Article

PVA-Chitosan-nCu complex improves yield and defense response in tomato

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Abstract

Currently the use of nanotechnology is revolutionizing agricultural production. Copper nanoparticles have been shown to influence the growth and development of different plant species, in addition to operating as stress resistance inducers. The objective of the present research was to evaluate the response in growth and yield, as well as the activation of the defense system of tomato plants. The treatments evaluated were a complex of polyvinyl alcohol-chitosan-copper nanoparticles (PVA-Cts-nCu), another complex of PVA-Cts and an absolute control (T0). The treatments were applied via foliar in tomato plants under greenhouse conditions. During the crop cycle, agronomic variables were determined, and the activity of enzymes related to stress tolerance such as β-1,3 glucanase, chitinase and phenylalanine ammonia lyase (PAL), as well as the expression of the PR1 gene. The PVA-Cts-nCu complex increased yield, number of fruits, average fruit weight, aerial fresh weight and root fresh weight, in addition, it promoted the defense system by increasing the PAL enzyme activity, as well as the overexpression of the PR1 gene.

Keywords: biostimulant, gene expression, stress, vegetables.

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Introduction

The agricultural sector currently faces significant challenges of increasing productivity to feed the growing world population and increasing the efficiency in resource use (Rouphael and Colla, 2020). This has led to the expansion of the field of nanotechnology research and its possible applications in the agricultural field (Usman *et al*., 2020). Nanoparticles (NPs) have a highly reactive surface, therefore, they are biologically active, this because their surface area is extremely high proportional to their volume (Pestovsky and Martínez, 2017). They are synthesized in different sizes, shapes, materials and reactivity, this gives them the ability to enhance the agricultural sector (Fatima *et al*., 2021).

Copper (Cu) is a micronutrient widely distributed in plant tissues, participates in physiological processes and is essential for plant growth (Rajput *et al*., 2018). It has been shown to activate key enzymes in the Calvin cycle, improving photosynthetic activity and increasing yield in plants (Pradhan *et al*., 2015). Cu NPs can function as reducers or oxidants in biochemical reactions within the cell so they can catalyze the production of reactive oxygen species (ROS) and induce oxidative stress (Somasundaran *et al*., 2010). The effects of Cu NPs vary, it has been reported that, at low concentrations, it increases the growth rate and germination of several plants, on the other hand, at high concentrations growth is delayed (Kasana *et al*., 2017). In addition, in foliar applications with Cu NPs, they favor the firmness of fruits and content of antioxidants such as vitamin C, lycopene and phenols (López-Vargas *et al*., 2018; Pérez-Labrada *et al*., 2019).

Chitosan (Cts) has been used to improve the natural defenses of plants, defense responses include lignification, activation of enzymes associated with the response to pathogens in plants, phytoalexin biosynthesis, ROS generation, biosynthesis of jasmonic acid, salicylic acid, abscisic acid and the expression of genes related to defense (El Hadrami *et al*., 2010). The application of chitosan increases the activity of β-1,3 glucanase and chitinase in different plant species, helping to hydrolyze the cell wall of phytopathogenic fungi (Rodríguez-Pedroso *et al*., 2009; González Peña *et al*., 2014).

Polyvinyl alcohol (PVA) has been widely used in the preparation of hydrogels and in the inhibition of bacterial growth, in addition, it has a high potential as a controlled release system (Yang *et al*., 2016). The PVA-Cts mixture has been shown to have *in vitro* effects on the antioxidant response by removing free radicals, as well as on the viability of *Staphylococcus aureus* 8325-4 and *Escherichia coli* RB (Yang *et al*., 2018).

There are currently few studies on the application of PVA-Cts-nCu complexes. These complexes applied in the form of hydrogels increase the nutraceutical quality in tomato fruits (Hernández-Hernández *et al*., 2018a). They also promote the increase of vigor and number of floral clusters in tomato (Hernández-Hernández *et al*., 2017). Applied in substrate, they increase the yield in jalapeño chili fruits (Pinedo-Guerrero *et al*., 2017). The objective of the present study was to evaluate the response of the PVA-Cts-nCu complex in the growth and quality of fruit, as well as the activation of the defense system of tomato plants.

