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The application of copper nanoparticles and potassium silicate stimulate the tolerance to *Clavibacter michiganensis* in tomato plants

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ABSTRACT

The damage by *Clavibacter michiganensis* in the tomato plants is of great economic importance worldwide due to the significant losses it generates in the crop. The advances in nanotechnology provide alternatives that can be applied in the control of pathogens. Nano-copper and silicon currently have a widespread use for the control of plant pathogens. The combined use of both is expected to have a synergistic effect on the tolerance of plants against pathogens. The objective of the present research was to determine the effect of the application of copper nanoparticles and potassium silicate on tolerance to *C. michiganensis* in tomato. The presence, severity, and the impact of the bacteria on the yield of the tomato crop were determined. Levels of activity of defense enzymes were determined as well as antioxidant compounds in the leaves and fruits of tomato to understand the changes at the biochemical level caused by the bacteria and by the treatments. The results showed that the application of copper nanoparticles and potassium silicate was effective in reducing the severity of *C. michiganensis*. Also, the loss of yield due to the bacteria was reduced in 16.1%. The biochemical analyzes showed that the application of copper nanoparticles and potassium silicate positively modified the activity of the enzymes SOD, PAL, GPX, and APX, as well as the concentration of reduced glutathione and total phenols in the leaves. The activity of the PAL and GPX enzymes, as well as the contents of lycopene and β-carotene, were elevated in the fruits. The joint application of copper nanoparticles and potassium silicate changed the levels of enzymatic and non-enzymatic compounds that are key in defense of tomato plants, increasing the tolerance to *C. michiganensis*.

1. Introduction

The tomato is one of the most important crops worldwide (Martí et al., 2018), however, the production of this is affected by external biotic factors among which are different pathogens. *Clavibacter michiganensis* is a bacterium that penetrates through the stomata and hydathodes causing severe damage in the crop, among which marginal necrosis in the leaves is observed, causing great losses (Chalupowicz et al., 2017). The control of this bacterium is of great importance, and although there are chemical products for the control of this pathogen, the efficiency is low, in addition to the risks they present for health (Nandi et al., 2018), so it becomes necessary propose new alternatives that are efficient.

An alternative is the induction of tolerance to pathogens from biostimulation, that is, the application of substances or microorganisms that do not directly provide essential elements for plants (du Jardin, 2015). Biostimulation in plants with non-essential elements includes the application of Al, Co, Na, Se and Si, elements that provide different benefits in crops under stress conditions (du Jardin, 2015). In addition, it has recently been shown that the application of nanoparticles (NPs) also generates positive effects in crops (Zuverza-Mena et al., 2017).

The effects of silicon have been well documented, this element is not considered essential and numerous beneficial effects have been reported for plants (Luyckx et al., 2017). Under conditions of abiotic stress, it can regulate the generation of ROS from the antioxidant system, which reduces oxidative stress (Kim et al., 2017). Or by physiological adjustments such as increased stomatal conductance and transpiration under conditions of salt stress (Rios et al., 2017). Particularly, the Si improves the resistance of the plants against both fungal and bacterial pathogens, from enzymes related to defense, stimulation

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of the production of antimicrobial compounds, regulation of signaling pathways, and activation of the expression of defense-related genes (Wang et al., 2017).

Meanwhile, nanotechnology can be used to increase crop productivity (De la Rosa et al., 2017; Wang et al., 2015). In recent years, nanotechnology has been used by means of the application of nanomaterials such as nano-fertilizers, nanoparticles, or nano-pesticides for nutrient management, genetic improvement, plant disease treatment, and plant growth promotion (De la Rosa et al., 2017). Nanoparticles (NPs) 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 (Bell et al., 2014). So much so that 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 (Zuverza-Mena et al., 2017). These new properties provide the material with an added value that has multiple applications in the agricultural industries, among others (Siddiqui et al., 2015). Specifically, the application of copper nanoparticles (Cu NPs) increases the yield and fruit quality of the tomato crop (Hernández-Hernández et al., 2017; Juarez-Maldonado et al., 2016), as well as the quality of the fruit of jalapeño pepper crop (Pinedo-Guerrero et al., 2017). Under conditions of salt stress, the application of Cu NPs increased the activity of antioxidant enzymes (APX, SOD, CAT, GPX), in addition to inducing better tomato fruit quality from the increase in lycopene (Hernández-Hernández et al., 2018).

Considering the biostimulation capacity of both silicon and nanoparticles, the combined use of both is expected to have a synergistic effect on the tolerance of plants against pathogens. Hence, the objective of the present research was to determine the effect of the application of copper nanoparticles and potassium silicate on tolerance to *C. michiganensis* in tomato.

