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Resistance Evaluation of Sweet Orange (*Citrus sinensis*) Cultivars to *Xanthomonas citri* subsp. *citri*

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HIGHLIGHTS

- The studies of enzyme activity showed differences among cultivars at different times.
- Cultivars with greater resistance will be better suited to be used in commercial production.
- Pera IAC is considered a more resistant cultivar, when compared to the remaining cultivars.

Abstract: Citrus canker, caused by the bacterium *Xanthomonas citri* subsp. *citri,* is responsible for significant economic losses of citrus in Brazil and elsewhere. The disease occurs in all regions of commercial production of sweet orange (*C. sinensis* L. Osbeck). Compared to chemical control, deploying canker-resistant cultivars is a viable and economical alternative for management of the disease. The objectives of this study were to compare the resistance to citrus canker of 14 genotypes of sweet orange, and to verify the levels of various enzymes involved in the canker resistance response, specifically peroxidase, catalase, and superoxide dismutase. The 14 genotypes included were Hamlin, Vermelha, Pera EEL, Pera IAC, Pera Bianchi/CC, Pera Ipiguá, Pera IAC 2000/1, Pera Ovale Siracusa, Pera Ovale, Pera IAC 2000/2, Pera M5, Pera Arapongas, Pera 58 and Pera 59. The varieties were evaluated under greenhouse conditions, through inoculation of leaves with *X. citri* subsp. *citri,* strain Xcc 306. After inoculation, measurements of lesion diameter and biochemical analyses were performed. Significant differences in resistance to *X. citri* subsp*. citri* between the citrus genotypes were observed, with cultivars Pera IAC and Pera EEL having significantly or numerically the smallest diameter canker lesions. Cultivar Pera IAC showed elevated levels of peroxidase, catalase and superoxide dismutase, enzymes known to be involved in resistance response to pathogen invasion. Cultivars with a strong resistance response to infection, and that produce smaller lesions are likely good candidates for commercial production or for use in breeding programs.

Keywords: Citrus genotypes; resistance; citrus canker; enzymatic response to infection.

INTRODUCTION

Brazil is the world's largest producer of oranges and frozen concentrated juice. With approximately 19 million tons of oranges produced and 1.1 million tons of exported frozen concentrated juice, the citrus industry is an important component of the country's economy [1]. Although citriculture generates considerable foreign exchange for Brazil, production has been hampered by several factors, among them are diseases [2], such as citrus canker, which is caused by the bacterium *Xanthomonas citri* subsp. *citri*. Citrus canker is present in all citrus growing regions of Brazil and affects most of the commercial cultivars of sweet orange (*C. sinensis* L. Osbeck), accounting for significant economic losses [3, 4, 5].

In Brazil, sweet orange cultivation accounts for approximately 93% of the entire are planted to citrus [6] and in the state of Paraná, sweet orange cultivation is dominated by the cultivars Pera, Folha Murcha, Valencia and IAPAR 73. Of these cultivars, the Pera oranges are the ones that have greater prominence, since they are more accepted in the fresh-fruit market and for frozen concentrated juice [4].

The identification and utilization of genetic resistance of host plants can be considered the most effective and economical strategy for disease control, however, citrus cultivars with high levels of resistance to citrus canker have not yet been identified. The susceptibility of citrus species to *X. citri* subsp. *citri* is widely variable [7]. Indeed, the response has been quite well characterized in citrus and its close relatives [8, 9, 10, 11, 12, 13]. Different field- and greenhouse-based methods have been used to screen citrus for canker resistance [8, 14, 15, 9, 10, 16, 13]. Conventional breeding approaches can be challenging in citrus [17], and although used, more recently transgenic approaches are being harnessed to develop canker resistant citrus [18, 19, 20, 21], and the latter may be a long-term solution to provide resistance to citrus canker. But screening of citrus germplasm and existing cultivars must be done to identify suitable material for commercial production and for introducing into breeding programs.

The pathogen *X. citri* subsp. *citri* infects several species of citrus. Indeed, the majority of species are reported to be susceptible to the pathogen [9, 13]. Several methods exist to assess susceptibility to citrus canker, including wounding and inoculation and injection infiltration [9, 22, 4], spray inoculation [8] and field screening [13]. Methods including wounding may affect results due to loss of some ‘field resistance’ by injury to the cuticle, epidermis and leaf integrity [9, 23, 11, 24, 25]. Nonetheless, wound inoculation can challenge other important sources of resistance. As noted, screening for resistance to citrus canker among a wide range of citrus germplasm and related species has been done in many regions where the disease is an issue [9, 10, 26, 11, 22, 13]. Although some relatively resistant species of citrus have been found (e.g., *C. reticulata*), others are very susceptible, and within a specific group there may be a range of susceptibility [9, 17, 13]. There are only a few reports in Brazil of specifically screening multiple cultivars or genotypes of a single species such as sweet orange under the same conditions [13, 4], but such information will be valuable to guide decisions for breeding programs and also to select more resistant cultivars of a particular species in canker endemic areas.

Enzyme activities in citrus leaves from plants both resistant to and susceptible to citrus canker have been explored previously, and differences have been observed [27]. Activities of catalase, peroxidase, and other enzymes and associated products occurred at different levels in citrus leaves differing in susceptibility to infection by strains of *X. citri* subsp. *citri*. Resistant plants tended to have higher levels of many enzymes compared to susceptible plants. More recently, Roeschlin and coauthors (2017) [28] demonstrated that the defense response of citrus by *X. citri* subsp. *citri* included upregulation and down regulation of various genes involved in susceptibility and resistance and resulted in various cellular changes including cell wall reinforcement, the accumulation of phenolic compounds and the induction of the salicylic acid (SA) signaling pathway. Various enzymes including peroxidase and catalase were involved.

Despite the widespread cultivation of citrus, and specifically sweet orange in Brazil, as noted above, only a few studies have been performed exploring relative resistance of sweet orange cultivars to citrus canker. And virtually no work has been done on the enzyme response in different sweet orange cultivars to infection with *X. citri* subsp. *citri*. Thus, the objectives of this work were to compare the resistance of 14 sweet orange cultivars under greenhouse conditions, to determine their relative resistance, and explore the production of some important enzymes involved in the host resistance response to infection with a pathogen.

MATERIAL AND METHODS

Plant material

The experiment was conducted in a greenhouse at the Núcleo de Pesquisa em Biotecnologia Aplicada (NBA) (23°23'57,8"S; 51°57'5,3"O, approximately 500 meters elevation) at the Universidade Estadual de Maringá (UEM, Maringá, Paraná State, Brazil). A total of 13 sweet orange cultivars (*Citrus sinensis*) and one tangerine, cultivar Vermelha (*Citrus reticulata* - used as resistant control), were grafted onto a *C. limonia* rootstock grown at the Viveiro de Mudas Pratinha nursery (Atalaia, Paraná State, Brazil) (23°08'25.0"S 52°04'14.7"W) for later inoculation and evaluation. The cultivars of sweet orange were Hamlin (susceptible control), Pera EEL, Pera Bianchi/CC, Pera IAC, Pera Ipiguá, Pera IAC 2000/1, Pera Ovale Siracusa, Pera Ovale, Pera IAC 2000/2, Pera M5, Pera Arapongas, Pera 58 and Pera 59. The experiment was conducted twice, once in February (trail 1, summer) and again in September (trial 2, spring).