Materials and methods

Synthesis of the PVA-Cts and PVA-Cts-nCu complex

For the preparation of the PVA-Cts-nCu complex, 1.5 L of PVA (BP-05, Nacional PIM Mexico SA de CV, molecular weight of 27 000-32 000 g mol^{-1}) at 1% (w/v) were mixed with 1.5 L of Cts (Marine Chemicals, India, molecular weight of 200 000 g mol^{-1}) at 0.5% (w/v). The nCu (1.85 g) was ground with 3 ml of Agrex® F (Agroenzymas® SA de CV) as a dispersant for NPs. The nCu exhibited a spherical and hemispherical morphology and an average size of 30 nm (Sierra-Ávila *et al*., 2014, 2015).

Establishment of the crop

The test was carried out in a tunnel-type greenhouse. A soilless growing system was used, the substrate was a mixture of peat moss (Premier® Premier Horticulture LTD) and perlite (Multiperl® Lagoon Perlite Group) (1:1 v/v). Cid F1 saladette-type hybrid tomato plants (Harris Moran®) of indeterminate growth were used, they were placed in polyethylene bags of 14 L.

The experiment was carried out applying the treatments PVA-Cts-nCu 500 mg L^{-1}, PVA-Cts 500 mg L^{-1} and an absolute control with distilled water (T0). The application of the treatments was foliar, with an approximate consumption of 75 ml of solution per plant throughout the production cycle. The seedlings received an application two days before the transplantation, subsequently it was applied at intervals of 20 days starting after the transplant. The Steiner solution (Steiner 1961) was used as a means of nutrition.

Variables of development and productivity in tomato

The evaluation of plant height and stem diameter was carried out 120 days after transplantation (DDT). At 120 DDT, the yield per plant was determined. In addition, the aerial fresh weight was determined, as well as the root fresh weight.

Enzyme extraction

The activity of the enzymes phenylalanine ammonia lyase (PAL), chitinase and β-1,3 glucanase was analyzed in leaves in three different samples, at the time of transplantation (48 h after the first application of the complex), at 40 DDT and 80 DDT. The leaves were collected with liquid nitrogen.

The extraction of biomolecules was performed according to Rodríguez-Pedroso *et al*. (2006). PAL activity was determined according to Sykłowska-Baranek *et al*. (2012). The results were expressed as U (production of μmol of trans-cinnamic acid equivalent per milliliter per minute) per total proteins (mg g^{-1}). Chitinase activity was determined according to Rodríguez-Pedroso *et al*. (2006). The results were expressed as U (production of μg ml^{-1} of glucose per minute per total proteins (mg g^{-1}). The β-1,3 glucanase activity was determined according to Rodríguez-Pedroso *et al*. (2006). Enzymatic activity was determined by measurement of the level of production of reducing sugars and was expressed in terms of production of μg ml^{-1} of glucose per minute per total proteins (mg g^{-1}).

Expression of the PR1 gene

The leaf samples were collected at three different times, starting at 48 h after the first application of the complex, after this first sampling the following were every 40 days. Young leaves completely expanded were collected with liquid nitrogen. RNA extraction was carried out using the TRIzol™ Reagent (Invitrogen) technique.

The cDNA was synthesized with the SensiFAST™ (Bioline) kit. The primers used correspond to an endogenous gene (Actin) and the PR1 gene. ACT (fwd 5'-CCCAGGCACACAGGTGTTAT-3'; rev 5'-CAGGAGCAACTCGAAGCTCA-3'); PR1 (fwd 5'-AAGTAGTCTGGCGCAACTCA-3'; rev 5'-GTCCGATCCAGTTGCCTACA-3'). The quantification of the PR1 gene was carried out in real-time PCR equipment (Applied Biosystems StepOne™ version 2.3) by the ΔΔCt method, measuring the fluorescence intensity of Sybr Green (Hernández-Hernández *et al*., 2018b).

