross-linking; it has significant water retention capacity, which is attributed to the presence of several functional groups (e.g., amino, carboxyl, amide, hydroxyl and sulfonic acid) in the polymers of the hydrogel network (Peppas and Khare, 1993). These hydrogels are used in pharmaceutical products (Singh et al., 2010; Kashyap et al., 2005), agriculture (Rudzinski et al., 2002), biomedical applications (Kashyap et al., 2005), the separation of biomolecules or cells (Wang et al., 2010), and biosensors (Krsko et al., 2009). Chitosan is a linear polysaccharide with good chemical functionality in hydrogel synthesis due to the greater crosslinking capacity and presence of a large number of amino groups ($-NH_2$) (Ravi-Kumar, 2000). Chitosan is a natural biodegradable polymer that is extracted from the shells of crustaceans, such as crabs and shrimp (Bautista et al., 2006). Several studies have shown that chitosan has antimicrobial properties (El-Hadrami et al., 2010), promotes changes in gene expression and increases flower production of orchids (Limpanavech et al., 2008), and operates as a resistance inducer (Khan et al., 2003; El-Hadrami et al., 2010). Chitosan hydrogels are prepared with the aim of encapsulating substances to produce dosing systems with applications in industry, agriculture, medicine, pharmacy and biotechnology. In the case of NPs, the use of hydrogels allows a greater control of the release of the materials in soils and substrates, with relatively little restriction for root exploration. To date, there are no reports on the agricultural use of nCu encapsulated in chitosan hydrogels, which could be advantageous from the standpoint of controlling release of NPs to the soil.

The objective of the present study was to evaluate different concentrations of nCu absorbed in chitosan hydrogels on tomato plants to known their effects on plant growth characteristics and antioxidant compound induction in the fruits.

## Materials and methods

This study was conducted in a hood-type greenhouse with a polycarbonate cover located in the Universidad Autónoma Agraria Antonio Narro, located in Saltillo, Mexico (25°21'5" Latitude North and 101°1'47" Longitude West, Elevation 1742 masl). The study was conducted in two stages that are described below. The nCu used in this study were synthesized at the Centro de Investigación en Química Aplicada (CIQA) using the methodology of Cadenas-Pliego et al. (2013). The chitosan hydrogels were also synthesized at the CIQA from Marine Chemicals brand chitosan (Mv=200,000) using cross-linked glutaraldehyde to obtain a hydrogel with a water absorptive capacity of 300 % based on its weight.

### Stage 1. Preliminary study to determine the range of concentrations of nCu suitable for application to the chitosan hydrogel substrate

In this first stage, seeds of the saladette tomato hybrid variety "Toro" with determinate growth were germinated in polystyrene trays. This variety was selected by its precocity and uniformity, also, because in the first stage it was not necessary the production of fruits. The seedlings developed for 30 days before being transplanted into 2 L pots with a mixture of peat moss and perlite 50:50 (v/v). Steiner nutrient solution was used (Steiner, 1961) with a targeted irrigation system to provide the nutrients required for growth. Microelements of the nutrient solution were applied in the form of chelates. Nutrient applications were adjusted by managing the concentration of Steiner solution according to the growth stage of the crop and its capacity of nutrient uptake: 25 % of concentration at two weeks after transplantation, 50 % of concentration at weeks 3 and 4, 75 % of concentration at weeks 5 and 6, and 100 % of concentration from week 7. This management was the same for the two experimental stages.

In the first stage, an exploratory study was conducted to determine the most suitable nCu concentration for plant growth, starting with a chitosan hydrogel with nCu absorbed at a concentration of 100 mg $kg^{-1}$. The five different treatments consisted of the application of the nCu-chitosan hydrogel substrate prior to transplantation: 0.3, 0.15, 0.06, 0.03 and 0.015 g $L^{-1}$, plus a control without application of nCu-chitosan hydrogel, which corresponded to 0.03, 0.015, 0.006, 0.003, 0.0015, and 0 mg nCu $L^{-1}$ of substrate. The treatments were prepared by placing the first 1 L of substrate followed with the nCu-chitosan hydrogel in the middle of each pot, and finally, the remaining substrate was added to complete the 2 L.

