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Effect of temperature and leaf wetness duration on infection of sweet oranges by Asiatic citrus canker

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Asiatic citrus canker, caused by *Xanthomonas smithii* ssp. *citri*, formerly *X. axonopodis* pv. *citri*, is one of the most serious phytosanitary problems in Brazilian citrus crops. Experiments were conducted under controlled conditions to assess the influence of temperature and leaf wetness duration on infection and subsequent symptom development of citrus canker in sweet orange cvs Hamlin, Natal, Pera and Valencia. The quantified variables were incubation period, disease incidence, disease severity, mean lesion density and mean lesion size at temperatures of 12, 15, 20, 25, 30, 35, 40 and 42°C, and leaf wetness durations of 0, 4, 8, 12, 16, 20 and 24 h. Symptoms did not develop at 42°C. A generalized beta function showed a good fit to the temperature data, severity being highest in the range 30–35°C. The relationship between citrus canker severity and leaf wetness duration was explained by a monomolecular model, with the greatest severity occurring at 24 h of leaf wetness, with 4 h of wetness being the minimum duration sufficient to cause 100% incidence at optimal temperatures of 25–35°C. Mean lesion density behaved similarly to disease severity in relation to temperature variation and leaf wetness duration. A combined monomolecular-beta generalized model fitted disease severity, mean lesion density or lesion size as a function of both temperature and duration of leaf wetness. The estimated minimum and maximum temperatures for the occurrence of disease were 12°C and 40°C, respectively.

Keywords: beta function, *Citrus sinensis*, epidemiology, monomolecular model, *Xanthomonas axonopodis* pv. *citri*, *Xanthomonas smithii* ssp. *citri*

Introduction

Brazil is the world leader in citrus production, with more than 210 million citrus trees. São Paulo is the main producing state, supplying 86% of Brazil's sweet orange production (FNP, 2004). However, citriculture in São Paulo has been steadily threatened by serious phytosanitary problems. One of the most severe is Asiatic citrus canker, caused by the bacterium *Xanthomonas smithii* ssp. *citri* (Schaad *et al*., 2005) (Xsc), formerly *X. axonopodis* pv. *citri* (Dunger *et al*., 2005). Present in the state since 1957 (Rossetti, 1977), exclusion and eradication measures have kept the disease under relatively good control, below 0·22% incidence (Fundecitrus, 2004).

Cultivars Valencia, Pera, Natal and Hamlin account for 90% of sweet oranges planted in São Paulo (FNP, 2004). Pera, Valencia and Natal are considered moderately susceptible, and Hamlin susceptible to citrus canker (Gottwald *et al*., 2002). Xsc can infect leaves, stems and fruit, causing defoliation, dieback and premature fruit drop.

In the absence of wounds, the main pathway of penetration of Xsc into citrus leaves is through the stomata, with the abaxial face of the leaf being more susceptible because of the higher density of stomata than on the adaxial face (McLean, 1921; Graham *et al*., 1992). Expanding leaf tissues (i.e. young leaves) are more susceptible than fully expanded (mature) leaves (Lee, 1922; Stall *et al*., 1982; Gottwald & Graham, 1992). Colonization by Xsc is restricted to the infected area, resulting in raised, corky, cortical lesions on the leaf surface; generally chlorotic halos surround the lesions.

In recent years, Asiatic citrus canker foci have been found in the northwest region of São Paulo, where temperatures are higher than in other regions. The occurrence of the disease in this area is obviously related to the proximity of sources of inoculum, but the effect of weather can also play a role. Peltier (1920) and Koizumi (1977) showed that environmental factors play an exceedingly important role in the susceptibility of citrus plants to canker. Low temperature negatively affected infection and

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development of citrus canker, and the optimum temperature for infection was found to be between 20 and 30°C.

Quantification of the effect of environmental factors on the development of epidemics, especially the influence of temperature, light and humidity on the processes of infection and colonization by leaf pathogens, is usually carried out in experiments conducted in growth chambers. Tests in growth chambers allow isolation of the effects of specific environmental factors, and can supply data explaining the development of epidemics in the field (Kranz & Hau, 1980; Rotem, 1988).

The objective of this work was to study the effects of temperature and leaf wetness duration on infection and development of citrus canker, under controlled environmental conditions, on the main cultivars of sweet orange grown in São Paulo.