Data analysis

A completely random design was used in the test in tomato plants. For the agronomic variables, 20 experimental units were analyzed for each treatment. In the quantification of PAL, chitinase and β-1,3 glucanase activity, five repetitions per treatment were analyzed and in the expression of the PR1 gene, four composed repetitions were analyzed (10 plants per repetition). To detect statistical differences between treatments, an analysis of variance (Anova) was performed, and a mean separation test was carried out according to Fisher's LSD test ($p \leq 0.05$). All statistical analyses were performed in the InfoStat v2018 statistical software.

Results and discussions

Effect of the PVA-Cts-nCu complex on developmental and productivity variables in tomato

The foliar application of the PVA-Cts-nCu complex had significant effects ($p \leq 0.5$) on the variables related to vigor in tomato plants (Table 1). The PVA-Cts-nCu treatment showed significant differences compared to the control, increasing the yield (60.68%), the average fruit weight (18.20%), the number of fruits (35.99%), the aerial fresh weight (26.99%) and the root fresh weight (80.87%). While the PVA-Cts treatment increased the average fruit weight (8.39%) and the root fresh weight (52.22%) compared to the control. No significant differences were observed in the variables of stem height and diameter.

Table 1. Effect of the PVA-Cts-nCu and PVA-Cts complex on tomato growth and productivity.

Treatment	Height (cm)	DT (mm)	Yield (g plant^{-1})	PPF (g fruit^{-1})	NF	PFA (g plant^{-1})	PFR (g plant^{-1})
T0	132.29 a	17.02 a	2573.31 b	58.62 c	43.9 b	2384 b	98.8 b
PVA-Cts-nCu	134.78 a	16.79 a	4134.95 a	69.29 a	59.7 a	3027.65 a	175.8 a
PVA-Cts	119.71 a	15.04 a	2690.92 b	63.54 b	42.35 c	2299.47 b	150.4 a

T0= control; DT= stem diameter; NF= number of fruits; PPF= average fruit weight; PFA= aerial fresh weight; PFR= root fresh weight, means with the same letter within each column are statistically equal (Fisher's LSD, $p \leq 0.05$).

The results obtained in this study demonstrate that the PVA-Cts-nCu and PVA-Cts complexes applied via foliar do not show toxicity in tomato plants. Rajput *et al*. (2018) mention that phytotoxicity will depend on the concentration, type and size of the NPs, in addition to the species in which they are applied, the growing conditions and the time of exposure.

Saharan *et al*. (2015) report an increase in biomass in tomato plants treated with Cu-Cts NPs. Adhikari *et al*. (2012) observed that the application of CuO NPs increased root growth in soybean and chickpea. In the present study, it was shown that the PVA-Cts-nCu complex improved growth by increasing the aerial and root fresh weight, very important parameters for the development of the crop. This effect might be induced by the synergy between nCu and chitosan. Since this biopolymer and nCu have shown to influence the growth and development of plants (El Hadrami *et al*., 2010; Hernández *et al*., 2017; Pinedo-Guerrero *et al*., 2017).

At the production stage, the PVA-Cts-nCu complex increased yield, number of fruits and average fruit weight. This coincides with what was reported by Pinedo-Guerrero *et al*. (2017), who point out that when applying the hydrogel of PVA-Cts-nCu direct to the soil in jalapeño chili, it increased the yield (8.27%) and the number of fruits (9.32%) per plant. Also, Hernández-Hernández *et al*. (2018a) reported that the application of hydrogels of PVA-Cts-nCu direct to the substrate increased the number of fruits in tomato plants (20%).

Pradhan *et al*. (2015) evaluated the application of Cu NPs in green soybean, where NPs influenced the activity of key enzymes in the Calvin cycle (fructose-1, 6-bisphosphate phosphatase, ribulose-5-phosphate kinase and NADP-glyceraldehyde-3-phosphate dehydrogenase). It is likely that when the activity of enzymes related to the transport of electrons in photosynthesis increases, the rate of production of photosynthates and the rate at which they are transported will be modified, thus increasing the production of fruits and their weight.