Among the genotypes evaluated in the experiment, 12 of the cultivars were Pera type sweet oranges, and they were compared to the cultivar Hamlin (also sweet orange) and cultivar Vermelha (a tangerine), used as susceptible and resistant controls, respectively [13]. Plants were watered via automatic irrigation. Periodic fertilization was applied (N, P, K and micronutrients) following standard recommendation for citrus. Approximately 50 days prior to inoculation, the plants were pruned to encourage new growth, and to obtain young leaves of homogenous age, at 75 to 100% leaf expansion [17]. The experiment had a completely randomized design.

Inoculum preparation, inoculation and evaluation of citrus resistance

The inoculum was prepared from a pure culture *of X. citri* subsp. *citri* (strain Xcc 306, GenBank accession number NC_003919), obtained from the pathogen culture collection at the Fundo de Defesa da Citricultura (Fundecitrus, Araraquara, São Paulo, Brazil) and kept in a refrigerator in phosphate buffer (0.075 M, pH 7.0). Isolate Xcc 306 is confirmed *X. citri* susbp*. citri* 'A' pathotype and has previously been sequenced by the Genome Project/FAPESP [29]. Subsequently, the bacterium was cultured in Petri dishes containing Mannitol Glutamate Yeast (MGY) agar (10 g mannitol, 2 g L-glutamic acid, 0.5 g potassium phosphate, 0.2 g NaCl, 0.2 g MgSO4H2O, 1 g extract of yeast, 15 g agar/L of distilled water), according to Behlau and coauthors (2012) [30]. Cultures were incubated for 48 hours at 28°C in the dark, when the bacterial colonies were transferred to 1.5 mL microtubes containing phosphate buffer (0.075 M, pH 7.0). The inoculum concentration was adjusted to 10^8 colony forming units/mL (CFU/mL) using a spectrophotometer at 600 nm (Mod. UV5, Mettler Toledo, USA). The resulting suspension was used for leaf inoculation [31].

Wound inoculation using a sterile needle (0.55 × 0.2 mm) was used to penetrate the surface of the leaf epidermis. Eight equidistant injury points were made on the abaxial surface of the leaves, four on each side of the mid-rib. Prior to each wounding, the needle was dipped in the bacterial suspension. Fifteen leaves were inoculated per plant, with three replicate plants per genotype, all assessed for each evaluation. During the first 24 hours after inoculation, the entire greenhouse was sprayed with water to ensure high humidity to favor infection of the citrus leaves with the canker bacteria.

Measurements of lesion diameters

The diameter of the lesions was measured using an electronic micrometer (Mitutoyo, Kawasaki, Japan) at 4, 8, 12, 16 and 20 days after inoculation (DAI). At each evaluation date the diameters of 3 lesions per plant were measured, totaling 126 lesion diameter readings per evaluation date. For the measurements to be performed in a standardized manner, the same lesions were measured on each occasion, always measuring the longitudinal diameter in relation to the leaves, and on the abaxial leaf surface, and disregarding the presence of a chlorotic halo (only the necrotic area of the lesion was measured).

Enzyme extraction and quantification

For the determination of enzyme activities, samples of leaf material were collected in September from trial 2 (in the spring) at four different times: 0 hours (0 H), 24 hours after inoculation (24 HAI), at 16 days from non-inoculated leaves on inoculated plants (16DSI) and at 16 days from inoculated leaves on inoculated plants at 16 days after inoculation (16 DAI). At each time, 6 leaves were collected per cultivar, taking two leaves per plant, which were immediately stored in styrofoam containers with ice for later enzyme extraction.

A 1 g sample from each leaf was individually macerated in liquid nitrogen and homogenized in an ice-cold mortar with 3 mL of 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM EDTA and

polyvinylpolypyrrolidone (PVPP) 5 % (w/v). After homogenization, the extract was obtained by centrifuging the sample at 10,000 g for 30 minutes at 4°C. The supernatant (containing the enzyme extract) was stored in a freezer at -80°C until determination of the protein content and measurement of enzyme activities (peroxidase, catalase and superoxide dismutase).

Total protein

Quantification of total proteins was performed using the Bradford test [32]. A 1 ml aliquot of the Bradford reagent was added to 20 µL of the enzyme extract obtained as described previously and diluted in distilled water at a ratio of 1:5. The samples were incubated for 5 minutes and a spectrophotometric measurement taken at 595 nm. For reference, 20 µl of distilled water was used with 1.0 ml of the Bradford reagent. Absorbance values were plotted on a standard curve of concentrations of bovine serum albumin (BSA) from 0 to 0.8 mg.ml^{-1} and the concentration of proteins expressed as mg protein.ml^{-1}.

Determination of guaiacol peroxidase (POD)

The activity of POD was determined at 30°C by a direct spectrophotometric method measuring the conversion of guaiacol to tetraguaiacol at 470 nm [33]. The reaction medium was prepared by adding 30 mL of 25 mM potassium phosphate buffer (pH 6.8), 35 µl of 0.1 mM H_2O_2 and 10 µl of guaiacol. The reference cuvette contained 2.6 mL of reaction medium. POD activity was measured at 470 nm for 5 minutes. Each reaction was initiated in the spectrophotometer by addition of 400 µL of enzyme extract in 2.6 mL of reaction medium. The results were expressed in µmols of tetraguaiacol.min^{-1}.mg protein^{-1}.

Determination of catalase (CAT).

CAT activity was determined following the Góth method [34], as modified by Tomanková and coauthors (2006) [35]. Initially, 50 µL of the enzyme extract was added to test tubes containing 50 µL of 0.1 M sodium phosphate buffer (pH7.0). The reaction was initiated with the addition of 0.5 mL of 60 mM H_2O_2. This solution was incubated in a water bath at 37°C for 4 minutes, when the reaction was terminated by adding 0.5 mL of 32.4 mM ammonium molybdate. The samples were immediately stored in the dark for 5 minutes. For each sample, a blank was prepared by adding 0.5 mL of 32.4 mM ammonium molybdate followed by the addition of 0.5 mL of 60 mM H_2O_2, omitting the reaction period. The amount of H_2O_2 consumed in the reaction was monitored in a spectrophotometer at 405 nm, and the difference between the absorbance of the blank and the sample was used to calculate the CAT activity, which was performed using the molar extinction coefficient of a standard curve of H_2O_2 (ε= 0.0085 mM^{-1}cm^{-1}) and the results expressed in µmol H_2O_2 min^{-1}mg protein^{-1}.

Determination of superoxide dismutase (SOD).

The activity of SOD was determined following the methodology of Giannopolitis and Ries (1977) [36]. The reaction medium was composed of 20 µl of enzyme extract, 1.33 ml of 50 mM phosphate buffer/1 mM EDTA (pH 7.8), 50 µl of 13 mM L-methionine, 20 µl of nitro blue tetrazolium (NBT) and 100 µL of 4 µM riboflavin. A blank for each sample was prepared following the methodology cited, but without the addition of the enzyme extract. The test tubes containing the samples were kept at 25°C under direct light for 10 minutes. The reduction of NBT was monitored in a spectrophotometer at 560 nm. The difference in NBT between the blank and the sample was measured for the determination of SOD activity, which consists of the inhibition of NBT by the enzymatic dismutation of superoxide. The results were expressed in unit of enzyme per gram of protein (U SOD.mg protein^{-1}.min^{-1}).