Materials and methods

Experiments were conducted in growth chambers (Conviron, model E-7), with three repetitions at different times. Tests were made at temperatures of 12, 15, 20, 25, 30, 35, 40 and 42°C, and leaf wetness durations of 0, 4, 8, 12, 16, 20 and 24 h, in a combined manner, resulting in 56 treatments, distributed in a split-plot design with temperatures as main plots and wetness durations as subplots, with eight plants per subplot.

Seedlings of sweet orange (*Citrus sinensis*) cvs Hamlin, Natal, Pera and Valencia were obtained from seeds cultivated in tubes 20 cm high and 1·5 cm in diameter. The production of seedlings in tubes allowed the experiments to be conducted in a closed room, inside growth chambers (0·65 $m^2 \times 0{\cdot}74$ m of growing space), following the phytosanitary safety standards concerning Asiatic citrus canker in São Paulo. Test tube stands with 40 holes were utilized as support for the plants in the tubes. Since Xsc infects only new citrus leaves, seedlings 20–25 cm tall were pruned to reduce their height by approximately one-third, to standardize sprouting.

Inoculum was prepared from Xsc isolate IBSBF 1421 supplied by Dr Júlio Rodrigues Neto from the Instituto Biológico de São Paulo, Campinas/SP. The bacterial culture, cultivated in nutrient agar medium (solid) for 48 h, was suspended in sterile distilled water and the suspension was calibrated using a colorimeter.

Seedlings of the sweet orange cultivars, with new injury-free leaves, were spray-inoculated on both leaf surfaces with a bacterial suspension of Xsc at a concentration of 10^6 cfu mL^{-1}. After inoculation, plants were wrapped in previously wetted plastic bags (wet-chamber conditions) and transferred to growth chambers at temperatures of 12, 15, 20, 25, 30, 35, 40 and 42°C, with a 12 h photoperiod (300 micromoles m^{-2} s^{-1}). Plants were kept under wet-chamber conditions for 0, 4, 8, 12, 16, 20 or 24 h. The leaf wetness duration of 0 h corresponded to the absence of a wet chamber after inoculation. In the treatments at 40 and 42°C, growth chambers were set to a temperature of 35°C during the night, from 48 h after inoculation, to avoid heat stress in the plants.

The variables evaluated in the experiment were incubation period, disease incidence, disease severity, mean lesion density and mean lesion size. Incubation period was defined as the period at which 50% of the plants presented symptoms. Disease incidence was assessed every 2 days. Disease severity and number of lesions were measured 30 days after inoculation in treatments at temperatures of 25°C or above; 40 days after inoculation in the treatment at 20°C; and 60 days after inoculation in treatments at 15 and 12°C. Under adverse conditions, symptoms may take up to 60 days to appear (Gottwald & Graham, 1992). Disease severity was measured by the disease quantification software, QUANT v.1·0 (Vale *et al.*, 2001), from digitized images of two leaves per plant with canker lesions.

Mean lesion size was calculated by dividing the total lesion area by the total number of lesions on each plant. Lesion density was obtained by dividing the number of lesions per leaf by the leaf surface area.

Statistical analysis

The effect of leaf wetness duration on disease severity was described by the monomolecular model $Y = b_1[1 - b_2 \exp(-b_3 M)]$, where Y represents disease severity, b_1, b_2, and b_3 are parameters to be estimated, and M represents the leaf wetness duration (Campbell & Madden, 1990).

The influence of temperature was described by the generalized beta function $Y = b_1[(T - b_2)^{b_3}(b_4 - T)^{b_5}]$, where b_2 and b_4 represent minimum and maximum temperatures, respectively, for the development of the disease, b_1, b_3 and b_5 are parameters to be estimated, T is the temperature and Y is disease severity (Hau & Kranz, 1990).

To describe the combined effect of temperature and leaf wetness duration on disease severity, mean lesion density and mean lesion size, a monomolecular-beta generalized function was fitted to the averages of each variable by nonlinear regression using the STATISTICA (2001) software (StatSoft, Inc.). Disease severity, mean lesion density and mean lesion size were described by the model $Y = b_1\{(T - b_2)^{b_3}(b_4 - T)^{b_5} b_6[1 - b_7 \exp(-b_8 M)]\}$, where b_2 and b_4 represent minimum and maximum temperatures, respectively, for the development of the disease, b_1, b_3, b_5, b_6, b_7 and b_8 are parameters to be estimated, T is the temperature, M is the leaf wetness duration, and Y is disease severity, mean lesion density or mean lesion size. Average values from the three repetitions were used in the nonlinear regression analysis.