2. Materials and methods

2.1. Crop development

A "El Cid F1" indeterminate growth tomato variety (Harris Moran, Davis, CA, USA) of the saladette type was used for this experiment. The transplant took place in black 10 L capacity polyethylene bags. The crop was established under greenhouse conditions, with average 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 106 days, and the yield of the crop considered the total of fruits harvested in that period. A substrate composed by a mixture of perlite-peat moss (1:1 on volume base) was used with a directed irrigation system. The Steiner nutrient solution was used for crop nutrition (Steiner, 1961) using the following micronutrients in chelated form using EDTA (2,2′,2″,2‴-[Ethane-1,2-diyldinitrilo] 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 in the combined application of silicon and copper nanoparticles (Cu NPs) in two doses namely low dose (LD) and high dose (HD), with a total of six treatments: 1) 50 mg L^{-1} Cu NPs + 184 mg L^{-1} Si (Cu NPs LD + Si LD); 2) 50 mg L^{-1} Cu NPs + 460 mg L^{-1} Si (Cu NPs LD + Si HD); 3) 250 mg L^{-1} Cu NPs + 184 mg L^{-1} Si (Cu NPs HD + Si LD); 4) 250 mg L^{-1} Cu NPs + 460 mg L^{-1} Si (Cu NPs HD + Si HD), these four treatments inoculated with Cmm, 5) A treatment inoculated with Cmm and without application of Si or Cu NPs (Cmm), and 6) A Control (T0). Five applications of treatments in total were made by foliar way, and at intervals of 14 days starting from two weeks after the transplant. As source of Si potassium silicate was used (K_2SiO_3). While the Cu NPs used were of average size of 42 nm and spherical shape and were synthesized according to the methodology of (Cadenas-Pliego et al., 2013). Each treatment consists of 16 plants, so the full experiment considers 90 plants of tomato.

2.3. Inoculation and severity of Clavibacter michiganensis

The bacterium *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) was obtained from the sap of diseased tomato plants. The sap was placed in Petri dishes with NYA culture medium and incubated at 29 °C for 48 h. The colonies obtained were analyzed by morphological and biochemical tests for their identification. The bacterium was increased in Petri dishes with NYA culture medium (nutritive broth 0.8%, yeast extract 0.2%, K_2HPO_4 0.2%, KH_2PO_4 0.025%, agar 1.5%). The Petri dishes were incubated at 29 °C for 48 h, after which the bacterial growth was collected.

The plants corresponding to the treatments with Cmm were inoculated at 28 days after transplanting (DAT). A solution of 1×10^6 colony forming units (CFU) per milliliter of Cmm was prepared. Cuttings were made on the leaves of the tomato plants and were immersed in 30 mL of bacterial solution for 5 min, and the remainder was sprinkled on the foliage. The severity of *Clavibacter michiganensis* in tomato plants was determined following the method of Baysal et al. (2003).

2.4. Biochemical variables

To determine proteins, catalase, ascorbate peroxidase, superoxide dismutase, glutathione peroxidase, phenylalanine ammonium lyase, reduced glutathione, ABTS, and DPPH antioxidant capacity, samples of leaf tissue were collected. For this purpose, after 73 days of transplantation, random plants were selected, and the third fully expanded young leaf was taken for biochemical analysis. Samples were stored at −80 °C until use. For the enzymatic and non-enzymatic determination, 200 mg of lyophilized leaves of each treatment and 20 mg of polyvinylpyrrolidone were weighed. After this, 1.5 mL of phosphate buffer with a pH of 7–7.2 (0.1 M) were added, and the mixture was then subjected to micro-centrifugation at 16,128 × *g* for 10 min at 4 °C. The supernatant was filtered with a nylon membrane (Ramos et al., 2010). Dilutions of the extract were prepared in a ratio of 1:20 with the phosphate buffer.

The quantification of total proteins was determined using Bradford's colorimetric technique (Bradford, 1976), and were expressed in mg g^{-1} of dry weight (DW). Catalase (CAT) (QE 1.11.1.6) enzymatic activity was quantified by the spectrophotometric method used by Dhindsa et al. (1981), and 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. Determination of Superoxide dismutase (SOD) (QE 1.15.1.1) enzymatic activity was carried out using the SOD Cayman 706002® kit, 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} per total proteins (mg g^{-1}). The Glutathione Peroxidase (GPX) (QE 1.11.1.9) enzyme was determined with the method adapted by Xue et al. (2001) using H_2O_2 as the substrate, 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 measurement of the enzymatic activity of ascorbate peroxidase (APX) (QE 1.11.1.1) was carried out according to what was established by Nakano and Asada (1987), the enzymatic activity was expressed as U per total proteins (mg g^{-1}), where U is equal to μmol QE of oxidized ascorbate per milliliter per minute. The measurement of the enzymatic activity of ascorbate peroxidase (APX) (QE 1.11.1.1) was carried out according to what was established by Nakano and Asada (1987), the enzymatic activity was expressed as U per total proteins (mg g^{-1}), where U is equal to μmol QE of oxidized ascorbate per milliliter per minute. Phenylalanine