Effect of the PVA-Cts-nCu complex on PAL activity

Foliar application of PVA-Cts-nCu had significant effects ($p \leq 0.5$) on PAL activity in leaves (Figure 1). The PVA-Cts-nCu treatment exhibits an increase in PAL activity at transplantation (301.70%) and at 80 DDT (55.04%) compared to the control. At 40 DDT, the PVA-Cts-nCu treatment increased PAL activity by 369.23% compared to PVA-Cts.

Chitosan and NPs are known as potential inducers to improve the defense response in plants to stress, resulting in an accumulation of metabolites related to defense (Zhang and Liu, 2015; Usman *et al*., 2020), as well as the generation of ROS and the increase in the activity of proteins related to defense (González-Peña *et al*., 2014).

The PAL enzyme is key in the synthesis of phenols and flavonoids, phenols are important precursors of compounds involved in the control of plant growth, in addition to being powerful antioxidants (Santos-Sánchez *et al*., 2019).

The increase in PAL can be derived from the direct interaction of nCu and Cts generating a synergistic effect. Cumplido-Nájera *et al*. (2019) applied Cu NPs foliarly increasing PAL activity in leaves by 1.78 times.

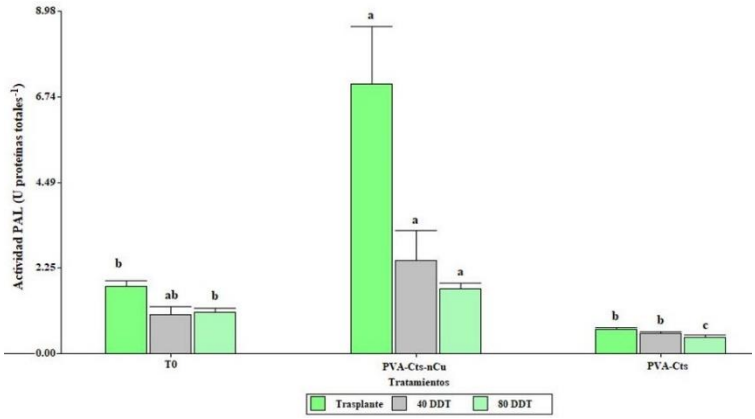

Figure 1. Enzymatic activity of phenylalanine ammonium lyase in tomato with application of PVA-Cts-nCu and PVA-Cts. Means with the same letter are statistically equal LSD Fisher, p≤ 0.05.

Falcón Rodríguez *et al*. (2012) reported that by spraying chitosan (0.5, 1 and 2.5 g L^{-1}) in tobacco plants, all concentrations increased the activity of PAL in the leaf. Rodriguez *et al*. (2006) found that previously treating rice seeds with chitosan hydrolysates (500 mg L^{-1}) stimulated the PAL activity in the leaf. In an essay by Hernández-Hernández *et al*. (2018a) with tomato plants under saline stress, it was found that the application of PVA-Cts-nCu without saline stress and PVA-Cts under saline stress increased the PAL activity in the leaf. González Peña *et al*. (2014) report that when spraying chitosan (100 mg L^{-1}) in tomato seedlings, an increase in PAL activity was recorded. Chandra *et al*. (2015) foliarly applied Cts NPs in *Camellia sinensis* L., finding a significant increase in the accumulation of peroxidase, polyphenol oxidase and PAL in the leaf.

Effect of the PVA-Cts-nCu complex on the β-1, 3 glucanase and chitinase activity

The foliar application of both treatments had no significant effects ($p \leq 0.5$) with respect to the control, however, the PVA-Cts-nCu showed an increase in the β-1, 3 glucanase activity with respect to PVA-Cts (162.85%) at 40 DDT. At 80 DDT, T0 increased the activity above PVA-Cts-nCu and PVA-Cts (Figure 2).