At 40 days after transplantation (dat), plant growth was measured using indices such as plant height, number of leaves, stem diameter and number of floral clusters. In addition, the abaxial stomatal conductance was measured in the third leaf using a Model SC-1 porometer (Decagon Devices, Inc.). Subsequently, the plants were collected, weighed and dried in a drying oven at 80 °C for 72 h to determine the biomass of the dry shoot (leaves and stems). These results were used to determine the range of the most suitable nCu concentration. The treatment with the best results was selected for evaluation in the second stage.

### Stage 2. Study of chitosan hydrogels with nCu applied to the substrate and their inductive capacity of antioxidants

Once the most suitable concentration of absorbed nCu applied to the chitosan hydrogel was determined in Stage 1, the treatment was evaluated in terms of its capacity to promote growth in adult plants and to induce antioxidant compounds in the fruits. For this assessment, a comparison was conducted with the same concentration of chitosan hydrogel but without any nCu content and with an absolute control without application of either chitosan hydrogel or nCu.

The same application system was used for the nCu absorbed on chitosan hydrogel as described for Stage 1, with the difference being that in this stage, 12 L pots were used for crop development. Hydrogels were applied in three parts, first by adding 3 L of substrate to the pot and then 0.33 g of hydrogel, repeating this process until the required 1 g of hydrogel and substrate was obtained. For the second stage, seeds from the ball-type tomato hybrid variety "Imperial" of indeterminate growth were used. This variety was selected by its uniformity and great production fruit capacity, also is commonly used under greenhouse conditions. The seeds were germinated in polystyrene trays and left to develop for 30 days before transplantation into 12 L pots.

For evaluating the effects of treatments, at 90 dat, the abaxial stomatal conductance and SPAD units were measured with a Model SC-1 porometer (Decagon Devices, Inc.) and a SPAD Minolta 502, respectively. At 120 dat, growth was measured using plant height, number of leaves, stem diameter, number of floral clusters, and fruit number and weight as indices. Subsequently, the plants were collected, weighed and dried in a drying oven at 80 °C for 72 h to determine the dry shoot biomass (leaves and stems).

In addition, the mineral contents (K, Ca, Mg, Fe, Cu and Zn) were measured in the different organs, including roots, leaves and fruits, using a plasma emission spectrophotometer (ICP; model Thermo Jarrel ASH). The plants were collected, dried in a drying oven at 80 °C for 72 h, ground and finally subjected to acid digestion according to the standard techniques of the AOAC (1990).

Quantification of total proteins and enzyme activity was conducted using the fresh tissue of leaves and fruits collected at 60 dat corresponding to first harvest of fruits. The samples were frozen at -20 °C and then lyophilized. Next, 200 mg of lyophilized tissue was ground in a mortar and placed in a microcentrifuge tube, to which 20 mg of polyvinylpyrrolidone and 1.5 mL of phosphate buffer pH 7-7.2 (0.1 M) were added. The extract obtained was centrifuged at 12,000 rpm for 10 minutes at 4 °C in a microcentrifuge (Labnet Int. Inc., Model Prism™ R), and the supernatant was collected and filtered with a nylon membrane (Ramos et al., 2010) and then diluted at a ratio of 1:20 with phosphate buffer for analysis according to the following methodologies.

Quantification of total protein: Plant tissue protein concentrations were determined using the Bradford (1976) colorimetric technique with bovine serum albumin (BSA) as a standard at a wavelength of 595 nm in a UV-Vis spectrophotometer (Thermo Scientific Model G10S).

Superoxide dismutase (SOD) (EQ 1.15.1.1): Measurement of SOD enzymatic activity was conducted using the Sigma 19160 determination kit. The reaction mixture (made in white Eppendorf microplates) contained 20 µL of protein extract, 200 µL of water soluble tetrazolium salt (WST) and 20 µL of enzyme working solution (XO/X). The plate was then incubated at 37 °C for 20 minutes and read using a plate reader (BioTek EL×808) at an absorbance of 450 nm. The SOD activity was expressed as a rate of inhibition assuming that WST inhibition is attributed to the neutralization of superoxide radicals by SOD.

