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METHODS FOR INOCULATING *Xanthomonas citri* subsp. *citri* IN SWEET ORANGE (*Citrus sinensis*) AND STUDY OF HOST RESPONSES TO INFECTION

MÉTODOS DE INOCULAÇÃO DE Xanthomonas citri subsp. citri EM LARANJA DOCE (Citrus sinensis) E ESTUDO DAS RESPOSTAS DO HOSPEDEIRO A INFECÇÃO

MÉTODOS DE INOCULACIÓN DE Xanthomonas citri subsp. citri EN NARANJA DULCE (Citrus sinensis) Y ESTUDIO DE LAS RESPUESTAS DEL HUÉSPED A LA INFECCIÓN

Diego Henrique Pereira Catani:

E-mail: diegocatani@gmail.com

Carlos Alexandre Zanutto*:

E-mail: cazanutto@uem.br * Corresponding author

Fernando Teruhiko Hata:

E-mail: fthata@uem.br

Nathan Victor Botta:

E-mail: ra117645@uem.br

Terezinha Aparecida Guedes:

E-mail: taguedes@uem.br

William Mário de Carvalho Nunes:

E-mail: wmcnunes@uem.br

ABSTRACT:
The Citrus Canker (CC) caused by *Xanthomonas citri* subsp. *citri* (Xcc), is responsible for severe damage to cultivated *Citrus* plants worldwide. The selection of resistant genotypes is the most effective and environmentally sustainable way to manage CC, providing long-term solutions. Breeding for disease resistance and, consequently, genotype selection requires efficient screening techniques. This study aims to identify an inoculation method that is easy to use on a large scale and responsive to screening for CC-resistant genotypes under greenhouse conditions. The study was conducted under a greenhouse at the Núcleo de Pesquisa em Biotecnologia Aplicada in two replicates from January to March of 2017 and 2018. Three inoculation methods were performed with five bacteria concentrations. The activities of three antioxidant enzymes were evaluated in the second experiment. The results indicated that needle wound inoculations with concentrations between $1.39x10^5$ and $1.52x10^5$ CFU ml^{-1} to inoculate *Xcc* were the most favorable to disease progress. Catalase e Peroxidase showed a significant increase of activity over time among treatments, especially to needle wound with bacteria.

KEYWORDS: *Citrus* canker; Inoculation methods; Bacterial concentration.

RESUMO:
O Cancro Cítrico (CC) causado por Xanthomonas citri subsp. citri (Xcc), é responsável por danos severos a plantas cítricas. A seleção de genótipos resistentes é a maneira mais eficaz e ambientalmente sustentável de gerenciar o CC, fornecendo soluções de longo prazo. Uma vez que o melhoramento de plantas para a resistência à doenças e a seleção de genótipos requer técnicas de seleção eficientes. Este estudo tem como objetivo determinar um método de inoculação que seja fácil de usar em larga escala e que responda à triagem de genótipos resistentes em condições de casa de vegetação. O estudo foi conduzido em casa de vegetação, em dois experimentos de janeiro a março de 2017 e 2018. Três métodos de inoculação foram realizados com cinco concentrações de bactérias. A atividade de três enzimas antioxidantes foi avaliada no segundo experimento. O resultado indicou que inoculações com ferida de agulha com concentrações entre $1,39x10^5$ e $1,52x10^5$ UFC ml^{-1} para inocular Xcc, foram as mais favoráveis para o progresso da doença. A catalase e a peroxidasse mostraram um aumento significativo da atividade ao longo do tempo entre os tratamentos, especialmente em feridas com bactérias.

PALAVRAS CHAVE: Cancro cítrico; Métodos de inoculação; Concentração bacteriana.

RESUMEN:
El cancro de los cítricos (CC), causado por Xanthomonas citri subsp. citri (Xcc), es responsable de graves daños a las plantas cultivadas de cítricos en todo el mundo. La selección de genotipos resistentes es la forma más eficaz y ambientalmente sostenible de gestionar el CC, proporcionando soluciones a largo plazo. Dado que el mejoramiento para la resistencia a las enfermedades y, en consecuencia, la selección de genotipos, requiere técnicas de selección eficientes, este estudio tiene como objetivo identificar un método de inoculación que sea fácil de usar a gran escala y que responda a la selección de genotipos resistentes al CC en condiciones de

invernadero. El estudio se realizó en invernadero en el Núcleo de Pesquisa em Biotecnologia Aplicada en dos réplicas de enero a marzo de 2017 y 2018. Se realizaron tres métodos de inoculación con cinco concentraciones de bacterias. Las actividades de tres enzimas antioxidantes se evaluaron en el segundo experimento. Los resultados indicaron que las inoculaciones con herida de aguja con concentraciones entre 1,39x105 y 1,52x105 UFC ml-1 para inocular Xcc, fueron las más favorables para el progreso de la enfermedad. La catalasa y la peroxidasa mostraron un aumento significativo de actividad a lo largo del tiempo entre los tratamientos, especialmente en la herida de la aguja con bacterias.

Palabras clave: Cancro de los cítricos; Métodos de inoculación; Concentración bacteriana.

INTRODUCTION

The Asiatic *Citrus* Canker (ACC), caused by *Xanthomonas citri* subsp. *Citri* (Schaad et al. 2006) (Xcc) is responsible for severe damage to cultivated *Citrus* plants worldwide, being an A2 quarantine in most *Citrus* production countries, such as Brazil and the USA (Carvalho et al. 2015; MAPA 2018; EPPO 2018). ACC typically occurs on seedlings and young and adult trees of susceptible hosts, infecting the leaves, shoots, twigs, and fruits through minor injuries caused by wind, thorns, insects, and physical or mechanical damage, followed by the presence of water. Xcc is naturally spread during rain splashing, and wind contributes to increasing the effectiveness of dispersal. Attacks of *Phyllocnistis citrella*, a Citrus leaf miner, increase susceptibility to the disease (Hall et al. 2010; Bock et al. 2010).

The typical symptom of ACC consists of necrotic lesions surrounded by oily with water-soaked margins and yellow chlorotic rings (Brunings and Gabriel 2003), which currently results in fruit quality and crop loss issues, which directly affects trade in fruits (Gottwald et al. 2009; Behlau et al. 2010), and in advanced disease conditions, defoliation of the plant, drying of branches and even fruit drop can occur (Gottwald et al. 1989).

Resistance is related within *Citrus*

relatives, widely varying between species, and screening for ACC-resistant genotypes has been achieved by various methods under greenhouse and field conditions. The selection of resistant genotypes is the most effective and environmentally sustainable way to manage ACC, providing long-term solutions; concomitantly, with the development of new germplasms, screening for ACC-resistant genotypes will become an important component of disease management (Peltier 1918; Kozumi 1981; Leite and Mohan 1984; Namekata et al. 1992; Gottwald et al. 1993; Burhan et al. 2007; Amaral et al. 2010; Francis et al. 2010; Stover et al. 2014; Carvalho et al. 2015; Gonçalves-Zuliani et al. 2016). Since breeding for disease resistance and consequently genotype selection requires efficient screening techniques, in addition to the evaluation of a large number of genotypes, artificial inoculation techniques can produce rapid results and, at the same time, a pattern of high levels of infection, ultimately resulting in a neat difference between susceptible and resistant genotypes (Salaheddin et al. 2005).