Data analysis

Unless otherwise stated, analyses were performed in SAS V9.4 (SAS Institute, Cary, NC, USA) [37]. In each of the two trials where lesion diameter was measured, there were a total of 630 observations (14 Cultivars × 5 Days × 42 Sheets × 3 Repetitions). A generalized mixed model (GLIMMIX) was used to analyze these data considering covariates and time. GLMMIX extend GLMs (Generalized Linear Models) by adding random effects. Random effects account for variability between groups or clusters of observations, or for repeated measures on the same subject. This allows for more complex modeling of data with hierarchical or nested structures [38].

The parameter estimates were based on a normal $NO(\mu, \sigma^2)$ distribution using an identity link function. The best model was selected using the response variables y_{ijk}, $i = 1, 2, \ldots, 14$ (number of cultivars), $j =$

$1, 2, \ldots, 5$ (days after inoculation), $k = 1, 2, \ldots, 42$ (number of leaves) based on the Akaike information criterion (AIC) and the Bayesian Schwarz criterion (BIC) [38].

The Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) are widely employed information-theoretic measures for model selection, both aiming to balance model fit with parsimony, yet differing in their penalization strategies. The AIC, derived from information theory, applies a relatively moderate penalty for additional parameters, thereby exhibiting a tendency to favor more complex models, particularly in analyses involving small to moderate sample sizes, where predictive accuracy is prioritized over strict parsimony [39, 40].

Conversely, the BIC, grounded in Bayesian principles, incorporates a sample size-dependent penalty that increases with data volume, leading to a stronger preference for simpler models and greater consistency in identifying the true model as sample size grows. The decision to employ AIC or BIC should be informed by the specific inferential or predictive objectives, the size of the dataset, and the acceptable trade-off between complexity and generalizability [40, 41].

The adjusted models for trial 1 and 2 are presented (Supplementary Table S1). The NO_3 model was chosen as the best model, since among those it is the most parsimonious [39, 40, 41]. A Tukey-Kramer test was implemented to explore differences between treatments. The enzyme activity analyses comprised the 14 Cultivars × 4 Replicates (for CAT, but × 4 Replicates for POD and × 6 Replicates for SOD) × 3 Repetitions. An analysis of variance was performed in R [42] for each enzyme and the means were compared using a Scott-Knott test (α = 0.05). Figures were generated in R for both the lesion diameter data and the enzyme data.

RESULTS

The first symptoms of citrus canker appeared on the eighth day after inoculation, in both evaluation periods, with the formation of eruptive lesions, around the point of inoculation.

Trial 1

The mean diameters are shown (Table 1). There were five distinct groupings based on the analysis. The means ranged from 1.35 (SD±0.17) cm for cultivar IAC to 1.47 (SD±0.28) cm for cultivar Hamlin. The estimates of the model and the Tukey-Kramer pairwise means separation is provided (Supplementary Table S2 and S3, respectively). Cultivars Hamlin, Vermelha and Pera 59 had the largest diameter lesions compared to the other cultivars, while cultivars Pera M5 and Pera IAC had the smallest. The characteristics of each cultivar lesion diameter sample at 20 days is presented in a box plot (Supplementary Figure S1). In all genotypes evaluated, there was a gradual increase in lesion size during the experiment period (Figure 1).

Trial 2

Cultivars Hamlin, Pera Ovale, Pera Siracusa and Pera Bianchi / CC had the largest diameter lesions compared to the other cultivars, while the cultivars Pera Ipiguá and Pera IAC had the smallest. In this trial there were four groupings of the cultivars. The mean diameters ranged from 1.19 (SD±0.16) cm for cultivar Pera IAC to 1.36 (SD±0.21) cm for cultivar Pera Ovale Siracusa. The estimates of the model and the Tukey-Kramer pairwise means separation is provided (Supplementary Table S4 and S5, respectively).

Lesion diameter increased with time in all varieties from 0.68 to 1.4 mm at 4 DAI to 1.12 to 1.64 mm at 16 DAI (Table 2, Figure 2). Cultivars Pera IAC, Pera EEL and Pera IAC 2000/2 had the smallest lesions, while cultivar Hamlin, Pera Ovale Siracusa and Pera M5 have the largest.

The characteristics of each cultivar lesion diameter sample at 20 days is presented in a box plot (Supplementary Figure S2). In some cases, there were transient reduction in rate of lesion growth (for example, cultivar Pera Ovale Siracusa), but in general lesion diameter growth tended to be fairly consistent or increase, invariable having maximum size at the end of the experiment.

There were some differences between the two trails in lesion diameter ranking of the 14 cultivars. For example, cultivar Vermelha ranked 13th in trial 1, but 5th in trial two. However, most cultivars ranked fairly consistently and were either the same or only one or two rankings different between trials.

Table 1. The mean diameter of citrus canker lesions (mm) at 20 days, the standard deviations, the range in size, and the coefficient of variation of the means for trial 1.

Ranking	Cultivar	Mean diameter[a]	Standard deviation	Range		Coefficient of variation[b]
				Minimum	Maximum	
1	Pera IAC	1.346 def	0.170	1.010	1.720	0.127
2	Pera EEL	1.352 cdef	0.192	0.990	1.740	0.142
3	Pera Ovale Siracusa	1.357 cdef	0.234	0.880	1.860	0.172
4	Pera M5	1.357 cdef	0.172	1.040	1.670	0.126
5	Pera IAC 2000/1	1.363 cde	0.206	0.800	1.710	0.151
6	Pera Bianchi/CC	1.366 cde	0.186	0.970	1.630	0.137
7	Pera Ipiguá	1.371 cde	0.158	1.050	1.650	0.115
8	Pera 58	1.378 cde	0.172	0.950	1.650	0.126
9	Pera Arapongas	1.389 bcde	0.197	1.030	1.700	0.142
10	Pera IAC 2000/2	1.403 bcde	0.194	1.020	1.740	0.138
11	Pera Ovale	1.403 bcd	0.198	1.060	1.730	0.141
12	Pera 59	1.420 bcd	0.201	1.030	1.790	0.142
13	Vermelha	1.424 bc	0.223	0.990	1.760	0.157
14	Hamlin	1.470 a	0.284	0.950	2.050	0.193

[a] If means have different letters, they are significantly different based on a Tukey-Kramer analysis (α=0.05).

[b] CV = coefficient of variation is a unitless measure of variation ([mean square error ÷ mean lesion diameter] × 100).