Measurement of catalase enzymatic activity (EQ 1.11.1.6): Catalase activity was quantified by measuring 2 reaction times using the spectrophotometric method of Dhindsa et al. (1981). The reaction mixture contained 100 µL of protein extract, 1 mL of $H_2O_2$ (100 Mm) and 400 µL of 5 % $H_2SO_4$ (added to stop the reaction). The reaction was performed in microcentrifuge tubes at a temperature of 20 °C under constant agitation. $H_2O_2$ consumption was monitored using a previously traced peroxide calibration curve at 270 nm, and en-

zymatic activity was expressed in mM of $H_2O_2 \cdot min^{-1} \cdot$total proteins (mg $g^{-1}$).

Glutathione peroxidase (EQ 1.11.1.9): To measure glutathione peroxidase activity, the method modified by Flohé and Günzler (1984) using $H_2O_2$ as a substrate was used. For the assay, 0.4 mL of reduced glutathione (0.1 M) and 0.2 mL of $Na_2HPO_4$ (0.067 M) were added to 0.2 mL of protein extract. This mixture was then pre-warmed in a water bath at 25 °C for 5 minutes, and 0.2 mL of $H_2O_2$ (1.3 mM) was subsequently added to initiate the catalytic reaction. This mixture was allowed to react for 10 min and was then stopped by the addition of 1 mL of 1% trichloroacetic acid, followed by incubation in an ice bath for 30 min. The sample was then centrifuged at 3000 rpm for 10 min. Finally, 0.48 mL of the supernatant was placed in a cell to which 2.2 mL of $Na_2HPO_4$ (0.32 M) and 0.32 mL (1 mM) of the stain 5,5' dithiobis(2-nitrobenzoic acid) (DTNB, Sigma) were added. Finally, the mixture was read in a UV-Vis spectrophotometer at 412 nm (Xue et al., 2001).

Glutathione: Glutathione assessment was performed colorimetrically through reaction with 5,5' dithiobis(2-nitrobenzoic acid) (DTNB, Sigma). For the assay, 0.48 mL of the protein extract was added to 2.2 mL of $Na_2HPO_4$ (0.32 M) and 0.32 mL of stain DTNB (1 mM) to a test tube. The reaction was mixed completely and then read using a UV-Vis spectrophotometer at 412 nm (Xue et al., 2001), and the absorbance obtained was interpolated using a calibration curve previously standardized with reduced glutathione.

Ascorbate peroxidase (EQ. 1.11.1.1): Quantification of the ascorbate peroxidase activity was performed according to the protocol established by Nakano and Asada (1987). For the assay, 0.1 mL of enzymatic extract, 0.5 mL of ascorbate (10 mg $L^{-1}$) and 1 mL of $H_2O_2$ (100 mM) were added for a final volume of 2 mL and were incubated at a temperature of 22 °C. The reaction was stopped after one minute by adding 0.4 mL of 5% $H_2SO_4$. The rate of ascorbate oxidation was quantified by the decrease in absorbance at 266 nm in a UV-Vis spectrophotometer.

Total phenols: One milliliter of a water:acetone solution (1:1) was added to 200 mg of lyophilized tissue to extract the total phenolic compounds (Yu and Dahlgren, 2001). The sample was mixed in a vortex for 30 seconds and later using a Bransonic brand ultrasonic cleaner (model 1510R-DTH) for 5 minutes, after which the sample was centrifuged at 4 °C at 12,500 rpm for 10 min, and the supernatant (phenolic extract) was collected. The quantification was performed colorimetrically using 50 µL of phenolic extract, 200 µL of the reagent Folin-Ciocalteu, 500 µL of $Na_2CO_3$ (at 20%) and 5 mL of distilled water, which were mixed with a vortex. The mixture was then incubated at 45 °C for 30 min to allow the reaction to occur (Sultana et al., 2009; Nsor-Atindana et al., 2012). Finally, readings were taken using a UV-Vis spectrophotometer at 750 nm. The results are expressed in mg of gallic acid $g^{-1}$ of fresh tissue. The calibration curve was performed with gallic acid.