For the genus *Xanthomonas*, two methods are commonly used to inoculate plants: by hydathodes and by artificial wounds. The following wound inoculation methods can be used:

injection infiltration, carborundum abrasion, pressurized spraying, hypodermic syringe, needle wound or pin pricking, and clipping with scissors and sandpaper (Topp et al. 1993; Salaheddin et al. 2005; Peñazová et al. 2018).

Considering the economic and social importance of Citrus worldwide, there is a compelling reason to ensure alternatives for the sustainable production of this crop. Thus, this study aimed to determine an inoculation method that is easy to use on a large scale and responsive to screening for ACC-resistant genotypes under greenhouse conditions to contribute to the selection of resistant citrus genotypes.

MATERIALS AND METHODS

Species of citrus. Plants of the sweet orange genotype Pera IAC (*C. sinensis* (L.) Osbeck) were evaluated in this study. The genotype was derived from Viveiro de Mudas Pratinha (VMP) (23°08'28.3"S 52°04'15.6"W) in Atalaia, Paraná, Brazil. Nursery trees were cultivated in a greenhouse, grafted onto Rangpur lime (*Citrus limonia* Osbeck) rootstock, and subsequently grown in a substrate composed of pine bark and vermiculite within plastic containers (15 x 35 cm, Wolff Klabin, MEC Prec).

Experiment location and management. This study was conducted in a greenhouse at the Núcleo de Pesquisa em Biotecnologia Aplicada (23°23’57.8” S, 51°57’5.3”W, 500 m in elevation) in two replicates from January to March of 2017 and 2018. Corresponding to Koppen’s climate, Alvares et al. 2014) have a tropical climate with hot summer temperatures and an average relative humidity of 66%. The average annual temperature is 16.7°C, reaching an average maximum of 33.6°C, which means that the temperature is above 22°C in the warmest month, but it is noted that under greenhouse conditions, temperatures might be as high as the estimated temperature.

Trees were subjected to standard nursery management practices, with periodic fertilization with N, P, K, and micronutrients. The water was supplied using an automated drip irrigation system. Chemical control was applied to Citrus leaf miner, psyllids, aphids, whiteflies, and mites. No bactericidal products or copper-based fertilizers were applied to the plants. Pruning was performed approximately 60 days before inoculation to obtain homogeneous leaves at the 75 to 100% stage of leaf expansion (Viloria et al. 2004).

Bacteria inoculum. The isolate selected for genotype inoculation was Xcc 306, which was stored at 4°C in a suspension of Phosphate Buffer Saline (PBS) (0,075 M, pH 7.0) (Xcc 306; GenBank accession number NC_003919) supplied by the Collection of Pathogen Cultures at the Fundo de Defesa da Citricultura (Fundecitrus, Araraquara, São Paulo, Brazil). The isolate Xcc 306 was previously confirmed to be the Xcc pathotype ‘A’ and was previously sequenced by the Genome Project/FAPESP (Da Silva et al. 2002).

Fresh Xcc was obtained by transferring the bacterial suspension to the nutrient agar Mannitol Glutamate Yeast (10 g of mannitol, 2 g of L-glutamic acid, 1 g of yeast extract, 0.5 g of potassium phosphate, 0.2 g of sodium chloride, 0.2 g of magnesium sulfate heptahydrate and 15 g of agar per liter of distilled water) in Petri plates and incubated for approximately 48 h at 28°C (Behlau et al. 2012).

Inoculation methods. Three methods were used:

A) *The needle-wound technique* consisted of immersing the needle (0.55×0.20 mm) in the bacterial suspension each time before injuring the leaf and performing eight equidistant stab punctures on the abaxial side.

B) *The cotton-rubbing technique* consisted of wetting leaves on the abaxial and adaxial sides and rubbing the cotton previously soaked in the bacterial suspension.

C) *The spray technique* consisted of wetting leaves with a spray bottle (200 ml) on the abaxial and adaxial sides, with the bacterial suspension sprayed to the point of dripping off;

Bacterial concentration and inoculation. Five bacterial concentrations were adjusted for use among the inoculation methods. To prepare the concentrations, a spectrophotometer (Mod. UV5, Mettler Toledo, USA), was photometrically adjusted in PBS to a bacterial concentration of $1x10^5$ CFU ml^{-1} (i.e., an optical density (OD) of 0.3 at 600 nm) (Belasque JR. and Jesus JR. 2006). Then, the concentrations of bacteria for the experiments were prepared using 5 ODs (i.e., ODs of 0.1, 0.2, 0.3, 0.4 and 0.5 at 600 nm), plus a control, using distilled water.

The three methods were inoculated on the same day to obtain the same conditions. Furthermore, the greenhouse was humidified by wetting the greenhouse ground at 1 day after the experiment implantation, and the procedure was repeated for 3 consecutive days, avoiding wetting the inoculated leaves, to provide a suitable environment for the establishment of the disease (Gonçalves-Zuliani et al. 2016).

After the bacteria were prepared for the experiments, their concentration was quantified from the correlation of colony forming units (CFU). Furthermore, needle-wound inoculated leaves were collected at 1 and 9 days after inoculation (DAI), and the bacterial population was quantified by CFU. Each treatment had 5 replicates. To obtain Xcc CFUs, a 1 cm diameter leaf disc with centralized ACC lesion was cut with a metallic cylinder, and external leaf tissue disinfection was performed (sodium hypochlorite for 1 min, anhydrous alcohol for 30 s, and 3 rinses with distilled water for 30 s). Subsequently, the bacteria were extracted in 1 ml of PBS, followed by 3 serial dilutions, disposed of in 5 Petri plates for each OD, and incubated for approximately 72 h at 28°C.

Disease assessment. To evaluate ACC progression in the host, 7 evaluations were made over time in the same lesions every 9 days (01, 09, 18, 27, 36, 45, and 54 DAI) to determine the disease evolution pattern. Disease severity was assessed by measuring the diameters of 8 lesions on 3 distinct plants, with 24 replicates for each evaluation for each treatment (3 inoculation methods with 5 bacteria concentrations) plus a control inoculated with distilled water via a needle-wound approach. The diameter was measured with the aid of a digital caliper (0.01 mm resolution, Mitutoyo, Japan) on the abaxial side of the leaf, not considering the characteristic yellow halo of the disease.