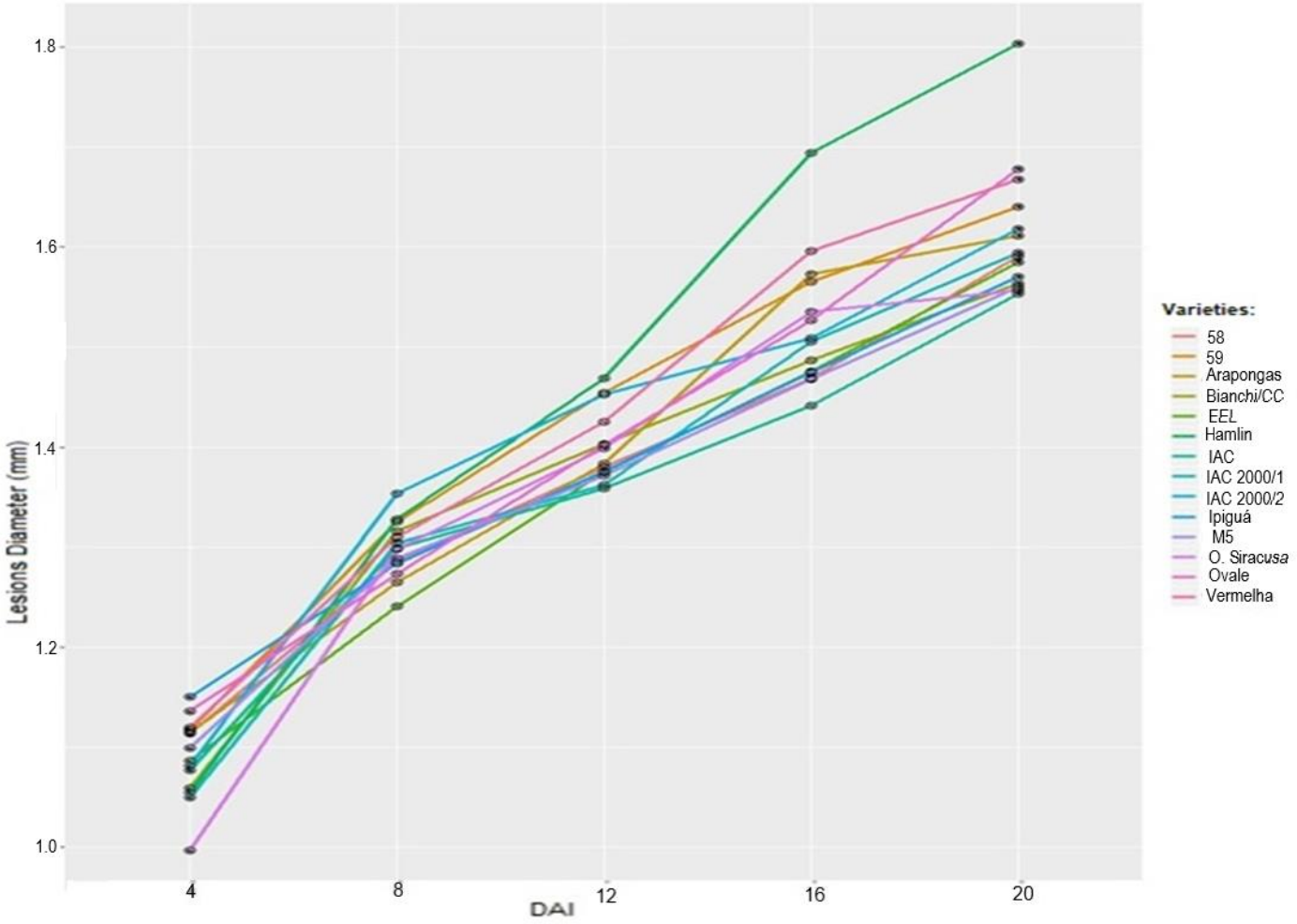

Figure 1. The change in measured mean lesion diameter for the 12 Pera sweet orange cultivars (and two controls) over the 20-day experiment in trial 1 (4, 8, 12, 16 and 20 days after inoculation – DAI).

Table 2. The mean diameter of citrus canker lesions (mm) at 20 days, the standard deviations, the range in size, and the coefficient of variation of the means for trial 2.

Ranking	Cultivar	Mean diameter[a]	Standard deviation	Range		Coefficient of variation[b]
				Minimum	Maximum	
1	Pera IAC	1.187 cd	0.159	0.800	1.510	0.133
2	Pera EEL	1.239 bcd	0.168	0.750	1.620	0.135
3	Pera Bianchi/CC	1.239 abcd	0.223	0.680	1.550	0.180
4	Pera IAC 2000/2	1.242 abcd	0.138	0.990	1.530	0.111
5	Vermelha	1.274 abc	0.160	0.950	1.570	0.125
6	Pera IAC 2000/1	1.286 abc	0.138	0.980	1.550	0.107
7	Pera Ipiguá	1.292 abc	0.135	0.990	1.640	0.105
8	Pera Ovale	1.307 abc	0.147	0.990	1.580	0.112
9	Pera 59	1.319 abc	0.139	0.970	1.610	0.106
10	Pera Arapongas	1.320 abc	0.158	0.820	1.650	0.119
11	Pera M5	1.331 ab	0.168	0.830	1.640	0.126
12	Pera 58	1.333 ab	0.145	1.070	1.650	0.109
13	Hamlin	1.337 ab	0.213	0.990	1.900	0.160
14	Pera Ovale Siracusa	1.363 ab	0.196	0.980	1.830	0.143

[a] If means have different letters, they are significantly different based on a Tukey-Kramer analysis (α=0.05).
[b] CV = coefficient of variation is a unitless measure of variation ([mean square error ÷ mean lesion diameter] × 100).