The variables for fruit quality were determined in fully ripe tomato fruits. A maturity rating scale was used when collecting the fruits, based on US standards for fresh tomato coloration, selecting those called "red" (those with over 90% of the surface red in color) (United States Department of Agriculture [USDA], 1997). Firmness was measured at the time of harvest in five fruits, with a second measurement taken 15 days after maintaining the fruit at room temperature (T max 25 °C and T min 18 °C), for this a fruit test penetrometer (FT20 Wagner Instruments) was used. The refractive index (% soluble solids) was determined with the pulp of the five fruits using a manual refractometer from 0 to 32% (Atago model ATC1E), along with electrical conductivity and pH using an HI 98130 potentiometer (Hanna Instruments), while redox potential was determined using an HI 2211 pH/Oxidation Reduction Potential (ORP) potentiometer HI 2211 (Hanna Instruments). The titratable acidity (% citric acid) was calculated using 10 mL of pulp from each fruit, to which 2 drops of phenolphthalein (1%) were added and titrated with 0.1 N NaOH (AOAC, 1990). Finally, the fruit lycopene content was quantified using the methodology cited by Fish et al. (2002) with a mixture of completely ripe fruit for the sample evaluated.

**Statistical analysis**

The experimental unit was a plant in a pot. For the two stages of the study, the growth and physiological variables were measured using 20 replicates for each treatment. For the minerals, biochemical and fruit quality variables five replications per treatment were performed. A completely randomized design was used for the experimental development. A factorial analysis considering two factors was performed to evaluate minerals only in the second stage. One factor was the organ evaluated (root, leaf and fruit), and the other factor was treatments applied (chitosan hydrogel only, nCu-chitosan hydrogel, and a control). Significant differences among treatments were detected using an analysis of variance (ANOVA) and Least Significant Difference (LSD) means separation test ($P \leq 0.05$). All of the statistical analyses were performed using the statistical software SAS v9.1.

## Results and discussion

**Stage 1. Preliminary study to determine the range of nCu concentrations suitable for application to the chitosan hydrogel substrate**

The results of the evaluation of the agronomic variables of tomato plants are shown in Tab. 1. Significant differences were observed (LSD, $P \leq 0.05$) in plant height, stem diameter, dry weight of the shoots and stomatal conductance; however, the variables number of

**Tab. 1:** Morphological variables of tomato plants treated with nCu-chitosan hydrogel.

| Treat | Height (cm) | NL | SD (mm) | NFC | FWS (g) | DWS (g) | SC (mmol $m^{-2}$ $s^{-1}$) |
|---|---|---|---|---|---|---|---|
| 0.03 mg $L^{-1}$ | 84.7 a | 15.5 a | 7.85 bcd | 3.75 a | 274 a | 31.5 c | 889 a |
| 0.015 mg $L^{-1}$ | 78.9 b | 14.7 a | 9.01 a | 3.60 a | 275 a | 38.0 a | 806 b |
| 0.006 mg $L^{-1}$ | 85.1 a | 14.2 a | 8.42 ab | 3.60 a | 283 a | 36.0 ab | 877 a |
| 0.003 mg $L^{-1}$ | 87.1 a | 15.4 a | 7.30 d | 3.95 a | 269 a | 33.9 bc | 815 b |
| 0.0015 mg $L^{-1}$ | 87.3 a | 14.5 a | 7.70 cd | 4.00 a | 267 a | 33.1 bc | 731 c |
| T0 | 82.8 ab | 15.2 a | 7.90 bc | 3.45 a | 262 a | 32.3 c | 664 d |

Treat: treatments on base to nCu concentration. T0: absolute control without application of either chitosan hydrogel or nCu. NL: number of leaves. SD: stem diameter. NFC: number of floral clusters. FWS: fresh weight of shoot. DWS: dry weight of shoot. SC: stomatal conductance. Data is the mean of 20 samples. Different letters by column indicate significant differences among treatments, at $P \leq 0.05$, according to the LSD test.