Antioxidant activities. Among the inoculation methods, the activities of three antioxidant enzymes were evaluated in the second experiment, from January to March 2018,

exclusively to an OD of 0.3 at 1 and 9 DAI. To obtain the extract samples, at 1 DAI, 2 leaves were collected from 5 replicates of distinct plants for each method (needle-wound, cotton-rubbing and spray) plus from plants inoculated with distilled water to obtain the activity of the enzymes without consumption by the disease, as well as from the control, from plants not inoculated. The procedure was repeated for 5 distinct plants at 9 DAI. Subsequently, leaf protein extraction was performed. The total soluble proteins in the extract were quantified to determine their correlation with the enzymes, and the enzyme activity was quantified with a spectrophotometer (Mod. UV5, Mettler Toledo, USA), determined by each specific method:

Protein extraction. Fresh leaves were collected, wrapped in aluminum foil, rapidly stored on ice, and taken to the Núcleo de Pesquisa em Biotecnologia Aplicada. To obtain the crude extract, 1 g of leaves was crushed in liquid nitrogen and homogenized with 3 ml of 100 mM phosphate buffer (pH 7.5) containing 3 mM DTT, 1 mM EDTA, and 0.01 g polyvinylpolypyrrolidone (PVPP). The samples were centrifuged at 10000 × g for 30 minutes at 4°C, and the supernatant was stored in a freezer at -80°C.

Total soluble proteins. Total soluble proteins (TSP) were measured by the Bradford method (Bradford 1976). Albumin (50 mg%) was used as a standard, and 20 µl of appropriately diluted sample extract was added to test tubes containing 1.0 ml of Bradford reagent. The reaction was observed in a spectrophotometer at 595 nm after 5 min of reaction. The TSP concentration was expressed in mg/mL^{-1}.

Superoxide dismutase (EC 1.15.1.1). Superoxide dismutase (SOD) activity was determined by the reduction of nitrotetrazolium blue (NBT) (Giannopolitis and Ries 1977) based on the ability of the enzyme to convert superoxide anions to hydrogen peroxide and molecular oxygen. The reaction medium (1.5 ml) contained 50 mM phosphate buffer (pH 7.8), 1 mM EDTA, 75 µM NBT, 13 mM L-methionine, 4 µM riboflavin, and 20 µl of extracted enzyme. The reaction mixture was placed under light for a period of 10 min at 25°C. The reduction of NBT was monitored with a spectrophotometer at 560 nm. An enzyme unit (U) for SOD was defined as the amount of enzyme required to cause 50% inhibition of the NBT reduction rate measured. The results are expressed as $U \times g^{-1} \times$ fresh biomass (FW).

Catalase (EC 1.11.1.6). Catalase (CAT) activity was determined by the H_2O_2 consumption of the reaction medium (Tomanková et al. 2006). The reaction medium (1.1 ml) contained 50 µl of 0.1 M phosphate buffer (pH 7.0) and 50 µl of the enzyme extract. After the test tubes were placed in a water bath at 38°C, the reaction was started by adding 0.5 ml of 60 mM H_2O_2 and quenched after 4 min with the addition of 0.5 ml of 0.34 mM ammonium molybdate. The activity of CAT was determined at 405 nm. The results are expressed as $\mu mol \times min^{-1} \times g \times FW$.

Peroxidase (EC 1.11.1.7). Peroxidase (POD) activity was determined by the oxidation of guaiacol. The reaction medium (2.6 ml) contained 25 mM phosphate buffer (pH 6.8), 2.58 mM H_2O_2, and 10 mM guaiacol. The reaction was started by adding 0.4 mL of enzyme extract diluted in 67 mM phosphate buffer (pH 7.0). After 5 min of reaction, guaiacol oxidation was monitored with a spectrophotometer at 470 nm. The POD activity was calculated using an extinction coefficient to tetraguaiacol of 25.5 mM^{-1} cm^{-1}. The results are expressed as $\mu mol \times min^{-1} \times g^{-1} \times FW$.

Scanning electron microscopy. To observe the bacterial distribution and gum on the leaf surface among the inoculation methods utilized in this study and at OD values of 0.3, 0.4 and 0.5, images were taken by Scanning Electron Microscopy (SEM) (Quanta 250, FEI Company, Oregon, USA) at 500x, 5000x, 10000x and 50000x magnification. To prepare the samples, 2 leaves were cut around the lesions (3 mm^2) with a stainless steel razor blade at 1 DAI to obtain 5 replicates of each inoculation method and 5 replicates of each bacterial concentration. Then, the samples were subjected to a fixative procedure in a modified Karnovsky solution (glutaraldehyde 2.5%, formaldehyde 2.5% in 0.05 M sodium cacodylate buffer, pH 7.2, and CaCl2 0.001 M) and maintained in a refrigerator.

The samples were dehydrated for 10 min each in a series of anhydrous alcohol diluted in distilled water (30, 50, 70, 80, 90, 100, 100 and 100%) and dried in a critical-point drier using a Baltec CPD 030 (Bal-Tec Inc. - Balzers - Liechtenstein) to replace the ethanol with carbon dioxide. Sections were mounted on aluminum stubs with abaxial and adaxial sides, sputter coated with gold (3 cycles of 5 min at 6 mA current in a Baltec SCD 050 metallizer (Bal-Tec Inc. - Balzers - Liechtenstein)), and subsequently examined under SEM at 20 kV.

Data analysis. To evaluate disease progression, a completely randomized design was used, with 3 inoculation methods as treatments and 24 repetitions represented by the lesion diameters for each experiment. For each repetition, the diameter obtained at 01 DAI, which represents the diameter of the needle wound, was subtracted from all 7 measurements. Subsequently, the area under the disease progress curve (AUDPC), calculated using the software Win AACPD, was determined to quantify disease progression over time, as described by Campbell and Madden (Campbell and Madden 1990), where Y represents the average diameter evaluated in mm; t is the time of each evaluation of the variable; (Y i + Y i-1) corresponds to the mean height of the rectangle between points Y i and Y i-1; and (t i - t i-1) is the difference in the base of the rectangle between points t i-1 and t I, which corresponds to the time interval, in days, between two sequential evaluations.

$$AUDPC = \Sigma\ [([Y\,i + Y_{i-1}]/2)\ (t\,i - t\,i_{-1})] \quad (1)$$

A pairwise comparison was made using Tukey's post hoc test for all data, using the function “Tukey HSD” from R software (v.3.5.2) (R Core Team 2017). In these analyses, the treatments were used as a factor. To verify the existence of groups of treatments, a general linear model was constructed through the function “glm”; subsequently, Tukey was applied to adjust pairwise comparisons through the function “HSD.test”.

