SHORT COMMUNICATION

REACTION OF GENOTYPES OF CITRUS SPECIES AND VARIETIES TO *XANTHOMONAS CITRI* subsp. *CITRI* UNDER GREENHOUSE CONDITIONS

A.M. Amaral[1,2], S.A. Carvalho[2], L.F.C. Silva[2] and M.A. Machado[2]

[1]EMBRAPA Recursos Genéticos e Biotecnologia, PqEB, Avenida W5 Norte, CP 02372, 70770-9002, Brasília, DF, Brazil
[2]Centro APTA Citros "Sylvio Moreira", CP 04, 13490-970, Cordeirópolis, SP, Brasil

SUMMARY

Resistance of citrus genotypes to Asian citrus canker, caused by *Xanthomonas citri* subsp. *citri* (*Xcc*) was evaluated on 582 accessions (319 varieties of sweet orange), including varieties with a potential commercial use. All accessions were grown in a greenhouse and were spray-inoculated with *Xcc* to screen for resistance, based on analyses of lesion expansion. Inoculum (10^8 CFU/ml) was applied on the abaxial side of all leaves and all plants were incubated up to 30 days. Area of lesions on inoculated leaf blades was scanned and compared with healthy foliar area. A disease index was obtained and used to classify response to citrus canker. Two experiments were carried out with different varieties and distinct periods. A wide range of reaction was observed, with approximately 13% of all accessions resistant to citrus canker, 42% moderately resistant, 20% susceptible, and 25% highly susceptible.

Key words: bacterial leafspot, citrus canker, disease resistance.

Citrus canker, one of the most devastating diseases of this crops around the world, is caused by *Xanthomonas citri* subsp. *citri* (*Xcc*) (ex Hasse, 1915) Gabriel *et al*., 1989 [formely *X. axonopodis* pv. *citri.* (Schaad et *al*., 2006)]. Citrus canker is a serious problem for Brazilian growers due to the frequent rainfalls and warm temperatures during most of the citrus vegetative period. In addition, damages caused by the disease were aggravated by the citrus leafminer (*Phyllocnistis citrella*) attacks, which enhance the coalescence of canker lesions due to the galleries made by the worm in the mesophyll tissue (Timmer *et al*., 2000; Gottwald *et al.*, 2005).

Plant eradication is the main strategy for protecting citrus orchards against the disease, especially because citrus canker has features that allow the efficient use of the technique, such as the absence of a vector and the clear visual symptom (Graham *et al*., 2004). In addition, the use of resistant varieties is also an interesting strategy, for long term control of diseases (Gottwald *et al*., 1993).

Citrus canker affects many types of citrus, with severity varying with the citrus variety. Washington navel sweet orange (*Citrus sinensis*) and grapefruit (*C. paradise*) are especially susceptible to the disease. On the other hand, mandarin (*C. reticulate*) and its hybrids, in general, show a moderate resistance to cancrosis. The variability in the susceptibility is influenced by the anatomy of the stomata, which determines the facilitation of bacteria penetration (Graham *et al.*, 1992). The response of plants from the citrus groups (i.e. the genera *Citrus, Poncirus* and *Fortunella*) is determined by leaf mesophyll (Gottwald *et al.*, 1993). Differences in ultrastructural changes in membranes and extracellular spaces of the host are also factors for variations in resistance between varieties (Zubrzycki and Zubrzycki, 1986).

The lack of information regarding resistance to *Xcc* of citrus genotypes in large collections, has impeded the use of such material in breeding programs or for eventual gene cloning and transformation. Such information regarding citrus plants that show resistance against *Xcc* would be very useful in citrus breeding programs especially because the use of genes from the citrus group (instead of the transgenic approach) tends to be less controversial in programs that aim to use the genetic transformation (Roose, 1996).

Although the resistance to citrus canker is frequently broken down in the field when the citrus leafminer is present, the high phenotypic variability in anatomy (fruits and leaves shape) and disease response present in citrus germplasm collections, indicates that plants resistant to *X. citri* subsp. *citri* (*Xcc*) can be screened and used to establish breeding programs (Herrero *et al*., 1996; Prasad *et al*., 1997; Shiotani *et al*., 2000). Furthermore, under conditions where the citrus leafminer is well controlled, the incorporation in the cropping system of accessions that show other potential characteristics would be valuable.

Our objective in this study was to evaluate, under

Corresponding author: S.A. Carvalho
Fax: +55. 1935461399
E-mail: sergio@centrodecitricultura.br

greenhouse conditions, a large number of citrus varieties and relatives, present in one of the world´s largest citrus germplasm collections, the Centro APTA Citrus Germplasm Collection, in Cordeiropolis, Brazil, for resistance to *Xcc* and indicate new genotypes to be included in breeding programs, gene cloning, or field introductions to eventually replace citrus canker susceptible varieties that have been in use in areas where the disease is present in Brazil.