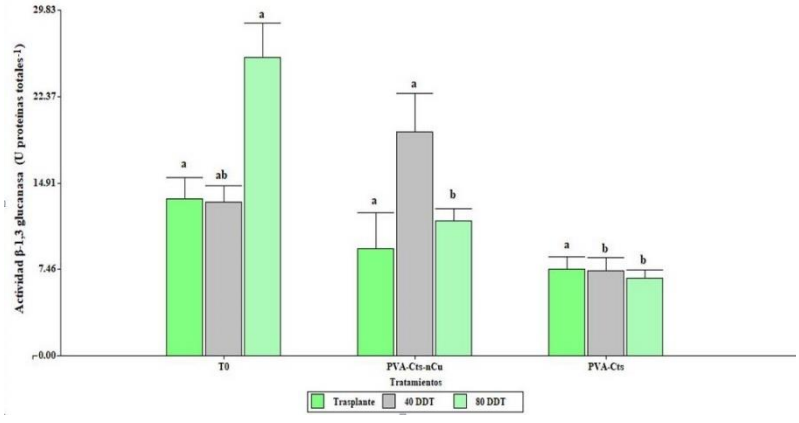

Figure 2. Enzymatic activity of β-1, 3 glucanase in tomato plants with the application of PVA-Cts-nCu and PVA-Cts. Means with the same letter are statistically equal Fisher's LSD, $p \leq 0.05$.

Foliar application of PVA-Cts-nCu had significant effects ($p \leq 0.5$) on chitinase activity (Figure 3). At 40 DDT, the PVA-Cts-nCu treatment increased the activity by 121% compared to PVA-Cts. At 80 DDT, T0 increased the activity above PVA-Cts-nCu and PVA-Cts. The PVA-Cts-nCu treatment increased the activity by 54.67% compared to PVA-Cts.

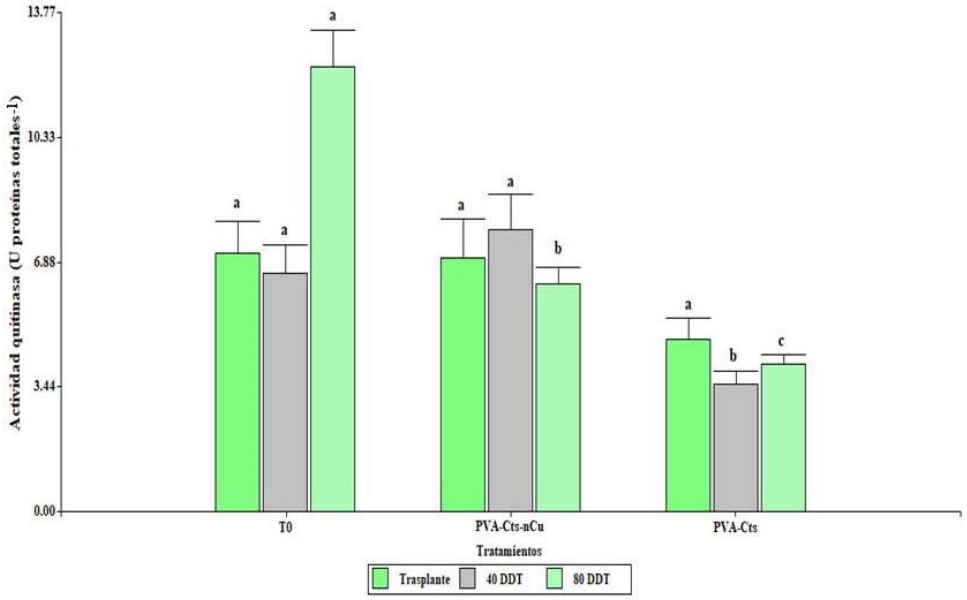

Figure 3. Enzymatic activity of chitinase in tomato plants with the application of PVA-Cts-nCu and PVA-Cts. Means with the same letter are statistically equal Fisher's LSD, $p \leq 0.05$.

Proteins related to pathogenicity (PR) such as β-1,3 glucanase and chitinase can be expressed constitutively and induced in response to an infection. Regarding the effectiveness of chitosan in the induction of the defense in plant, in leaves of *Camellia sinensis* L., it increased the activity of β-1,3-glucanase 29.26%, as well as the content of peroxidase and polyphenol oxidase (Chandra *et al*., 2015). When applying NPs Cts and Cts in tomato plants, these increased the relative expression of genes related to the synthesis of chitinase and β-1,3 glucanase compared to the control (Chun and Chandrasekaran, 2019).