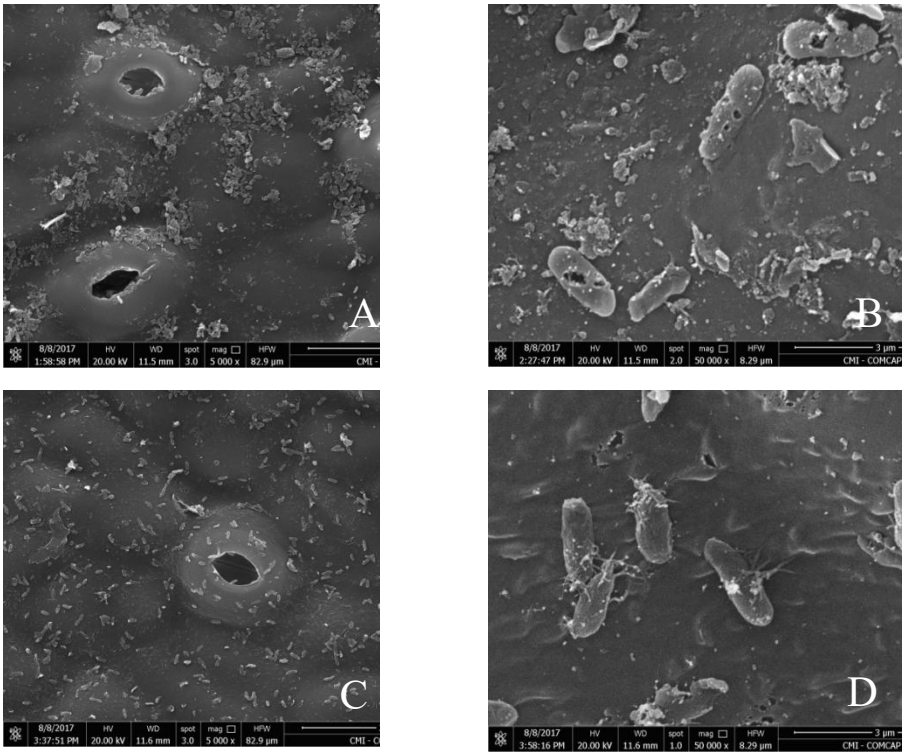
RESULTS

Inoculation methods. A positive and significant relationship between bacteria concentration and diameter lesion ($F = 15.24,\ P < 0.001$). In addition, it was observed that the needle-wounding technique presented a stable and reproducible pattern among ACC lesions; nevertheless, the inoculation methods, cotton-rubbing and spray techniques, presented no disease lesions over time in the conditions of this study (Figure 1).

The symptoms were characteristic of ACC, showing progressive evolution over time, being observed at first at 09 DAI as small yellow aqueous canker symptoms around the wound, and except for control, with the absence of canker, it was not possible to visually distinguish differences of lesion diameters among treatments. Furthermore, it was noted symptoms of hypersensitivity response (HR) around the wounds at 18 DAI on the second experiment, for all treatments, which led us to keep the experiments data analysis for AUDPC separated, even though the disease was able to overcame the defense and generate ACC as well as the first experiment.

Bacterial concentration. The bacterial concentrations were obtained by the optical density of a spectrophotometer adjusted to $1x10^5$ CFU ml^{-1} at an absorbance of 0.3 at 600 nm, with no centrifugation, and the bacteria quantification was obtained by the correlation of UFCs. There was a significant difference among the bacterial solution concentrations ($F = 545.36$, $P < 0.001$) used for inoculation, where the OD 0.1 ($3.44x10^4$ CFU ml^{-1}) and OD 0.2 ($4.60x10^4$ CFU ml^{-1}) treatments had fewer bacteria than did the other treatments, and the OD 0.3 ($1.39x10^5$ CFU ml^{-1}) treatment had fewer bacteria than did the OD 0.5 ($1.55x10^5$ CFU ml^{-1}) treatment, however, the concentration of OD 0.4 ($1.52x10^5$ CFU ml^{-1}) did not contrast with the OD 0.3 or OD 0.5 (Figure 1).

Figure 1 - Bacteria on the abaxial side of the leaf surface after 1 DAI (day after inoculation) for cotton-rubbing (A and B) and spray (C and D) techniques (OD 0.3: $1.39x10^5$ CFU ml^{-1}). Showing bacteria distribution (5000x of magnitude: A and C), dead bacteria from cotton friction (50000x: B), and first impressions of gum formation from bacteria (50000x: D)



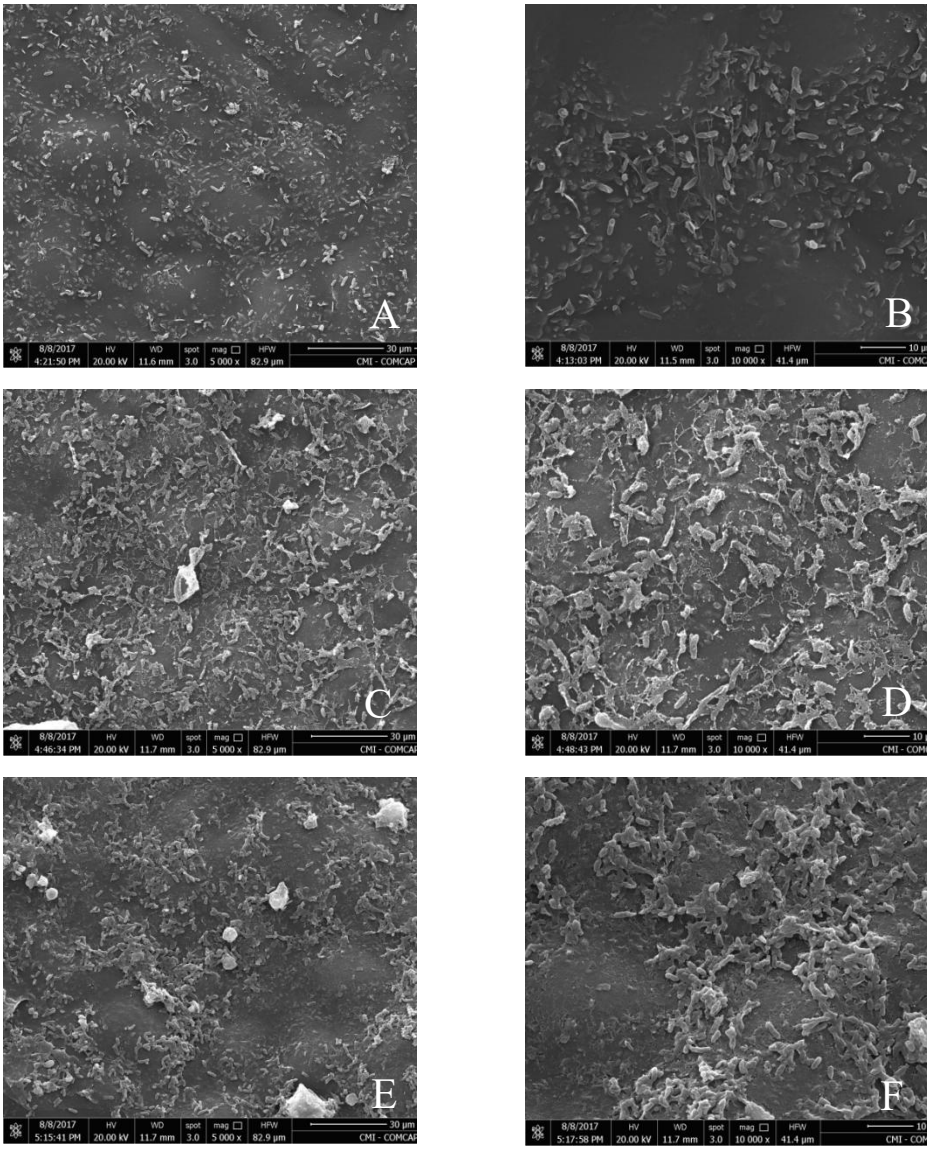
Source: Authors (2024).