leaves, number of clusters, shoot fresh weight, fresh weight of leaves and fresh weight of stems did not differ. For stem diameter and shoot dry weight, the best treatments were nCu at 0.015 and 0.006 mg $L^{-1}$, which were greater than the control by 6.5-14% and 11-17% for each variable, respectively. This result is consistent with ADHIKARI et al. (2012), who report more biomass in the root and shoots of soybeans and chickpeas after seed treatment with CuO NPs at 60 mg $L^{-1}$ and 100 mg $L^{-1}$, respectively. The best treatments for stomatal conductance were 0.03 and 0.006 mg nCu $L^{-1}$, which were greater than the control by approximately 32% (LSD, $P \leq 0.05$). For plant height, the 0.015 mg nCu $L^{-1}$ treatment was the lowest, with the remaining treatments with nCu being greater than or equal to that treatment. UMBER et al. (2015), report that the application of 100 mg $kg^{-1}$ $TiO_2$ NPs in soil significantly increased plant dry weight and height in lettuce. Based on these results, treatment with 0.006 mg $L^{-1}$ of nCu was selected for evaluation in the second stage.

**Stage 2. Study of chitosan hydrogels with nCu applied to the substrate and their inductive capacity of antioxidants**

The results corresponding to the growth and physiological variables of the second experimental stage are shown in Tab. 2. Differences were detected among treatments in the number of floral clusters, number of fruits, weight of fruits, shoot fresh weight, shoot dry weight, stomatal conductance, and SPAD units. There were no differences in height, number of leaves, or stem diameter (LSD, $P \leq 0.05$).

Compared to the control, treatment of chitosan hydrogel with nCu showed the following significant increases: 11% more clusters, 29% more fruits, 25% higher fruit weight, 20% higher shoot fresh weight, 29% higher shoot dry weight, 7% more stomatal conductance and values of of SPAD units 9% highest. Applications using chitosan hydrogels without nCu were never greater than the control (Tab. 2). This response is possibly due to the capacity of the nCu to induce physiological or biochemical adjustment responses due to their size, surface area and capacity to easily traverse cellular barriers to interact with intracellular structures (SHOBHA et al., 2014). The exact mechanisms for response induction for nCu have not been understood, but the formation of reactive oxygen species (ROS) and release of $Cu^{2+}$ are mentioned in the literature (PHOGAT et al., 2016).

It has been reported that the application of solution of 60 mg $L^{-1}$ CuO NPs at the seeds significantly increased the root and shoot biomasses in soybeans, while application of 100 mg $L^{-1}$ in chickpeas obtained similar results (ADHIKARI et al., 2012). The application of $TiO_2$ NPs significantly increased shoot dry weight and height in lettuce; however, the authors used soil concentrations of 100 mg $kg^{-1}$ (UMBER et al., 2015). In contrast, TRUJILLO-REYES et al. (2014) reported that the application of nCu decreased water content, root length and dry biomass in lettuce plants grown in solution with the NPs. Additionally, NHAN et al. (2014) reported negative effects for growth, root biomass and shoot biomass in cotton grown in solution after application of $SiO_2$ NPs at concentrations of 10 to 2000 mg $L^{-1}$. These findings indicate that NPs have different effects depending on their concentration, the type of NPs and even the vegetable species to which they are applied.

In the case of SPAD units, a significant increase (LSD, $P \leq 0.05$) of approximately four SPAD (9%) units compared to the control was observed (Tab. 2). However, the opposite was reported for lettuce after application of nCu at concentrations of 10 and 20 mg $L^{-1}$, decreasing the quantity of SPAD to 17% (TRUJILLO-REYES et al., 2014). The results obtained in the present study can be explained by the low dose of nCu used (0.006 mg nCu$L^{-1}$ of substrate) compared to the dose used by TRUJILLO-REYES et al. (2014), as it is known that Cu is very toxic, even in its ionic form, and that its toxicity increases in the form of NPs (SHOBHA et al., 2014).