Results

Disease incidence was similar among all the sweet orange cultivars tested. Symptoms of Asiatic citrus canker were observed at all temperatures evaluated, except 42°C. The new leaves, in most plants, did not withstand the wet heat at 42°C for longer wetting periods (above 12 h), and in the remaining plants buds also underwent heat stress, even after temperature was reduced during the night period. In the treatment with 0 h leaf wetness duration,

the disease manifested in four out of a total of 56 plants (one at 15°C, one at 20°C and two at 25°C).

At 20–35°C, all leaf wetness periods of 4–24 h produced 100% disease incidence. At the extreme temperatures, disease incidence was clearly affected. At 12°C, disease incidence was lower than 30%. At 15°C, maximum incidence was 80% for longer periods of wetness (20 and 24 h). However, at 40°C, only plants at short leaf wetness durations presented lesions (below 50%).

Mean incubation period, at which 50% of plants showed symptoms, was also strongly influenced by temperature and leaf wetness duration The mean incubation periods for leaf wetness durations of 4 and 24 h were 28 and 19 days, respectively, at 20°C, compared with 16 and 10 days, respectively, at 25–35°C. At 12 and 15°C, the incubation period was 30 days for the 24 h wetness duration.

The estimated parameters and coefficients of determination of the monomolecular model fitted to wetness duration at 30°C are shown in Table 1. In general, disease severity increased with the extension of wetness duration for all temperatures tested (Fig. 1).

The coefficients of determination and the parameters estimated by the generalized beta function fitted to disease severity at different temperatures with a 24 h wetness duration are shown in Table 2. Severity gradually increased with temperature up to 30–35°C, above which it markedly decreased (Fig. 2).

The monomolecular-beta generalized function allowed construction of response surfaces for disease severity (Fig. 3), mean lesion density (Fig. 4) and mean lesion size (Fig. 5) for each cultivar. The coefficients of determination and the parameters estimated in each fit are shown in Table 3. The minimum and maximum temperatures estimated by the monomolecular-beta equation were 12 and 40°C, respectively (Table 3). In general, as the temperature increased, disease severity, density of lesions and lesion size increased, up to approximately 35°C, after which they decreased (Figs 3–5). The maximum estimated severity at 24 h wetness duration occurred between

Table 1 Estimated parameters and coefficients of determination (R^2) of a monomolecular model[a] fitted to data of disease severity of citrus canker on sweet orange cultivars under different leaf wetness durations (0, 4, 8, 12, 16, 20 or 24 h) at 30°C

	Model parameters			
Cultivar	b_1	b_2	b_3	R^2
Hamlin	2·02	−0·02	0·08	0·82
Natal	0·67	0·12	0·32	0·89
Pera	1·10	0·18	0·07	0·97
Valencia	0·88	0·02	0·14	0·94

[a] $Y = b_1[1 - b_2 \exp(-b_3 M)]$; Y, disease severity (%); b_1, b_2, b_3, model parameters; M, duration of leaf wetness (h).

Table 2 Estimated parameters and coefficients of determination (R^2) of the generalized beta function[a] fitted to data of disease severity of citrus canker on sweet orange cultivars at different temperatures (12, 15, 20, 25, 30, 35 or 40°C) and 24 h leaf wetness duration

	Model parameters					
Cultivar	b_1	b_2	b_3	b_4	b_5	R^2
Hamlin	0·0004	12·0	1·99	40·2	1·01	0·99
Natal	0·003	12·0	1·41	42·0	0·78	0·86
Pera	0·02	11·9	0·99	40·0	0·31	0·92
Valencia	0·003	15·0	1·47	40·0	0·93	0·93

[a] $Y = b_1[(T - b_2)^{b_3}(b_4 - T)^{b_5}]$; Y, disease severity (%); T, temperature (°C); b_1, b_3, b_5, model parameters; b_2, b_4, minimum and maximum temperatures (°C), respectively.