However, at higher concentrations ($4.7x10^9$ to $6.0x10^9$ CFU ml^{-1}), at which point the bacteria were collected into microtubes from UFCs and inoculated in hosts without dilution, it

was decided to maintain the concentrations of ODs obtained by the spectrophotometer to preserve the bacterial gum and prevent symptoms of high hypersensitivity.

A positive and significant relationship was observed between bacterial concentration and time (*F = 614.85, P < 0.001*) (Figure 2), with a progressive increase in bacteria (*F = 411.22, P < 0.001*) to OD 0.1, four times more bacteria ($1.35x10^5$ CFU ml^{-1}), and OD 0.2, three times more bacteria ($1.38x10^5$ CFU ml^{-1}) at 9 DAI than at the initial concentration.

Figure 2 - Bacteria and gum distribution on the adaxial side of the leaf of the spray technique after 1 DAI (Day After Inoculation) for Optical Density (OD) 0.3 (1.39x105 CFU ml-1) (A and B), OD 0.4 (1.52x105 CFU ml-1) (C and D) and OD 0.5 (1.55x105 CFU ml-1) (E and F), with 5000x (A, C and E) and 10000x (B, D and F) of magnitude



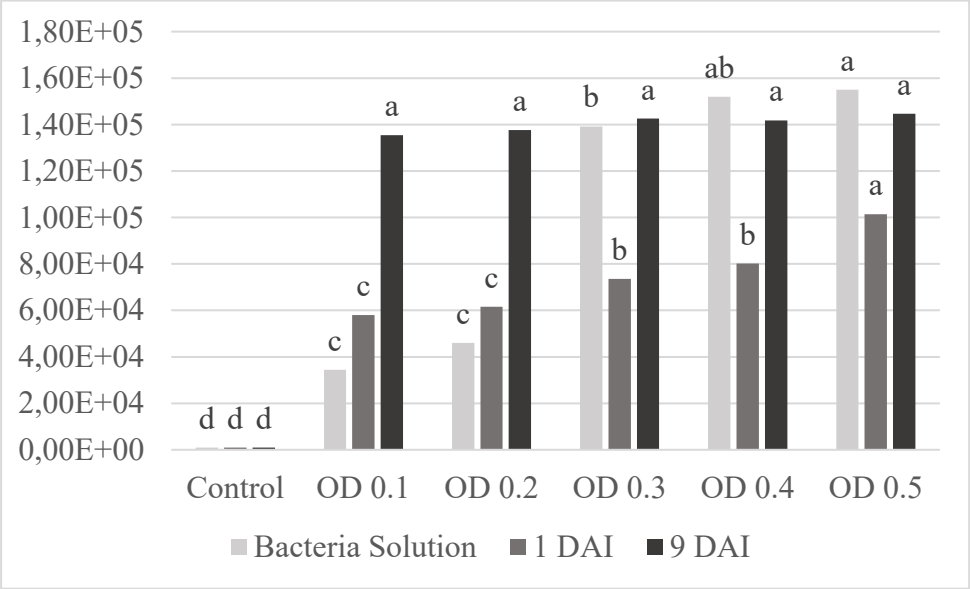
Source: Authors (2024).

After the bacteria were extracted from the leaves at 01 DAI (*F = 252.59, P < 0.001*) (Figure 3), the OD of 0.5 ($1.01x10^5$ CFU ml^{-1}) was significantly greater than that of the other

treatments, and the OD of 0.3 ($7.36x10^4$ CFU ml^{-1}) and OD of 0.4 ($8.02x10^4$ CFU ml^{-1}) were greater than the OD of 0.1 ($5.80x10^4$ CFU ml^{-1}) and OD of 0.2 ($6.16x10^4$ CFU ml^{-1}), respectively.

In the third evaluation, at 09 DAI (*F = 540.75, P < 0.001*) (Figure 4), except for the control, all the treatments had significantly equal bacterial concentrations, ranging from $1.35x10^5$ to $1.44x10^5$ CFU ml^{-1}.

Figure 3 - The means of bacteria concentration for the treatments Control, OD 0.1, OD 0.2, OD 0.3, OD 0.4, OD 0.5, at the inoculation day (Bacteria Solution) (F = 614.85, P < 0.001), 1 DAI (F = 252.59, P < 0.001) and 9 DAI (Days After Inoculation) (F = 540.75, P < 0.001) are presented with letters from a Tukey pairwise comparison

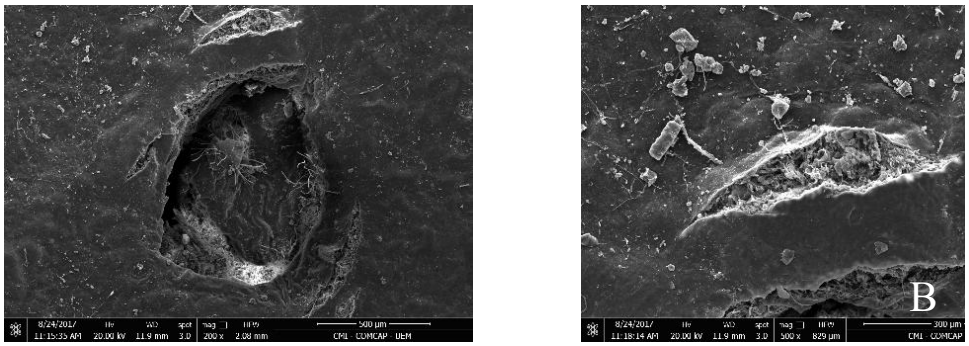


Source: Authors (2024).

Area under the disease progression curve (AUDPC). There was a significant AUDPC for lesion diameter in both greenhouse experiments. In the first experiment (*F = 1331.60, P < 0.001*), an OD of 0.1 presented a significantly lower mean AUDPC of 98 than did the other treatments, which ranged from 105 to 110 (Figure 5).