The mineral content results in the different tomato plant organs are shown in Tab. 3. Significant differences between the plant organs were observed for all of the minerals evaluated (LSD, $P \leq 0.05$). The Cu, Fe and Zn contents were highest in the root compared to the leaf and fruit. The Ca and Mg contents were highest in the leaf, and finally, the K content was highest in the fruit. These results are within the expected range, as has been reported by different authors the concentrations of the tomato plant organs are different even among growth stages (BETANCOURT and PIERRE, 2013; QUESADA-ROLDÁN and BERTSCH-HERNÁNDEZ, 2013).

Regarding the effects of the different chitosan hydrogel treatments, significant differences were observed only regarding Cu concentration. There were no significant differences between the three treatments evaluated for K, Ca, Mg, and Fe (LSD, $P \leq 0.05$) (Tab. 3). The

**Tab. 3:** Mineral concentration (μg $g^{-1}$ [dry weight]) in tomato plants treated with nCu-chitosan hydrogel at a concentration of 0.006 mg nCu $L^{-1}$.

| Factor | Treatment | K | Ca | Mg | Fe | Cu | Zn |
|---|---|---|---|---|---|---|---|
| Organ | Root | 18147 b | 5323 b | 1922 b | 303 a | 6.19 a | 73.23 a |
| | Leave | 17707 b | 13381 a | 2877 a | 144 b | 3.71 b | 27.67 b |
| | Fruit | 24532 a | 755 c | 1873 b | 115 b | 2.47 c | 19.88 b |
| Hydrogel | nCu | 21202 a | 6828 a | 2324 a | 171 a | 3.59 b | 32.95 a |
| | Chitosan | 19044 a | 6175 a | 1942 a | 165 a | 3.97 b | 46.41 a |
| | T0 | 20140 a | 6456 a | 2407 a | 226 a | 4.81 a | 41.43 a |

nCu: nCu-chitosan hydrogel at a concentration of 0.006 mg nCu $L^{-1}$. Chitosan: chitosan hydrogel without nCu. T0: absolute control without application of either chitosan hydrogel or nCu. Data is the mean of five samples. The data of hydrogel factor are the mean of roots, leaves and fruits. Different letters by column indicate significant differences among treatments, at $P \leq 0.05$, according to the LSD test.

**Tab. 2:** Morphological, fruit yield and physiological variables of tomato plants treated with nCu-chitosan hydrogel at a concentration of 0.006 mg nCu $L^{-1}$.

| Treat | Height (cm) | NL | SD (mm) | NFC | NF | FWF (kg) | FWS (kg) | DWS (g) | SC (mmol $m^{-2}$ $s^{-1}$) | SPAD units |
|---|---|---|---|---|---|---|---|---|---|---|
| nCu | 267 a | 30.4 a | 11.4 a | 9.75 a | 25.4 a | 3.56 a | 1.45 a | 172.8 a | 490.5 a | 45.0 a |
| Chitosan | 276 a | 29.9 a | 11.2 a | 9.35 ab | 20.9 b | 2.90 b | 1.23 b | 127.6 b | 459.2 b | 40.3 b |
| T0 | 257 a | 28.6 a | 11.0 a | 8.75 b | 19.6 b | 2.84 b | 1.20 b | 133.6 b | 458.2 b | 41.1 b |

Treat: treatments. nCu: nCu-chitosan hydrogel. Chitosan: chitosan hydrogel without nCu. T0: absolute control without application of either chitosan hydrogel or nCu. NL: number of leaves. SD: stem diameter. NFC: number of floral clusters. NF: number of fruits. FWF: fresh weight of fruits. FWS: fresh weight of shoots. DWS: dry weight of shoot. SC: stomatal conductance. Data is the mean of 20 samples. Different letters by column indicate significant differences among treatments, at $P \leq 0.05$, according to the LSD test.