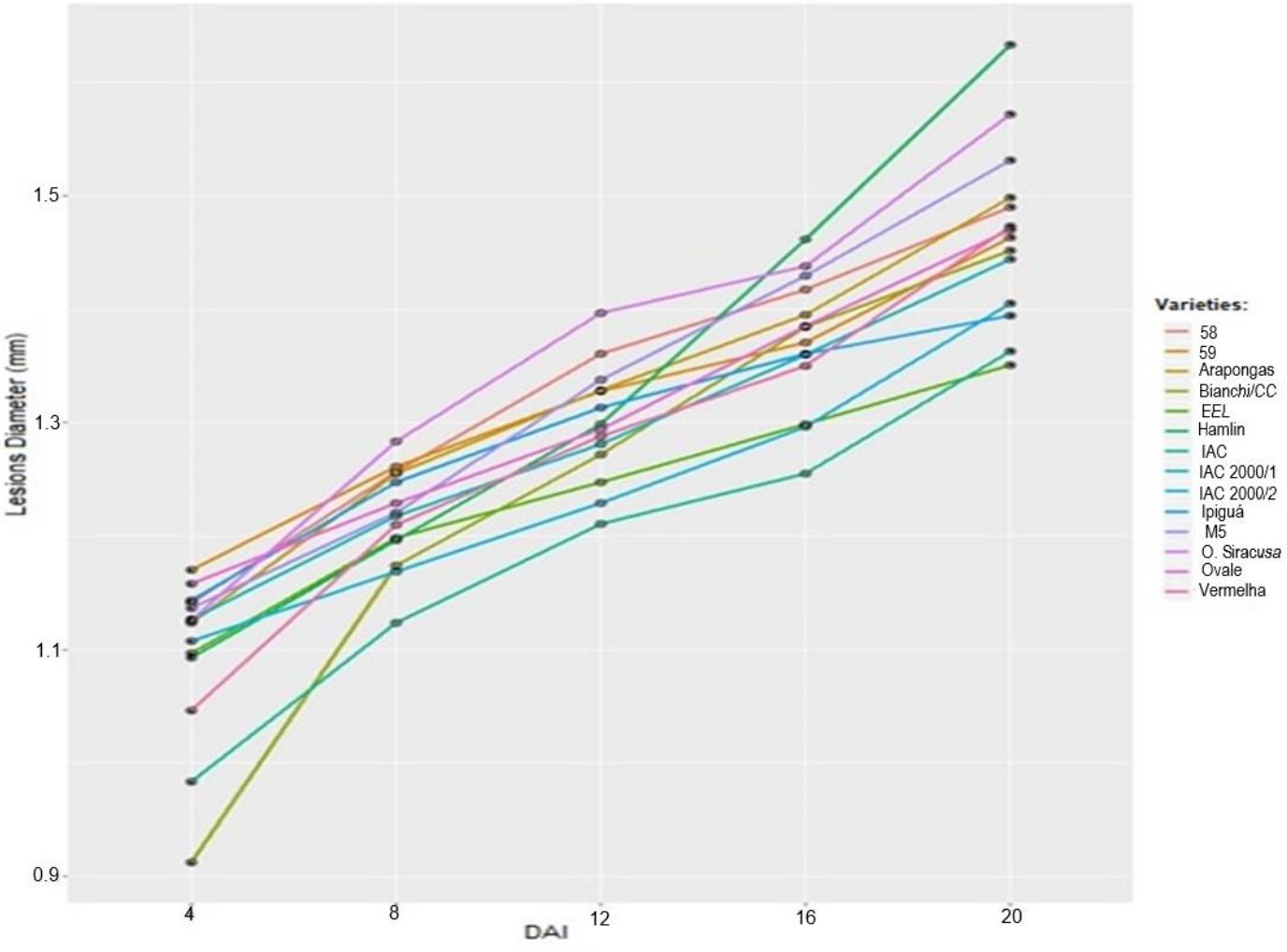

Figure 2. The change in measured mean lesion diameter for the 12 Pera sweet orange cultivars (and two controls) over the 20-day experiment in trial 2 (4, 8, 12, 16 and 20 days after inoculation – DAI).

Peroxidase activity

At 0 hours cultivars Hamlin, Pera Bianchi/CC, Pera 58 and Pera 59 had the highest POD activity (Figure 3a). The remaining cultivars all had significantly lower and similar POD activity as a group. At 24 HAI, cultivars Hamlin, Pera Bianchi/CC, Pera IAC, Pera Arapongas, Pera 58 and Pera 59 had the highest POD activity (Figure 3b). Remaining cultivars had significantly lower and similar activity. The 16 DSI sampling showed cultivars Hamlin, Pera Vermelha, Pera Bianchi/CC, Pera IAC, Pera Ovale Siracusa, Pera Arapongas, Pera 58 and Pera 59 all had relatively elevated activity of POD compared to the other cultivars that had significantly lower activity (Figure 3c). The 16 DAI sampling showed that although there were differences between cultivars Hamlin, Vermelha, Bianchi/CC, Pera IAC, Ovale Siracusa, Pera Arapongas, Pera 58 and Pera 59, all had elevated activity of POD compared to the other cultivars (Figure 3d).

Comparing measurements at 0 hours (healthy) and 24 HAI, cultivars Hamlin, Pera IAC Pera and Pera Arapongas had numerically higher POD activity at 24 HAI. Comparing activity at 16 days (DAI [inoculated] and DSI [healthy]) cultivars Hamlin, Pera Bianchi/CC, Pera IAC and Pera 58 had numerically elevated POD activity. Boxplots showing the activity of POD in the 14 cultivars comparing the four times (0 H, 24 HAI, 16 DSI and 16 DAI) for each cultivar are presented for direct comparisons (Supplementary Figure S3).

Catalase activity

Only cultivar Hamlin had relatively elevated CAT activity at 0 hours (Figure 4a). At 24 HAI, only Pera IAC and Pera Arapongas had elevated CAT activity (Figure 4b). On uninoculated leaves at 16 DSI, CAT activity was elevated on cultivars Hamlin, Pera Bianchi/CC, Pera Ovale Siracusa, Pera Arapongas, Pera 58 and Pera 59 relative to the remaining cultivars that were all grouped with least activity (Figure 4c). At 16 DAI cultivars Hamlin, Vermelha, Pera Bianchi/CC, Pera IAC, Pera IAC 2000/1, Pera Ovale Siracusa, Pera Arapongas, Pera 58 and Pera 59 had elevated activity of CAT compared to the other cultivars (Figure 4d).