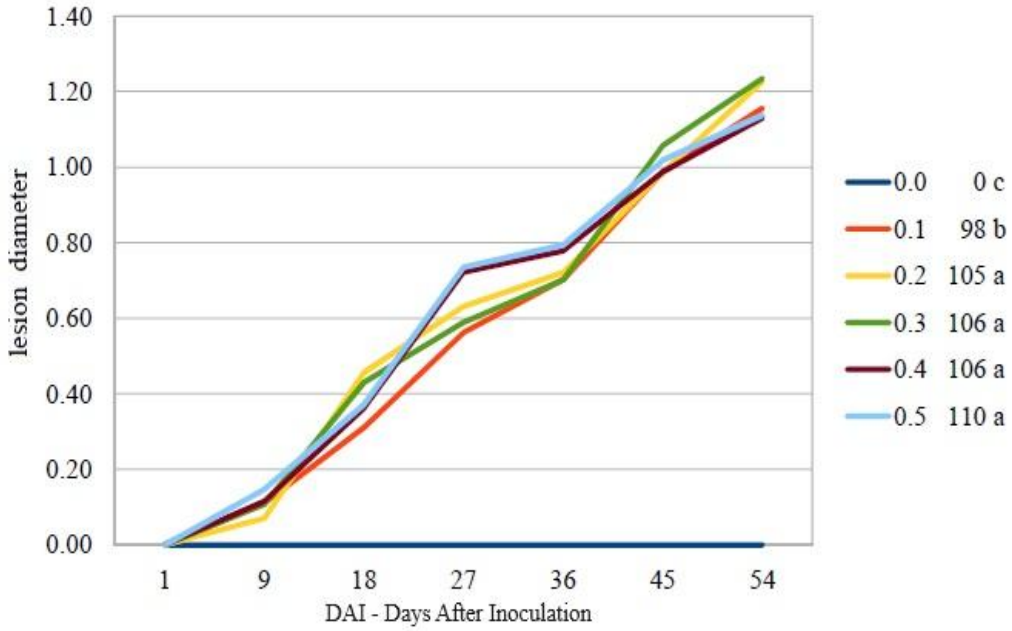
In the second experiment (*F = 820.81, P < 0.001*), an OD of 0.4 ($1.52x10^5$ CFU ml^{-1}) presented an AUDPC mean of 145, which was greater than that of the other treatments (OD of 0.1, 0.2 and 0.5), which means that the OD ranged from 133 to 137, except for OD of 0.3 (140), which was different from that of the control (Figure 6).

Figure 4 - Asiatic Citrus Canker (ACC) symptom after 9 DAI (Days After the Inoculation) with needle-wounding technique (OD 0.3: $1.39x10^5$ CFU ml^{-1}), with 200x (A) and 500x (B) of magnitude



Source: Authors (2024).

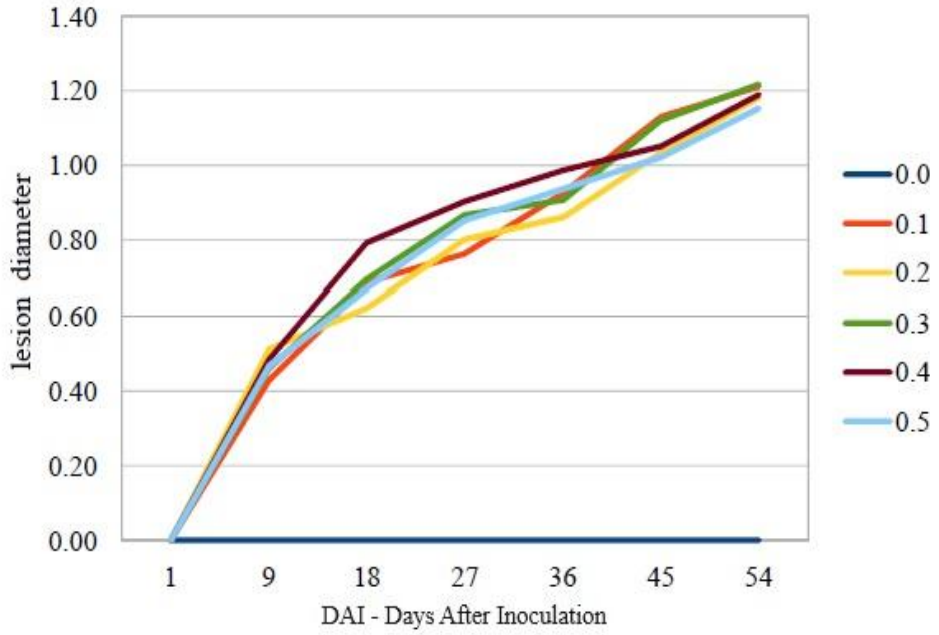
Figure 5 - The means of diameter for the first experiment is presented in lines over 7 evaluation times for the treatments OD 0.0 (control), OD 0.1, OD 0.2, OD 0.3, OD 0.4, OD 0.5. AUDPC is presented with letters from a Tukey (F = 1331.60, P < 0.001) pairwise comparison



Source: Authors (2024).

Antioxidant activities. There was distinct behavior among the enzyme activities, where CAT and POD showed significant increases in activity from 1 to 9 DAI among treatments, especially for needle wounds with bacteria (T2). In contrast, SOD showed a significant decrease in activity from 1 to 9 DAI for treatments inoculated with Xcc, with an interesting decrease observed at 1 day after the spray technique (T4).

Figure 6 - The means of diameter for the second experiment is presented in lines over 7 evaluation times for the treatments OD 0.0 (control), OD 0.1, OD 0.2, OD 0.3, OD 0.4, OD 0.5. AUDPC is presented with letters from a Tukey (F = 820.81, P < 0.001) pairwise comparison



Source: Authors (2024).

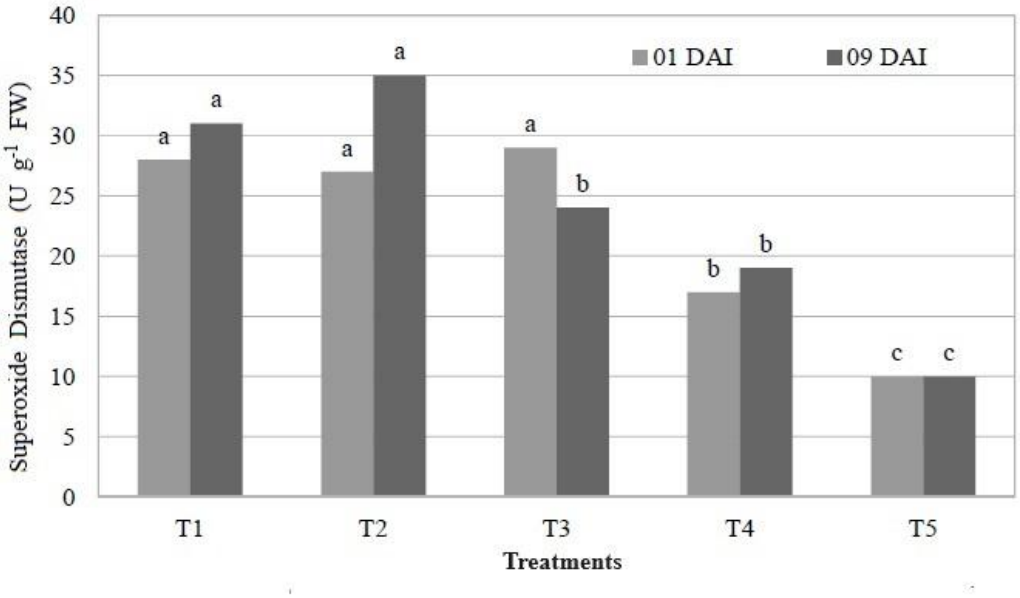
Superoxide dismutase. At 1 DAI ($F = 79.93$, $P < 0.001$), SOD activity was very responsive to the presence of Xcc on the leaf surface (Figure 7), where the spray (T4) (10 U g^{-1} FW) and cotton-rubbing (T3) (17 U g^{-1} FW) techniques significantly decreased SOD activity compared with the other treatments, which ranged from 27 to 29 U g^{-1} FW.