Cu content was highest in the control and lowest in both treatments with chitosan hydrogels, regardless of the presence of nCu. These results suggest that chitosan could negatively influence Cu absorption and translocation. However, TRUJILLO-REYES et al. (2014) reported that the application of 20 mg $L^{-1}$ of Cu/CuO NPs increased the quantity of Cu in the roots and leaves of lettuce. This difference between the two studies may be due to the difference in nCu concentrations used and for the presence of chitosan hydrogels. As explained by TRUJILLO-REYES et al. (2014), nCu can be absorbed by the root due to its tiny size and then later translocated to the leaves of lettuce plants, causing increases in the Cu concentrations in the roots and leaves. The same authors also reported effects on the concentrations of different minerals in lettuce tissues (Al, Zn, Mn, Mg, P, S and Ca); however, in the present study, differences were only observed in the Cu concentration. This finding confirms the feasibility of using nCu, provided it is applied at appropriate concentrations. Of note, NHAN et al. (2014) reported that the application of $SiO_2$ NPs had significant effects on the concentrations of Na, Mg and Cu in the shoots of cotton plants. These results indicate that the effects on mineral concentration of the different plant tissues can vary depending on NP type and concentration.

The results of the content of antioxidant compounds in the leaves and fruits of tomato plants are shown in Tab. 4. Catalase activity measured in the leaves had significant differences among treatments (LSD, $P \leq 0.05$). For this variable, the nCu-chitosan hydrogel treatment was more than five times higher than the control and more than two times higher than chitosan hydrogel without nCu. This finding agrees with that reported by TRUJILLO-REYES et al. (2014), as they mention that when applying 10 mg $L^{-1}$ of nCu, catalase activity increased but ascorbate peroxidase activity decreased in the root. This result may be due to the oxidative stress caused by the nCu, as suggested by the same authors.

In terms of the variables ascorbate peroxidase, total phenols, proteins, glutathione peroxidase, glutathione and superoxide dismutase evaluated in the leaves, there were no significant differences among treatments. Although TRUJILLO-REYES et al. (2014) observed a decrease in the activity of ascorbate peroxidase in lettuce leaves after applying 20 mg $L^{-1}$ of nCu and NHAN et al. (2014) reported an increase in the activity of superoxide dismutase in cotton by applying 10 and 500 mg $L^{-1}$ $SiO_2$NPs, these differences could be due to the high concentrations of NPs used in the studies mentioned compared to the present study. There were also no differences in the variables evaluated in the fruit (LSD, $P \leq 0.05$) (Tab. 4).

The results of the evaluation of variables for tomato fruit quality are shown in Tab. 5. There were differences (LSD, $P \leq 0.05$) in fruit firmness, pH, titratable acidity and lycopene at harvest; however, there were no differences observed in firmness and soluble solids measured 15 days after harvest. Compared to the control, there was an increase in lycopene of approximately 12% after applying the nCu-chitosan hydrogel. However, treatment of the chitosan hydrogel without nCu had a positive effect on fruit firmness at the time of the harvest, being 16% greater than the control. Nevertheless, at 15 days after harvest, firmness was equal between the three treatments (LSD, $P \leq 0.05$). The treatment of the chitosan hydrogel without nCu also increased the fruit titratable acidity, resulting in a value 25% greater than the control. It was also observed that the chitosan hydrogel and the nCu-chitosan hydrogel treatments decreased the ORP compared to the control. This result is positive because, according to BENAVIDES et al. (1999), a reduction in the potential redox values denotes a greater antioxidant potential, indicating a higher nutritive quality in fruit.

## Conclusions

Application of nCu in chitosan hydrogels had positive effects on almost all of the variables related to the vigor of tomato plants, as

**Tab. 4:** Antioxidant compounds in tomato leave and fruits treated with nCu-chitosan hydrogel at a concentration of 0.006 mg nCu $L^{-1}$.

| Organ | Treat | Protein (mg $L^{-1}$) | CEA | SOD | GPX | Glu | APX | TF |
|---|---|---|---|---|---|---|---|---|
| Leaves | nCu | 316.2 a | 5.06 a | 66.1 a | 10085 a | 267.7 a | 55.3 a | 921 a |
| | Chitosan | 263.6 a | 2.33 b | 51.9 a | 9471 a | 239.7 a | 56.9 a | 1007 a |
| | T0 | 246.4 a | 0.90 b | 52.3 a | 10224 a | 204.3 a | 55.4 a | 869 a |
| Fruit | nCu | 102.4 a | 1.46 a | 35.2 a | 9587 a | 207.0 a | 53.5 a | 580.4 a |
| | Chitosan | 79.2 a | 2.23 a | 37.1 a | 9676 a | 135.0 a | 50.8 a | 410.2 a |
| | T0 | 100.3 a | 0.79 a | 39.4 a | 9586 a | 141.7 a | 50.5 a | 483.8 a |