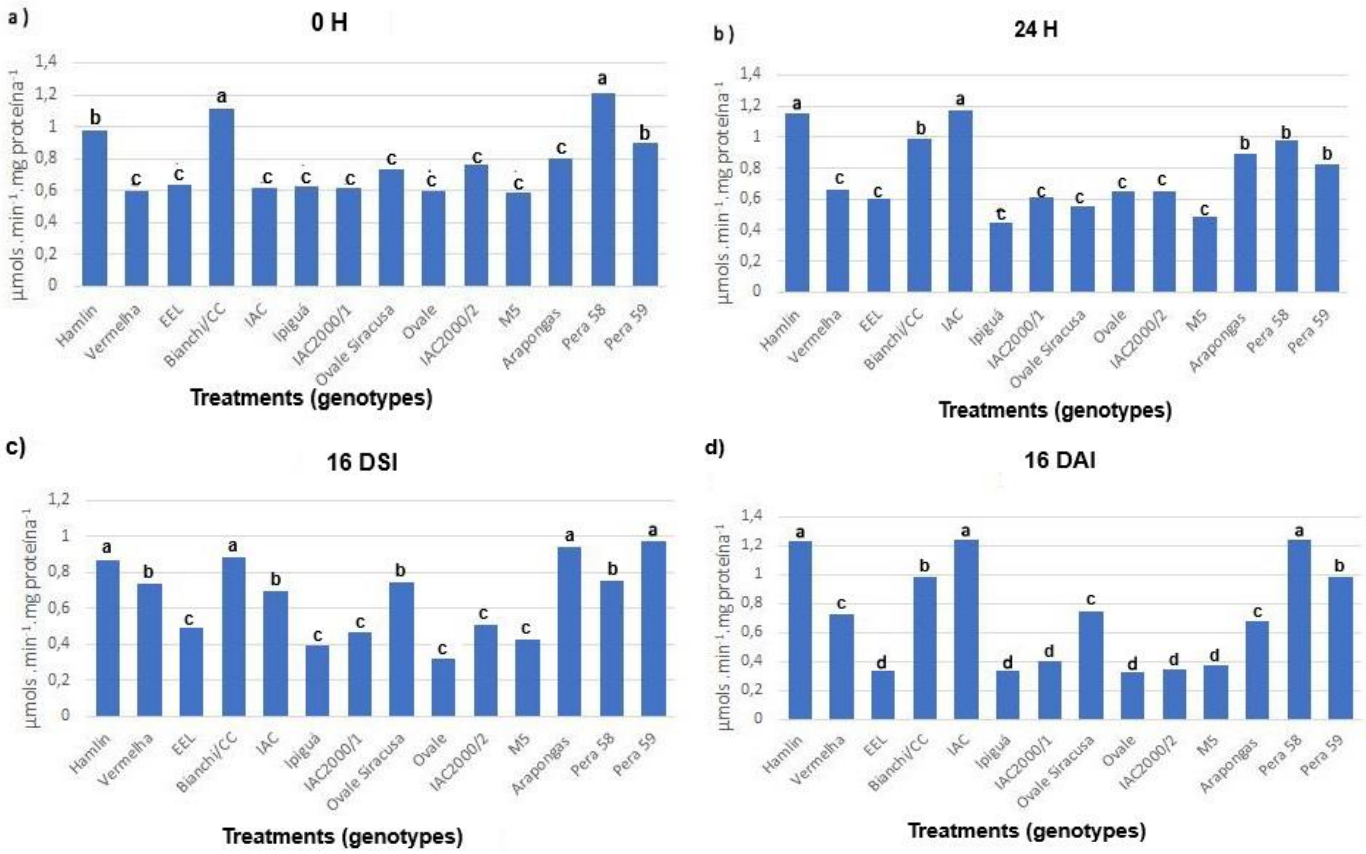

Figure 3. Peroxidase activity in the leaves of 12 Pera sweet orange cultivars (and two controls). a) 0 H - 0 hour, b) 24 H - 24 h after inoculation with Xanthomonas citri subsp. citri. c) 16 DSI - leaves not inoculated from inoculated plants at 16 days after inoculation, d) 16 DAI – inoculated leaves from inoculated plants 16 days after inoculation. If means have different letters, they are significantly different based on a Scott-Knott analysis (α=0.05).

Comparing measurements at 0 hours (healthy) and 24 hours, cultivars Pera IAC and Pera Arapongas had numerically higher CAT activity at 24 HAI. Comparing activity at 16 days (DAI [inoculated] and DSI [healthy]) cultivar Pera IAC had numerically elevated CAT activity, but there was little evidence for differences that suggested other cultivars had elevated activity. Boxplots showing the activity of CAT in the 14 cultivars comparing the four times (0 H, 24 HAI, 16 DSI and 16 DAI) for each cultivar are presented for direct comparisons (Supplementary Figure S4).

Superoxide dismutase activity

At 0 H cultivars Hamlin, Pera Bianchi/CC, Pera Ovale Siracusa, Pera Arapongas, Pera 58 and Pera 59 had the highest SOD activity compared to the other cultivars that grouped in two separate classes of lower activity (Figure 5a). At 24 HAI, cultivars Hamlin, Pera Bianchi/CC, Pera IAC and Pera 58 had elevated SOD activity relative to the other cultivars (Figure 5b). At 16 DSI cultivar Hamlin, Vermelha, Pera Bianchi / CC, Pera IAC, Pera Ovale Siracusa, Pera Arapongas, Pera 58 and Pera 59 showed higher activity compared to the remaining cultivars (Figure 5c). At 16 DAI cultivar Hamlin, Vermelha, Pera Bianchi / CC, Pera IAC, Pera Ovale Siracusa, Pera 58 and Pera 59 showed higher activity compared to the remaining cultivars (Figure 5d).

Comparing measurements at 0 hours (healthy) and 24 hours, cultivars Hamlin, Pera IAC and M5 had numerically higher SOD activity at 24 HAI. Comparing activity at 16 days (DAI [inoculated] and DSI [healthy]) only cultivar Pera IAC had numerically elevated SOD activity, but there was little evidence for differences that suggested other cultivars had elevated activity. Boxplots showing the activity of SOD in the 14 cultivars comparing the four times (0 HAI, 24 HAI, 16 DSI and 16 DAI) for each cultivar are presented for direct comparisons (Supplementary Figure S5).

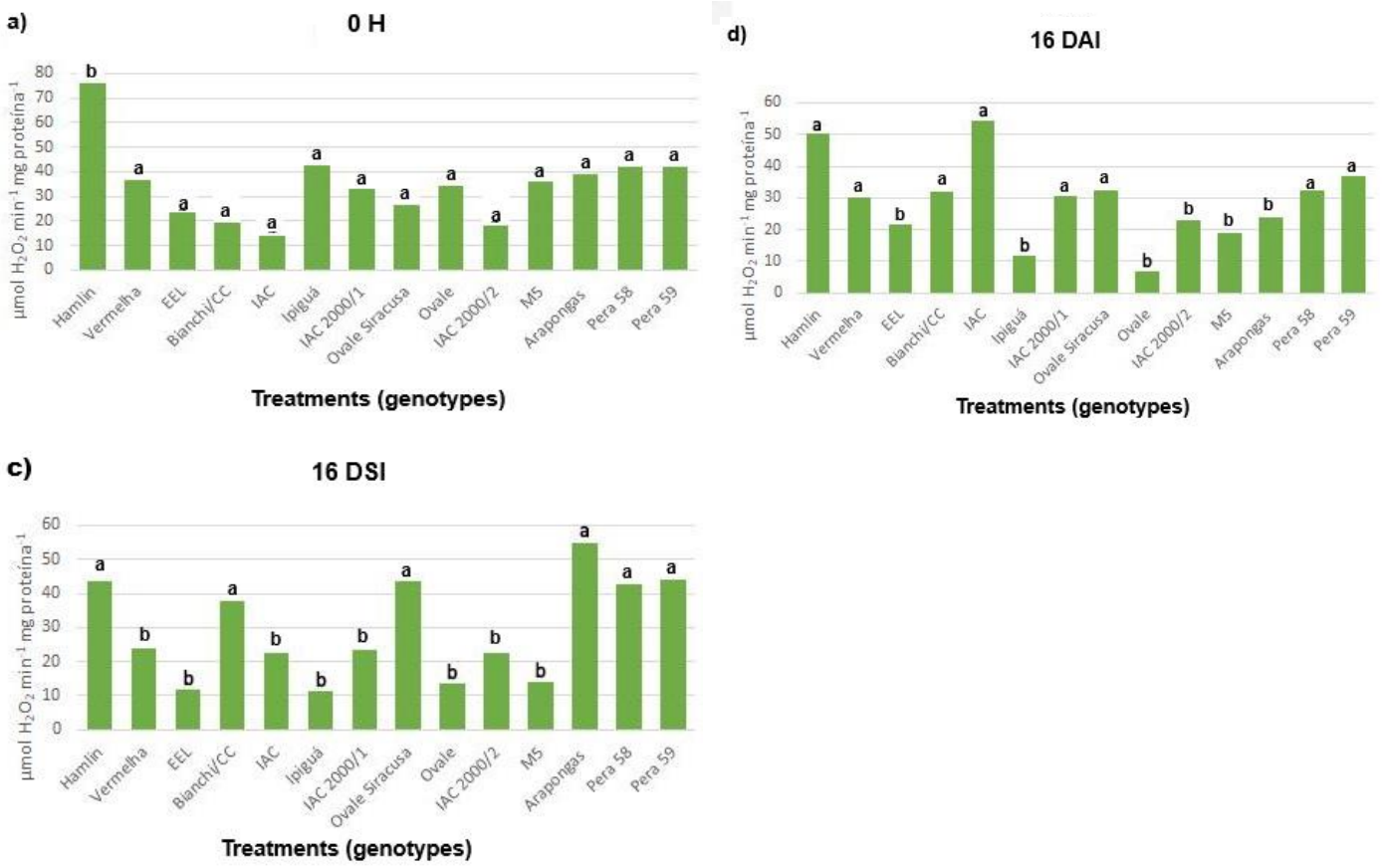

Figure 4. Catalase activity in the leaves of 12 Pera sweet orange cultivars (and two controls). a) 0 H - 0 hour, b) 24 H - 24 h after inoculation with Xanthomonas citri subsp. citri. c) 16 DSI - leaves not inoculated from inoculated plants at 16 days after inoculation, d) 16 DAI – inoculated leaves from inoculated plants 16 days after inoculation. If means have different letters, they are significantly different based on a Scott-Knott analysis (α=0.05, except for 'a' where α=10.1).