At 9 DAI, the spray (T4) (10 U g^{-1} FW) and cotton-rubbing (T3) (19 U g^{-1} FW) techniques showed no difference over time; however, likewise 1 DAI, they were significantly different from each other ($F = 41.28$, $P < 0.001$), in contrast to the control and the absence of bacteria in needle wounds (T1). Nevertheless, needle wounds with bacteria (T2) (19 U g^{-1} FW) showed a significant decrease of 20% over time ($F = 41.62$, $P < 0.001$), becoming equally significant to the cotton-rubbing technique (T3) at 9 DAI.

Catalase. At 1 DAI ($F = 4.57$, $P < 0.001$), the activity of CAT in the needle wound inoculated with bacteria (T2) (109 µmol min^{-1} g^{-1} FW) was higher than cotton-rubbing technique (T3) (68 µmol min^{-1} g^{-1} FW), however the other treatments, which means ranged from 79 to 99 µmol min^{-1} g^{-1} FW, presented a not significant contrast among all (Figure 8).

Nevertheless, at 9 DAI ($F = 5.03$, $P < 0.001$), all treatments except the control treatment showed a significant increase over time, and needle wounds inoculated with bacteria (T2) (131 µmol min^{-1} g^{-1} FW) showed a 60% increase in activity ($F = 4.57$, $P < 0.001$). Despite this, the activity did not differ among the other treatments ($F = 5.03$, $P < 0.001$), whereas the control, cotton-rubbing (T3) and spray (T4) techniques ranged from 108 to 110 µmol min^{-1} g^{-1} FW, which were significantly different from the absence of bacteria in the needle wound (T1), where the activity increased to 148 µmol min^{-1} g^{-1} FW.

Figure 7 - Least square means (lsmeans) ranking of Superoxide Dismutase (U g^{-1} FW) activity for the treatments needle wound absent of bacteria (T1), needle wound with bacteria (T2), cotton-rubbing technique (T3) and spray technique (T4), presented with letters from a Tukey pairwise comparison at 1 DAI (Day After Inoculation) (F = 79.93, P < 0.001) and 9 DAI (F = 41.28, P < 0.001)



Source: Authors (2024).

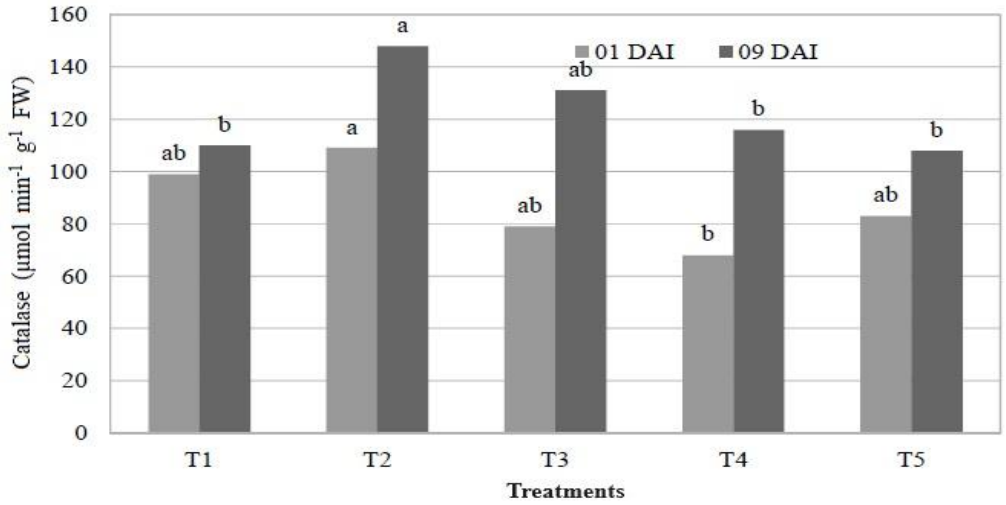
Peroxidase. At 1 DAI (*F = 14.45, P < 0.001*), the values for the control, needle wound without bacteria (T1) and cotton-rubbing technique (T3) treatments were significantly equal, with means ranging from 1.43 to 1.66 µmol min^{-1} g^{-1}, but significantly greater than those for the needle wound inoculated with bacteria (T2) and spray technique (T4) treatments, which were significantly equal, with means ranging from 0.93 to 0.98 µmol min^{-1} g^{-1} (Figure 9).

The activity of POD was very responsive to leaf lesions at 9 DAI (*F = 51.43, P < 0.001*). The activity of needle wound absent of bacteria (T1) (3.46 µmol min^{-1} g^{-1} FW) was significantly greater than that in the other treatments and was two times greater than that at 1 DAI.

Respectively, cotton-rubbing technique (T3) (2.11 µmol min^{-1} g^{-1} FW) showed a higher significance than (T2) and spray technique (T4), which were significantly equal at 1 DAI however, in this evaluation, POD activity was also equal to that of the control, with values ranging from 1.39 to 1.61 µmol min^{-1} g^{-1} FW. The enzyme activity significantly increased over time for all treatments except for the control, even with the establishment of the disease at the needle wound site inoculated with bacteria (T2) (*F = 75.02, P < 0.001).*

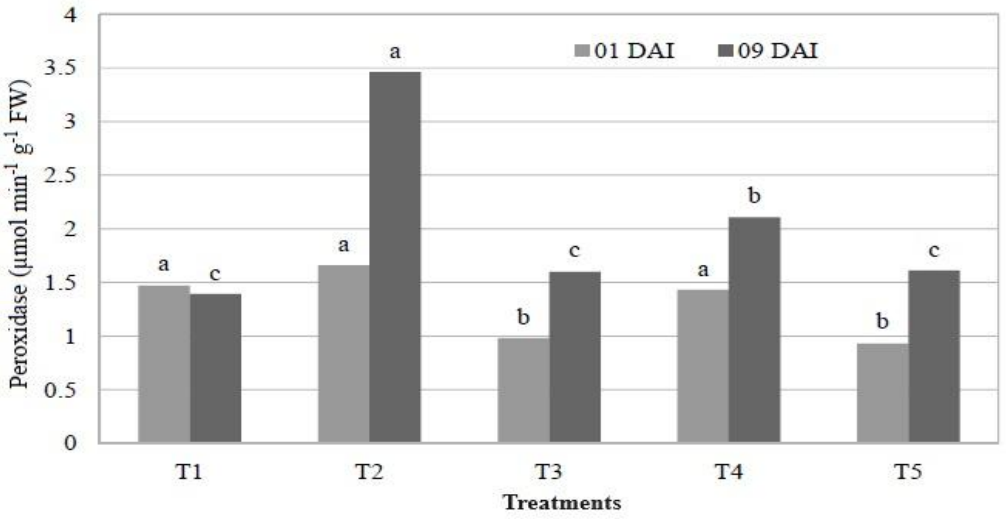
Figure 8 - Least square means (lsmeans) ranking of Catalase (µmol min^{-1} g^{-1} FW) activity for the treatments needle wound absent of bacteria (T1), needle wound with bacteria (T2), cotton-rubbing technique (T3) and spray technique (T4),presented with letters from a Tukey pairwise

comparison at 1 DAI (Day After Inoculation) (F = 4.57, P < 0.001) and 9 DAI (F = 5.03, P < 0.001)



Source: Authors (2024).