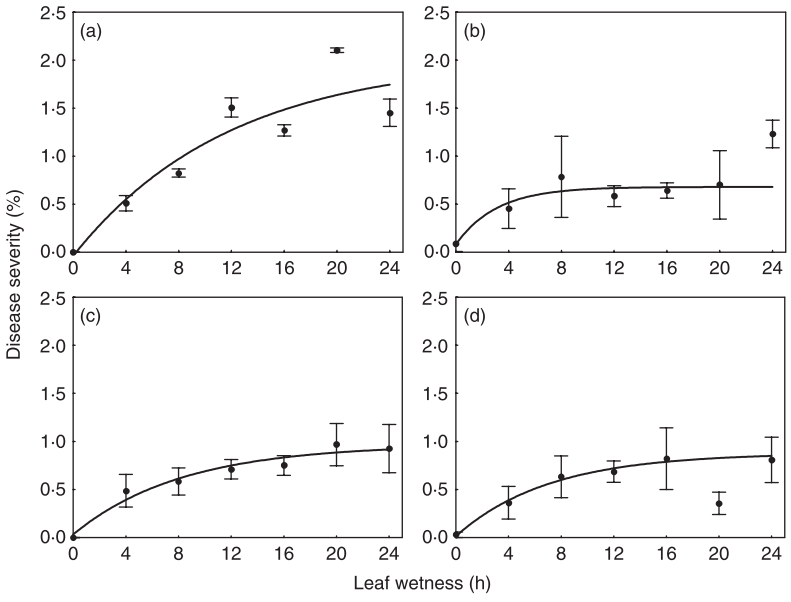

Figure 1 Effect of leaf wetness duration (0, 4, 8, 12, 16, 20 or 24 h) on disease severity of citrus canker on sweet orange cvs Hamlin (a), Natal (b), Pera (c) and Valencia (d) at 30°C. Each point represents the mean of three repetitions. Vertical bars represent standard errors. Lines show the monomolecular model fitted to data. Estimated parameters are given in Table 1.

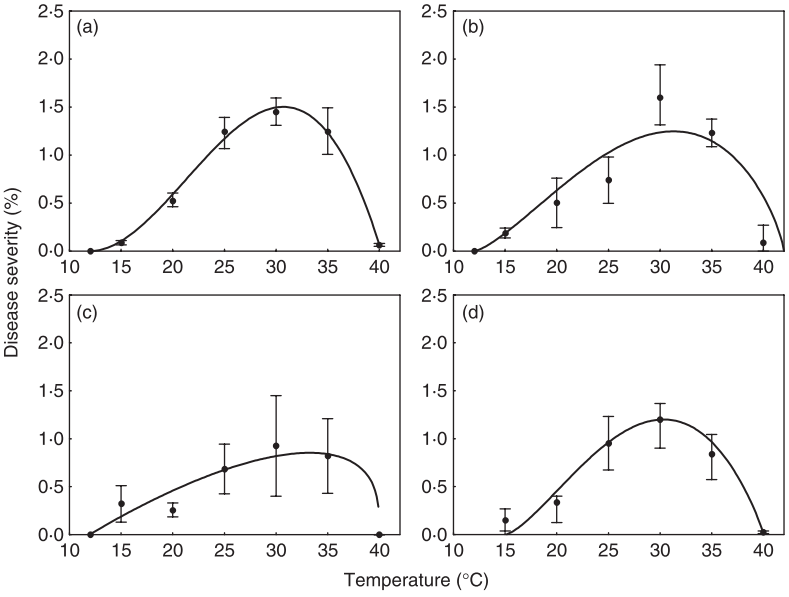

Figure 2 Effect of temperature (12, 15, 20, 25, 30, 35 or 40°C) on disease severity of citrus canker on sweet orange cvs Hamlin (a), Natal (b), Pera (c) and Valencia (d) with a leaf wetness duration of 24 h. Each point represents the mean of three repetitions. Vertical bars represent standard errors. Lines show the generalized beta function fitted to data. Estimated parameters are given in Table 2.