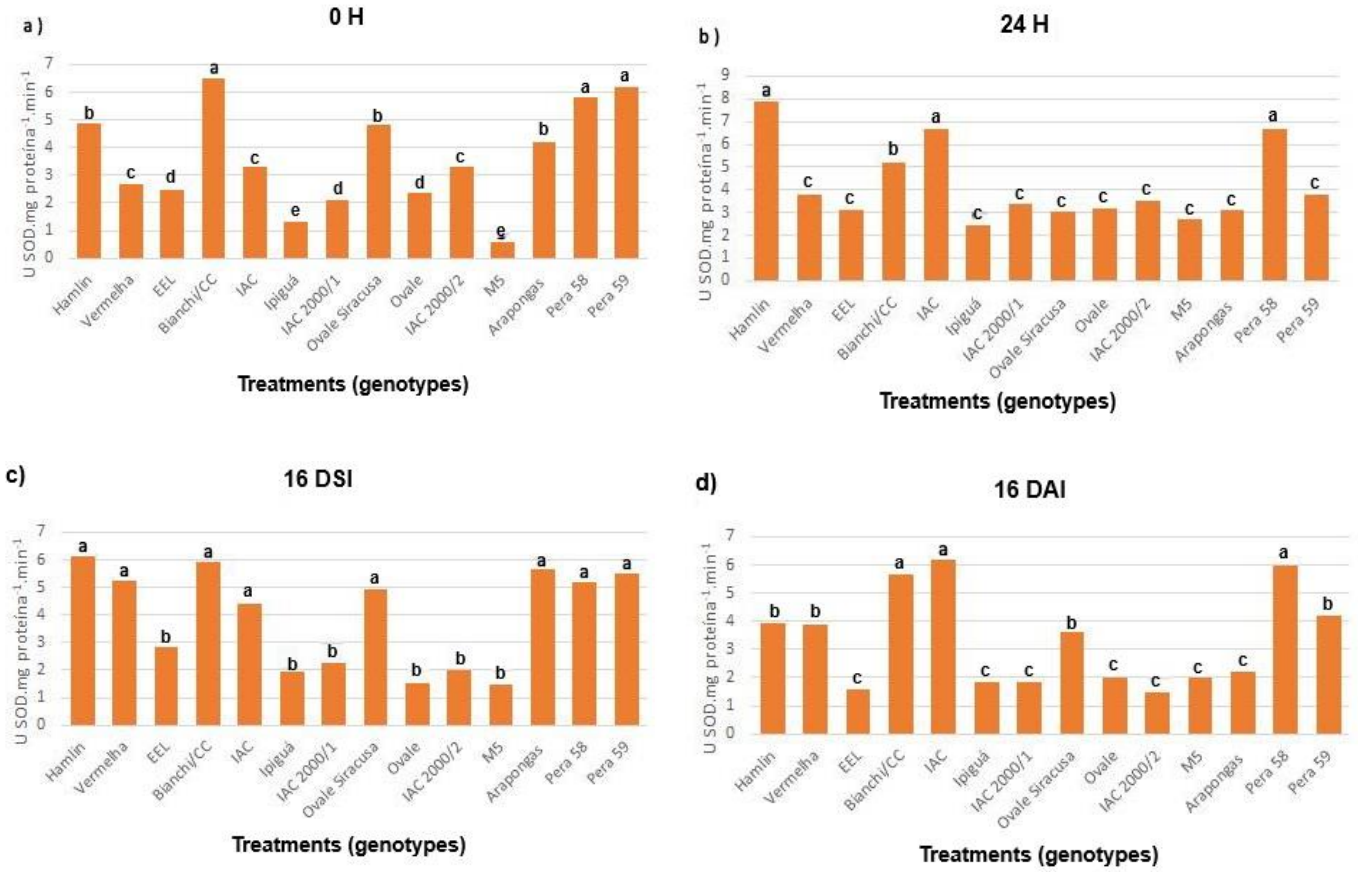

Figure 5. Superoxide dismutase activity in the leaves of 12 Pera sweet orange cultivars (and two controls). a) 0 H - 0 hour, b) 24 H - 24 h after inoculation with *Xanthomonas citri* subsp*. citri.* c) 16 DSI - leaves not inoculated from inoculated plants at 16 days after inoculation, d) 16 DAI – inoculated leaves from inoculated plants 16 days after inoculation. If means have different letters, they are significantly different based on a Scott-Knott analysis (α=0.05).

DISCUSSION

X. citri subsp. *citri* is able to infect its host by entering through wounds or hydathodes, colonizing in the mesophyll parenchyma and apoplastic areas of the host, multiplying there, and secreting systems, effectors, cell-wall-degrading enzymes, toxins, and bacterial adhesions which cause cell death, electrolyte loss, and tissue maceration [47]. Characteristic symptoms of the disease were observed in other experiments with citrus species artificially inoculated with *X. citri* subsp. *citri* [4]. The first symptoms of citrus canker appeared on the eighth day after inoculation, in both trials, with the formation of erumpent lesions around the point of inoculation. The results corroborate with studies conducted by Belasque Jr. and coauthors (2008) [43], where plants inoculated with needle wounds showed the first symptoms of citrus canker after one to two weeks after inoculation.

The cultivars showed a range of resistance to citrus canker. In the trial 1, cultivars Hamlin, Vermelha and Pera 59 had larger diameter lesions compared to the other cultivars, while cultivars Pera M5 and Pera IAC had the smallest. In trial 2 cultivars Hamlin, Pera Ovale Siracusa and Pera Bianchi / CC had the largest diameter lesions while the cultivars Pera Ipiguá and Pera IAC had the smallest. In all cultivars evaluated, there was a gradual increase in lesion size during the experiment period. There were no dramatic differences in the maximum and minimum lesion sizes for each cultivar. Nociti and coauthors (2006) [44] observed the increase in lesion diameter over a period of 94 days and found a variation of 1.28 to 5.15 mm in mean lesion size. Similarly, Gonçalves-Zuliani and coauthors (2016) [4] observed an increase in canker lesion size over time with Pera Orange genotypes inoculated with strain Xcc 306 of *X. citri* subsp. *citri*. Our results corroborate those of Gonçalves-Zuliani and coauthors (2016) [4].