To evaluate the differential response of several varieties and clones of citrus plants to *Xcc,* buds were collected from the germplasm collection at the Centro APTA Citros "Sylvio Moreira"/IAC, in Cordeiropolis, SP, Brazil. Using the Rangpur lime (*C. limonia*) as rootstock, the plants were grown in 3.8 litre containers with vermiculite and pine rind as substrate in a screen protected nursery, following the other management procedures described by Carvalho and Machado (1997) for commercial nursery tree production.

The study was divided into two assays, that included different varieties of citrus and its relatives, which refer to: Assay I) various genotypes of *Citrus* and its relatives, which were inoculated in the summer of 2002; Assay II) only sweet oranges and mandarins and their hybrids, all with potential commercial use (according to their fruit characteristics), and inoculated during the summer of 2004.

A highly virulent strain of *X. citri* subsp. *citri* was incubated at 26°C for 48 h on NA. A loopful of bacterial suspension was transferred to liquid Circlegrow^{TM} (BIO101, Carlsbad, CA) culture medium and the culture incubated for 48 h at 26°C at 120 rpm (Swings et *al.*, 1993). There, bacterial cells were concentrated by centrifugation at 3,000 *g* for 10 min at room temperature, harvested, and resuspended in ddH_2O and diluted to a final optical density of 0.55 at 600 nm. This resulted in a bacterial suspension of approximately 10^8 colony-forming units (CFU) per ml.

For resistance studies citrus plants were exposed to *Xcc*, in a greenhouse located in Campinas-SP, Brazil, provided by Dr. Julio Rodrigues Neto (Instituto Biológico). All plants (~12 months old) were pruned to promote uniform flush of susceptible new foliage and active growing. Although expanding leaves of 60 to 80% of final size were the main target for inoculation, whole plants were also sprayed with *Xcc* until complete coverage of the abaxial side (approximately 5 ml of suspension per plant). The plots, i.e. group of three plants per variety, were arranged in a randomized block design with three replications. The four leaves with the highest number of lesions from each plant were sampled.

Symptoms on each genotype were evaluated four weeks after inoculation by sampling the four leaves that, with no injuries caused by thorns or insects (i.e., leaves where the canker symptoms were not increased by mechanical lesions), developed the highest number of canker lesions. Each leaf was scanned (abaxial side) and had its lesions identified by color screening (distinct chlorotic spot and/or slight necrotic flecks, distinct but pale necrotic spot or greyish-white lesion, and dark necrotic spot, with or without chlorotic halo) using Image Analysis Software for Plant Disease Quantification (APS ASSESS). Basically, each sample had a "disease index" calculated as:

$$\text{Disease index} = \frac{\text{Sum of area with lesions per leaf}}{\text{Total foliar area}}$$

The prediction of field resistance was based on an adaptation of previously suggested classification for infection of peach by *Xanthomonas arboricola* pv. *pruni* (Ham-

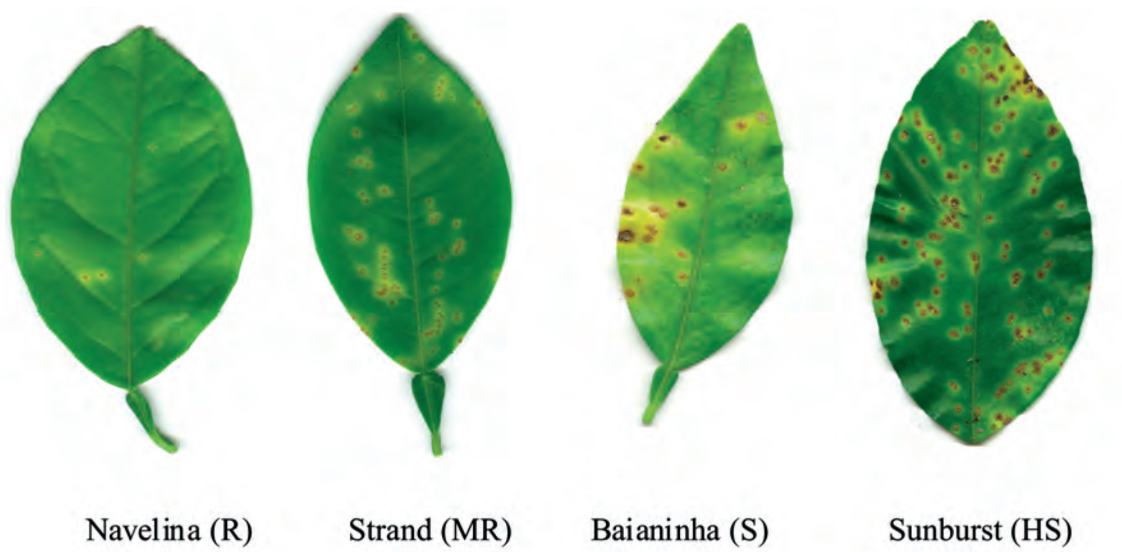

Fig. 1. Leaf samples of citrus varieties classified according to the response to inoculation with *Xanthomonas citri* subsp. *citri*. R, resistant; MR, moderately resistant; S, susceptible; HS, highly susceptible.

merschlag, 1990), with four classes: resistant (0-5% area with lesions), moderately resistant (5-10%), susceptible (10-15%), and highly susceptible (> 15%). Accordingly, accessions were grouped as described above.