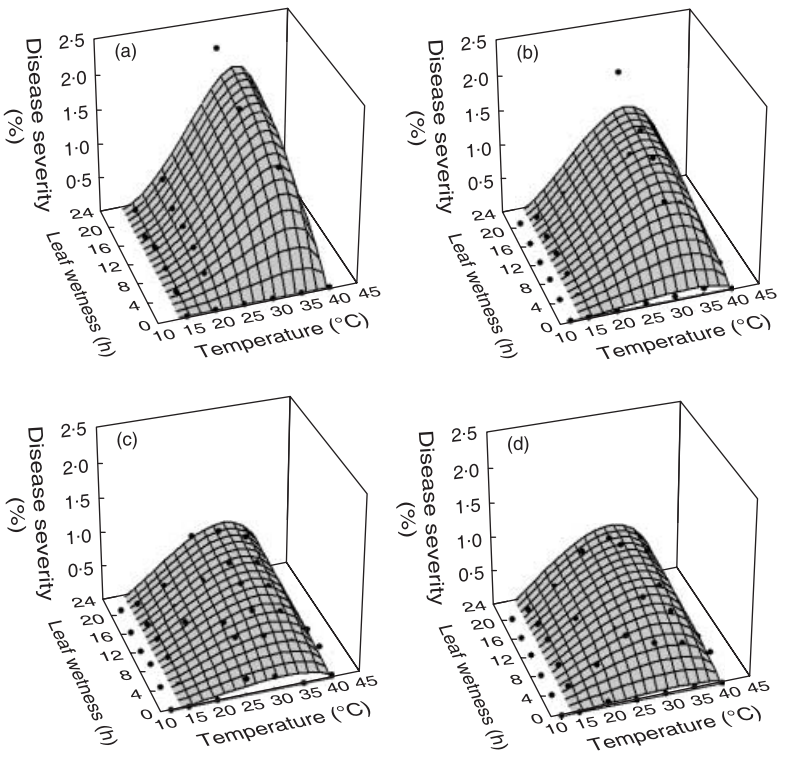

Figure 3 Combined effects of temperature (12, 15, 20, 25, 30, 35 or 40°C) and leaf wetness duration (0, 4, 8, 12, 16, 20 or 24 h) on disease severity of citrus canker on sweet orange cvs Hamlin (a), Natal (b), Pera (c) and Valencia (d). Each point represents the mean of three repetitions. Lines show the monomolecular-beta generalized function fitted to data. Estimated parameters are given in Table 3.

30 and 35°C (Table 4). Hamlin developed the greatest lesion density (1·13 lesions cm^{-2}) and Pera the lowest (0·5 lesions cm^{-2}). Pera presented the biggest lesions (2·39 mm^2) and Valencia the smallest (1·17 mm^2) (Table 4).

Discussion

Temperature had a greater effect than leaf wetness duration on the incidence of Asiatic citrus canker in sweet orange cvs Hamlin, Natal, Pera and Valencia. The optimal temperature range for disease development in this study was from 25 to 35°C, with short incubation periods and maximum incidence (100%) occurring at this optimum. Extreme temperatures, higher than 40°C or lower than 20°C, interfere in the infection process, reducing incidence. At optimal temperatures, all plants developed symptoms, even with a period of leaf wetness as short as 4 h. The citrus canker infection process requires a short wetness duration. Given that disease incidence of 100% occurred at the optimal temperature with a leaf wetness

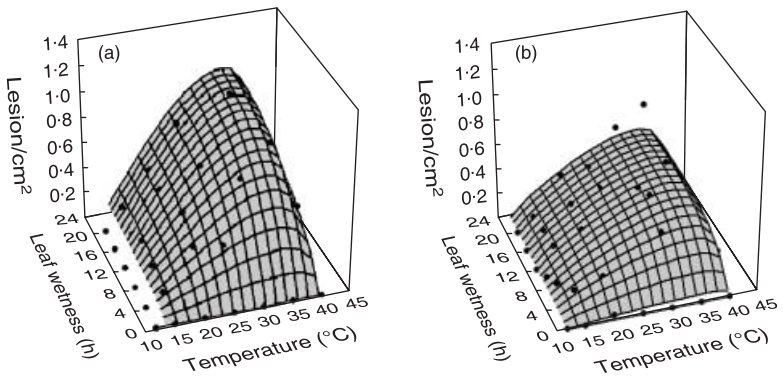

Figure 4 Combined effects of temperature (12, 15, 20, 25, 30, 35 or 40°C) and leaf wetness duration (0, 4, 8, 12, 16, 20 or 24 h) on lesion density (number of lesions per cm^2) of citrus canker on sweet orange cvs. Hamlin (a), Natal (b), Pera (c) and Valencia (d). Each point represents the mean of three repetitions. Lines show the monomolecular-beta generalized function fitted to data. Estimated parameters are given in Table 3.