Figure 9 - Least square means (lsmeans) ranking of Peroxidase (µmol min^{-1} g^{-1} FW) activity for the treatments needle wound absent of bacteria (T1), needle wound with bacteria (T2), cotton-rubbing technique (T3) and spray technique (T4), presented with letters from a Tukey pairwise comparison at 1 DAI (Day After Inoculation) (F = 14.45, P < 0.001) and 9 DAI (F = 51.43, P < 0.001)



Source: Authors (2024).

DISCUSSION

The needle wound inoculation method was efficient, showing a direct relationship between bacterial concentration and diameter of the lesions, proving to be reliable for experiments that aim to study citrus genotypes regarding resistance to infection by the bacteria, as observed in previous studies (Belasque JR. and Jesus JR. 2006; Vernière et al. 2003). The symptoms in the experiments were characteristic of ACC, as described by Gottwald et al. (1993) and Gonçalves-Zuliani et al. (2016), showing progressive evolution over time. On the other hand, the cotton swab and spray inoculation methods were not efficient in promoting infection by the bacteria and leading to the formation of disease lesions over time under the conditions of this study.

The differences among the bacterial solution used for inoculation was related to a critical population threshold of approximately $1x10^3$ to $1x10^5$ bacteria per canker lesion and a quorum sensing mechanism (Da Silva et al. 2002). However, the ODs of 0.3, 0.4, and 0.5 presented a significant decrease in the number of bacteria in contrast to the inoculation solution, and these results indicate that the decrease in the bacterial concentration might be involved in host defense.

Except for the control, the third evaluation, at 09 DAI, showed that all the treatments had significantly equal bacterial concentrations, showing that bacteria were able to establish in the host and cause disease even at the lowest concentration used in this study.

In the conditions of this study, these results indicate that concentrations between $1.39x10^5$ and $1.52x10^5$ CFU ml^{-1} to inoculate Xcc were the most favorable for disease progression, even under conditions of host stress. In the first experiment, an OD of 0.1 presented a lower mean AUDPC than did the other treatments, indicating that inoculations with bacteria at concentrations less than $4.60x10^4$ CFU ml^{-1} might influence the decrease in symptoms of ACC.

The monitoring the Superoxide Dismutase (SOD) activity results, indicates that the decrease in SOD activity might be involved in host defense, as observed when used the needle wounds with bacteria (T2). A decrease in SOD activity was also observed in grapefruit and sweet orange, with a peak in activity occurring 4 days after inoculation and a rapid decline in activity similar to or less than that of the controls for the rest of the infection process (Kumar et al. 2011a; Kumar et al. 2011b).

Related to the activity of Catalase (CAT), all treatments except the control treatment showed a significant increase over time and as expected, needle wounds inoculated with bacteria was the one that increased activity the most. Nonetheless, the activity increased in all the treatments, showing that the lesion itself was able to induce CAT activity. Similar results showed an increase in activity in grapefruit, starting at 2 DAI and extending until 16 DAI (Kumar et al 2011a). In addition, besides control not showing a significant difference at 9 DAI to spray technique, it showed that the presence of Xcc on the leaf surface was sufficient to induce CAT activity ($F = 6.37$, $P < 0.001$), with an increase of 75% over time.

The Peroxidase (POD) activity at 1 DAI, the control, needle wound without bacteria and cotton-rubbing technique treatments were equal, but significantly greater than those for the needle wound inoculated with bacteria and spray technique treatments, which were equal. The result of the cotton-rubbing technique being equal to that of the control might be related to the prejudice of damage in Xcc cells, as observed in the SEM images (Figure 1).

Moreover, the analysis quantified the real-time activity of POD but not what POD was consumed to protect the host, which could be a reason for the decrease in POD activity in needle wounds inoculated with bacteria (T2) or subjected to the spray technique (T4). Similarly, POD activity increased in the absence of bacteria (T1) but decreased in the presence of bacteria (T2). POD activity was very responsive to leaf lesions, which demonstrated a sensible response of POD activity in the presence of ACC. However, at 1 DAI, in this evaluation, POD activity was also equal to that of the control, which suggests that over time, the enzyme activity of the diseased host could provide a POD activity pattern similar to that of its natural state.

Moreover, the enzyme activity significantly increased over time for all treatments except for the control, even with the establishment of the disease at the needle wound site inoculated with bacteria (T2) ($F = 75.02, P < 0.001$), similar to the increase in response to Xcc observed by Kumar et al. (2011a, 2011c) in grapefruit, sweet orange and kumquat. Furthermore, it was noted from cotton-rubbing ($F = 17.78, P < 0.001$) and spray techniques ($F = 38.40, P < 0.001$) that only the presence of Xcc on the leaf surface was sufficient to induce POD activity, with an increase ranging from approximately 60% over time.

The screening of Citrus genotypes relies on needle-wound inoculation under greenhouse conditions and bypasses physical mechanisms related to resistance, such as epicuticular wax and epidermis and stomatal edge and aperture, thus, any screening performed under these conditions is aimed at expressing mesophyll resistance, however, this approach may result in more rapid and greater disease severity than relying on natural inoculum. Despite this, this technique provides a better standardization and discerning measure for assessing genotype resistance (Graham et al. 1992; Gottwald et al. 1993; Wang et al. 2011; Gonçalves-Zuliani et al. 2016). Furthermore, the prune procedure and the presence of CLM might expose mesophyll under field conditions, which increases the chances of Xcc infection through injury (Hall et al. 2010; Carvalho et al. 2015).

FINAL CONSIDERATIONS

Therefore, affirm that needle-wound inoculation at concentrations between $1.39x10^5$ and $1.52x10^5$ CFU ml^{-1} to inoculate Xcc resulted in rapid and greater disease severity, providing standardization and discerning measure for the screening of citrus genotypes, even under conditions of hypersensitivity response. Furthermore, in addition to the presence of Xcc on the leaf surface, which induces CAT and POD activity, similar to plant defense against natural inoculum, enzyme metabolism was also activated in response to needle wounding, with a peak at 9 DAI, be used as a tool to the screening of resistant genotypes.

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Conflict of interest:

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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