Treat: treatment. nCu: nCu-chitosan hydrogel at a concentration of 0.006 mg nCu $L^{-1}$. Chitosan: chitosan hydrogel without nCu. T0: absolute control without application of either chitosan hydrogel or nCu. CEA: catalase enzymatic activity (mM of $H_2O_2$ $min^{-1}$ total proteins [mg $g^{-1}$]). SOD: superoxide dismutase (inhibition rate [%] of water soluble tetrazolium salt). GPX: Glutathione peroxidase (IU mg $protein^{-1}$). Glu: glutathione (IU mg $protein^{-1}$). APX: ascorbate peroxidase (µmol oxidized ascorbate $min^{-1}$ $mg^{-1}$ $protein^{-1}$). TF: total phenols (mg Gallic Ac $g^{-1}$ fresh tissue). Data is the mean of five samples. Different letters by column indicate significant differences among treatments, at $P \leq 0.05$, according to the LSD test.

**Tab. 5:** Fruit quality variables of tomato treated with nCu-chitosan hydrogel at a concentration of 0.006 mg nCu $L^{-1}$.

| Treatment | Firmness (kg $cm^{-2}$) | Firmness 15 dah (kg $cm^{-2}$) | Soluble solids (°Brix) | pH | ORP (mV) | TA (% CA) | Lycopene (µg $g^{-1}$) |
|---|---|---|---|---|---|---|---|
| nCu | 4.36 ab | 3.47 a | 5.11 a | 4.79 a | 61.7 b | 0.38 b | 0.0419 a |
| Chitosan | 4.64 a | 3.56 a | 5.04 a | 4.70 b | 65.8 b | 0.45 a | 0.0370 b |
| T0 | 4.00 b | 3.34 a | 5.03 a | 4.66 b | 102.1 a | 0.36 b | 0.0372 b |

nCu: nCu-chitosan hydrogel at a concentration of 0.006 mg nCu $L^{-1}$. Chitosan: chitosan hydrogel without nCu. T0: absolute control without application of either chitosan hydrogel or nCu. dah: days after harvest. ORP: oxidation reduction potential. TA: titratable acidity. CA: citric acid. Data is the mean of five samples. Different letters by column indicate significant differences among treatments, at $P \leq 0.05$, according to the LSD test.

the floral cluster numbers, fruit numbers, fruit weight, fresh and dry shoots, stomatal conductance and SPAD units all significantly increased.

Additionally, the application of nCu-chitosan hydrogels increased the catalase activity in the leaves and the lycopene content in the fruit, along with the pH in the pulp of the tomato fruits. Treatment with chitosan increased the firmness of fruit at the time of harvest, but after 15 days, there were no differences in this variable. Application of chitosan only and chitosan hydrogels with nCu decreased the ORP of the fruit extract, indicating greater antioxidant capacity. With respect to titratable acidity, treatment with chitosan increased the value of this variable compared to the control; however, in contrast, nCu treatment was not different from the control. The lycopene concentration in the fruit was higher in the chitosan treatment with nCu.

Application of the chitosan only and nCu-chitosan hydrogels also affected the Cu concentration in the tissues of the tomato plants, reducing the content of this mineral. However, there were no differences in the concentrations of other minerals.

The application of Cu nanoparticles in chitosan hydrogels at a concentration of 0.06 g $L^{-1}$ had positive effects on tomato growth, yield and nutritional characteristics.

These results indicate that application of Cu nanoparticles in chitosan hydrogels can be used as a tool to increase the yield of tomato crop as well as the beneficial compounds of the tomato fruits. However, nowadays the lack of sufficient knowledge about the effects of nCu on other plants, ecological systems or human health do not make advisable to use this material commercially. More studies are necessary that include the assessment of the effects of nCu on other species and agricultural systems.

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