Cultivars Hamlin, Pera Bianchi/CC, Pera IAC, Pera 58 and Pera Arapongas had numerically higher POD activity at 24 HAI and/or 16 (DAI and DSI). The increase in POD activity may be related to a hypersensitivity response, in which stress plants accumulate more H_2O_2 in the intercellular space due to cellular apoptosis, that aims to contain the pathogen and prevent the disease from spreading to surrounding areas. The cell wall

provides the initial interface between the pathogen and host. Lignin formation in cell walls of plants is a resistance response to disease. Cell wall components such as lignin play a role in plant response to pathogens since the POD is involved in the synthesis of lignin. The high POD activity increases lignin biosynthesis and carried to the greater resistance to infections by pathogen. [48]. This provides some evidence for activation of the biochemical defense mechanisms in these cultivars via elevated POD activity after inoculation with *X. citri* subsp. *citri*. However, cultivar Hamlin is considered to be susceptible. Studies suggest that the increase in peroxide production may occur gradually when compared to other cultivars that are resistant and is unable to contain the spread of the bacterium at the infection site [45, 46]. These same authors observed an increase in POD activity in cultivar Hamlin inoculated with *X. axonopodis* pv. *citri* from 1 DAI to 20 DAI, with a peak in production at 14 DAI. The authors also observed that despite an increase in POD activity, this variety had characteristic symptoms of the disease at 4 DAI, with the bacterial population remaining high when compared to resistant cultivars.

Wang and coauthors (2011) [49], evaluated a canker resistant and canker susceptible; cultivar, and found that the resistant cultivar had high and constant activity of POD after inoculation with *X. citri* subsp. *citri*, while the susceptible cultivar exhibited a reduction in the activity of POD 7 DAI. Other studies indicate that PODs participate in a number of physiological processes related to defense, such as cross-linking of cell wall proteins and oxidation of phenolic compounds during lignin formation. Under conditions of stress, in plants, POD activity increases in the intercellular spaces, stimulating cell wall hardening [50]. Our observations may reflect this response.

Differences were observed among the evaluated cultivars in CAT activity at the different sample times, regardless of inoculation status. CAT catalyzes the decomposition of H_20_2 to detoxify harmful metabolic products in the cell, and inhibits NADPH oxidation [48]. Comparing measurements just after inoculation, cultivars Pera IAC and Pera Arapongas had numerically higher CAT activity at 24 HAI. At 16 days DAI cultivar Pera IAC had numerically elevated CAT activity. Wang and coauthors (2011) [49], studying two contrasting citrus cultivars inoculated with *X. citri* subsp. *citri*, observed that the resistant cultivar Meiwa had higher levels of CAT when compared to the susceptible cultivar Newhall. Our results concur with these observations regarding resistant and susceptible cultivars.

Cultivars Hamlin, Pera IAC and Pera M5 had numerically higher SOD activity at 24 HAI, but at 16 DAI only cultivar Pera IAC had numerically elevated SOD activity. The results corroborate the observations of Kumar and coauthors (2011b) [46]. In that study cultivar Hamlin had a peak in SOD activity at 1 DAI and 8 DAI, with a subsequent decrease in SOD activity at 14 DAI. Slight increases in SOD activity were discernable in cultivars Vermelha, Pera EEL, Pera Ipiguá, Pera IAC 2000/1, Pera Ovale and Pera M5. The slight increase may be related to the entry of the pathogen into the plant through inoculation, as observed in the studies of Kumar and coauthors (2011b) [46]. Elevated SOD activity may be related to the development of *X. citri* subsp. *citri* in the plant tissue, since the presence of pathogenic bacterial activity is known to promote SOD activity, as well as the accumulation of peroxide in the cells [45, 46].

Plants employ sophisticated cell-based defense mechanisms to counteract pathogenic threats. Upon pathogen recognition, an apoplastic oxidative burst rapidly generates reactive oxygen species (ROS), primarily superoxide (O_2^-) and hydrogen peroxide (H_2O_2), constituting an early defense barrier. In current models of plant immunity, ROS and related radicals participate in covalent cell wall cross-linking, initiation of programmed cell death [47], and regulation of resistance-associated gene expression [51].

Elevated ROS levels reinforce cell wall integrity and restrict pathogen proliferation, contributing to hypersensitive responses (HRs) and transcriptional reprogramming [52]. Conversely, excessive ROS accumulation is cytotoxic, necessitating tight homeostatic control via antioxidant metabolites and enzymes [51]. In plants, ROS are produced by plasma membrane-localized NADPH oxidases, class III peroxidases (POD), and their associated pathways, metabolic processes including photosynthesis, photorespiration, and respiration [48].

ROS detoxification is mediated primarily by superoxide dismutase (SOD) and catalase (CAT), which act in concert with ROS-producing systems to maintain redox balance [52]. Long-term analyses of Citrus Canker pathogenesis have demonstrated that increased POD activity correlates with reduced ROS accumulation. Moreover, elevated SOD, POD and CAT activities are associated with enhanced resistance to Citrus Canker, as confirmed in the present study.

Resistance in citrus and related genera has been widely studied, with some germplasms, such as calamondin (C. mitis), kumquats (Fortunella spp.), and certain mandarins (C. reticulata), showing partial resistance [49]. However, under artificial inoculation or when grown alongside sweet oranges, all known genotypes develop citrus canker symptoms, and no fully resistant variety has been identified [47, 51]. Consequently, conventional breeding has achieved minimal progress, and molecular approaches—such as

the introduction of antibacterial genes—have only reduced disease incidence without conferring complete resistance [53, 54]. The molecular basis of pathogenesis remains unclear, and no resistance genes have been isolated, making the development of resistant cultivars particularly challenging [53].

Although genome editing offers promising avenues for improving disease resistance, its application in perennial fruit crops like citrus faces six major limitations: (i) difficulty in identifying highly efficient sgRNAs, (ii) incomplete optimization of Cas protein expression and activity, (iii) low efficiency of delivery systems, (iv) limited precision in plant genome editing, (v) slow and inefficient plant regeneration, and (vi) challenges in achieving transgene-free edits [51, 53, 54].

Therefore, studies that search for varieties that express some level of resistance among the currently available varieties are a way to reduce the effects of the disease, involving lower costs and with cultivars available for planting. As in the case of this study, the Pera IAC cultivar was identified as a good option, offering the greatest resistance to Citrus Canker among the cultivars studied, thus providing an option available to producers.

CONCLUSION

In summary, there were significant differences in the diameter of scab lesions among the 14 cultivars of sweet orange inoculated with *X. citri* subsp. *citri*. Those cultivars with greater resistance (smaller lesions) will be better suited to be used in commercial production where citrus canker epidemics are a major issue. They may also be useful in citrus canker resistance breeding programs. The studies of enzyme activity showed differences among cultivars at different times, and the results also indicated POD, CAT and SOD were elevated in some cultivars after inoculation with *X. citri* subsp. *citri*, when compared to in healthy leaves. Among the cultivars, only Pera IAC showed elevation in the activity of all three enzymes at 16 days after inoculation. Pera IAC is considered a more resistant cultivar, when compared to the remaining cultivars.

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