Effect of PVA-Cts-nCu on the expression of the PR1 gene

The results of the expression of the PR1 gene are shown in Figure 4. The PVA-Cts-nCu treatment overexpressed the PR1 gene (268.3, 0.72 and 8 times with respect to T0) at transplantation, 40 and 80 DDT. Like PVA-Cts-nCu, the PVA-Cts treatment overexpressed the PR1 gene (3.9, 1.5 and 123.2 times with respect to T0) at transplantation, 40 and 80 DDT. These results suggest that the PVA-Cts-nCu complex could play an important role in the activation of genes that encode the production of PR proteins, related to the salicylic acid signaling pathway and systemic acquired resistance (RSA) linked to stress tolerance (AbuQamar *et al*., 2009).

There is evidence that chitosan can activate the defense response in plants, increasing the activity of PAL enzyme that is directly related to the synthesis of salicylic acid, thus inducing the synthesis of PR proteins, involved in the mechanisms of RSA (Rodríguez-Pedroso *et al*., 2006; Sánchez *et al*., 2010; González Peña *et al*., 2014).

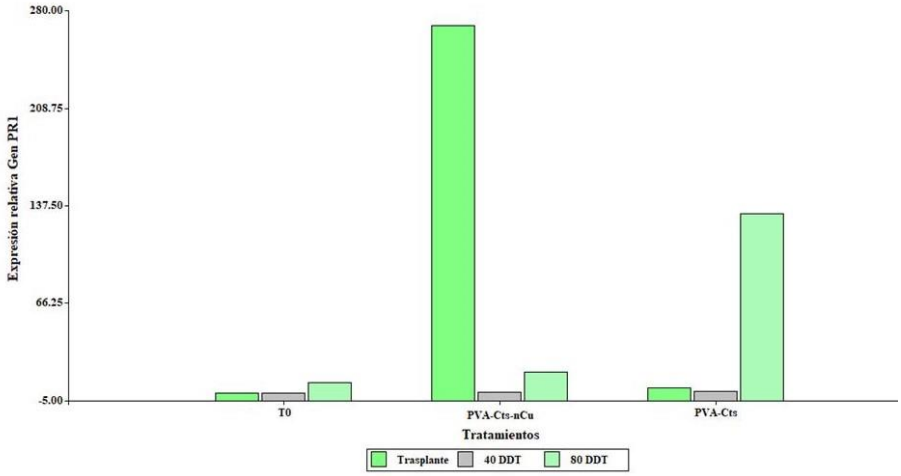

Figure 4. Relative expression of the PR1 gene in plants tomato leaves treated with PVA-Cts-nCu and PVA-Cts.

Chun and Chandrasekaran (2019) applied Cts NPs foliarly in tomato plants inoculated with *Fusarium andiyazi*, these increased the relative expression of the PR1 gene in the leaf compared to the control. Hernández-Hernández *et al*. (2018b) applied hydrogels of PVA-Cts-nCu direct to the substrate in tomato under conditions of saline stress and found that the expression of the PR1 gene was repressed under saline condition and without saline stress, however, the JA gene related to the jasmonate pathway was overexpressed and it is mediated by induced systemic resistance (RSI).

These results show that the PVA-Cts-nCu and PVA-Cts complexes may be potential inducers to mediate the expression of PR1 and JA genes in tomato plants activating the signaling cascade of synthesis of phenolic compounds and enzymes that will prepare the plant for the effects of stress, either by the salicylic acid pathway (RSA) or the jasmonic acid pathway (RSI).

Conclusions

This study showed that polyvinyl alcohol-chitosan-nanocopper and polyvinyl alcohol-chitosan increased the variables related to vigor in plants, yield, in addition to activating the defense mechanism of plants by increasing enzymes such as PAL, β-1, 3 glucanase and chitinase, in addition to increasing the expression of the PR1 gene linked to stress tolerance in plants.

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