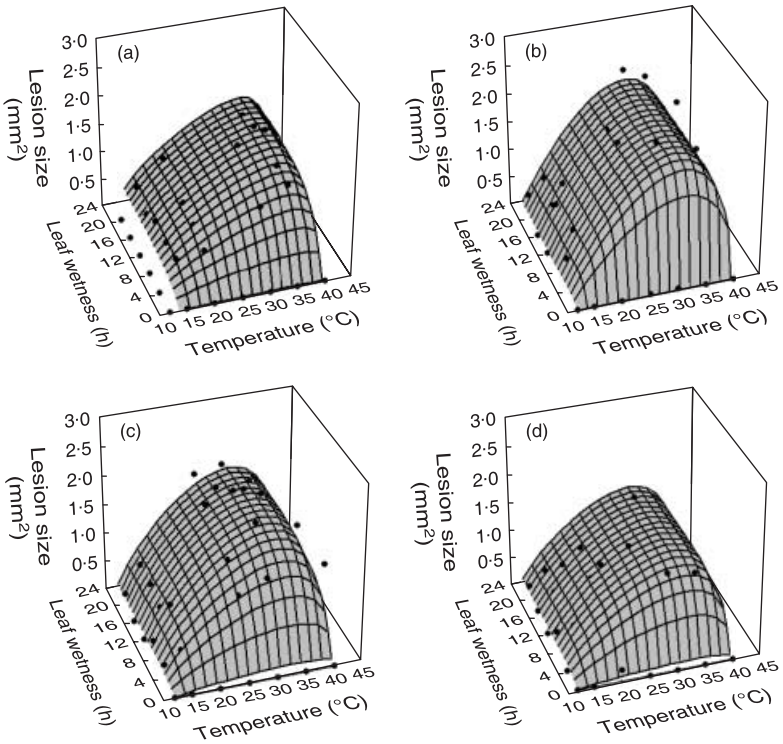

Figure 5 Combined effects of temperature (12, 15, 20, 25, 30, 35 or 40°C) and leaf wetness duration (0, 4, 8, 12, 16, 20 or 24 h) on lesion size (mm^2) of citrus canker on sweet orange cvs Hamlin (a), Natal (b), Pera (c) and Valencia (d). Each point represents the mean of three repetitions. Lines show the monomolecular-beta generalized function fitted to data. Estimated parameters are given in Table 3.

duration of 4 h, it is suggested that the minimum leaf wetness period required for infection by Xsc is less than 4 h. Further experiments, using shorter leaf wetness durations, are required to establish the minimum leaf wetness requirement for infection.

Only at 0 h wetness duration were disease symptoms not observed in the majority of plants. The few plants that had canker lesions at 0 h wetness may have developed symptoms as a result of the ability of the bacterium to infect plants once inoculated inside the substomatic

Table 3 Estimated parameters and coefficients of determination (R^2) of the monomolecular-beta generalized function[a] fitted to data of disease severity, mean lesion density and mean lesion size of citrus canker on sweet orange cultivars under different temperatures and leaf wetness durations

Variable/cultivar	Model parameters								R^2
	b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	
Disease severity									
Hamlin	0·001	11·9	1·98	40·0	0·44	1·97	−0·004	0·11	0·81
Natal	0·06	12·0	1·58	40·0	0·56	0·07	0·09	0·11	0·77
Pera	0·02	12·0	1·36	40·1	0·54	0·33	0·15	0·08	0·90
Valencia	0·01	12·0	1·21	40·0	0·52	0·95	0·11	0·13	0·83
Lesion density									
Hamlin	0·14	12·0	1·08	40·1	0·31	0·17	−0·02	0·14	0·72
Natal	0·13	11·2	0·90	40·0	0·20	0·19	0·18	0·20	0·75
Pera	0·15	11·0	0·63	40·1	0·29	0·27	0·23	0·27	0·62
Valencia	0·06	11·0	1·0	40·0	0·25	0·25	0·15	0·57	0·51
Lesion size									
Hamlin	0·02	12·0	0·63	40·0	0·12	0·09	−0·21	0·25	0·72
Natal	0·07	11·3	0·70	40·1	0·22	0·02	0·49	0·69	0·57
Pera	0·02	11·9	0·65	40·0	0·16	0·12	0·34	0·41	0·64
Valencia	0·01	11·6	0·62	40·0	0·10	0·14	0·02	0·53	0·62

[a] $Y = b_1\{[(T - b_2)^{b_3}(b_4 - T)^{b_5}b_6[1 - b_7\exp(-b_8M)]\}$; Y, disease severity (%) or density of lesion (lesions cm^{-2}) or lesion size (mm^2); T, temperature (°C); M, duration of leaf wetness (h); b_1, b_3, b_5, b_6, b_7, b_8, model parameters; b_2, b_4, minimum and maximum temperatures (°C), respectively.

Table 4 Maximum disease severity, maximum lesion density and maximum lesion size and their respective temperatures estimated by a monomolecular-beta generalized function (24 h wetness duration)

Cultivar	Disease severity (%)	T_{sev}[a] (°C)	Lesion density (lesions cm^{-2})	T_{ld}[a] (°C)	Lesion size (mm^2)	T_{ls}[a] (°C)
Hamlin	1·86	34·9	1·13	33·9	1·57	35·5
Natal	1·44	32·7	0·59	34·8	1·86	33·2
Pera	1·05	32·1	0·50	31·0	2·39	34·5
Valencia	1·01	31·6	0·54	34·2	1·17	36·0

[a] T_{sev}, T_{ld}, T_{ls}, estimated temperatures for maximum disease severity, maximum lesion density and maximum lesion size, respectively.

chamber, as observed by Gottwald & Graham (1992) and Graham *et al*. (1992). In these plants, during inoculation, Xsc cells may have been introduced directly into the interior of the substomatic chamber.

The average period of disease incubation in different cultivars was similar, varying from 10 to 16 days at 25 and 35°C, respectively. Temperatures below 20°C resulted in incubation periods longer than 20 days. Gottwald & Graham (1992) reported that citrus canker symptoms may appear after 60 days under adverse conditions for Xsc infection and incubation.

The combination of the equations obtained for the temperature data (generalized beta) and leaf wetness (monomolecular) allowed for the construction of response surfaces of Asiatic citrus canker severity for each of the sweet orange cultivars as a function of temperature and leaf wetness, indicating high severity in the 30–35°C range at wetness durations of greater than 16 h.

At 15 and 20°C, small increases in disease severity between 0 and 24 h of leaf wetness were observed; however, the increases become more marked at 30 and 35°C (Fig. 3). Thus, as the temperature increased, long wetness duration became more effective at increasing severity. Above 35°C, the efficiency of the long wetness duration diminished, and the difference between severity at 0 and 24 h again became less marked.

Between 15 and 40°C, disease severity increased until the inflection point was reached (30–35°C), followed by marked decreases approaching zero. The curve thus formed was asymmetrically skewed to the right (Fig. 3). As wetness increased, the curve extended along the ordinate axis.

Mean lesion density and mean lesion size (Figs 4 and 5) increased as far as the inflection point around 35°C, after which they markedly decreased. Therefore, the increased density and size of lesions were less influenced by leaf wetness duration, with the slope of the response surface to the right showing the greater influence of temperature for such components.

The maximum disease severity estimated for cv. Hamlin was higher (1·86%) than that of the other cultivars tested (Table 4), confirming its greater susceptibility (Gottwald *et al*., 2002). The greater severity in cv. Hamlin was the result of a higher number of lesions: 1·13 lesions cm^{-2}, approximately twice the number in other cultivars.

Disease incidence, disease severity, lesion density and lesion size showed their greatest values between 25 and 35°C for the sweet orange cutivars used. This result differs from that reported by Peltier (1920), who found the optimum between 20 and 30°C, although *C. sinensis* was not used in this earlier study.

Since temperature apparently exerts a greater influence on the infection and development of Asiatic citrus canker

than the duration of leaf wetness under controlled conditions, epidemics in the field are probably more influenced by differences in temperature, especially since the minimum wetness duration necessary for infection is short.

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