Under greenhouse conditions and in a broad range of responsiveness, all citrus accessions tested proved to

Table 1. Response of accessions of sweet orange genotypes to infection by *Xanthomonas citri* subsp. *citri* strain, based on foliar area affected by canker lesions.

Resistant	
Assay I	Baia Monte Parnazo, Caipira L, Caipira R, Do Céu-1, Enterprise, Jaffa, Lue Gin Gong, Navelina, Orvalho de Mel, Pera Mutação, São Sebastião, Tobias 1392, Vermelha.
Assay II	Biondo di Caccia, Harris, Kyomi, Midsweet, Natal África do Sul, Navelina SPA 12, Orange Fukuhara, Osaceola, Ovale Mut Proc., Pera Comprida, Pera EEL, Pera Dibbern, Pera GS, Pera Olimpia, Pera Ovale, Pera Perão, Shamouti, SRA 561, Tarocco 12, Tarocco Blood, Valencia 1230, Valencia Taquari, Werley 435/96.
Moderately resistant	
Assay I	Açoriana, Agrodoce, Artebanta, Bahia IPEAL no.7, Baia Cabula, Baia Monte Parnazo de Franca, Baía Vale del Cauca, Baiana Retiro, Baianinha 1-38-8, Boa Vista, Branca, Buckeye Navel , Caipira 1401, Caipira Comum, Caipira Doce, Caipira, Cameta, Champagne, Cipó, Cleópatra, Coco. Coronel, Deliciosa, Do Rei, Dulce del Mediterrâneo, Enterprise, Flórida Sweet, Fontes, Golden Nugget Navel, Hamlin, Hart's Late, Homossassa, Ibicaba, Imperial, João Nunes, Lamb Summer, Lanceta Amarga, Lanceta Barão, Lima Verde, Lisa, Macaé, Magnum Bonum, Melão, Mortera, Non Pareil, Nova Califórnia, Ovale (viveiro EEL), Parson Brown, Pele de Moça, Pera de Abril, Pera Olímpia 1516, Pera Roberto Gullo, Perão do Rio 3, Piralima Variação, Robertson Navel, Rubi, Sanguínea de Piracicaba, Santa Catarina, Santa Lúcia, São Miguel, Setúbal, Shamouti (D), Surprise Navel, Tobias 1393, Vasconcelos, Washington da Flórida, Washington Navel.
Assay II	Azeda Beja, Baia Caracara, Baia Lane Late, Baia Leng, Bema IVIA 43, Berna, Berna IVIA-43-I (SB), Bidwells Bar, Cadeneira Punchosa, Castellana IVIA-64-3, *C. sinensis,* Comuna IVIA–105, Diva, Early Oblong, Espanole, Hamlin Jaraguá, Harvard, Isle of Pines, Kawatta Blood, Khailily White, Lane Late SPA 23, Conveto, Fraga Proc. 49/97, Lima, Miyakawawase, Moro 27, Moro 29, Moro 30, Moro 31, Natal Folha Murcha, Navelina, Olivelands, Orange Barile SRA 559, Orange Barlerin SRA 568, Orange Clanor SRA 391, Orange Hall SRA 394, Orange Navelina SRA 332, Orange Yoshida Navel SRA 558, Ouro, Pardilho, Pera Bianchi, Pera IAC, Pera Ipiguá, Pera Mel, Pera Ovale Siracusa, Pera Ovo, Pera Piragi, Rico, Rotuma Blood, Setubalense Proc. 49/97, Strand, Sanguino, Tarocco 23, Tarocco 27, Tarocco 32, Tarocco 34, Tobias, Torregrosso, Valencia, Valencia Berry, Valencia Chaffei, Valencia Late Florida, Vanilla, Vasconcellos, Wetumpka.
Susceptible	
Assay I	Agridoce, Corumbá, Albertina, Bahia IPEAL-5, Baianinha 1-39-6, Baianinha 23-4-55, Baianinha BB, Baianinha Ivers, Cacau, Caipira, Caipira B, Caipira C, Caipira Piaui, Coroa, Do, Céu-2, Hamlin Variegada, Jaboticaba, Lamb Summer, Lara Campos, Lisa, Paulista, Lue Gin Gong, Mangaratiba, Maracanã, Navelência, Ouro, Parson Brown, Pera, Pera CENA 1, Pera CENA 3, Pera Ipiguá, Pera Redonda, Pera Rio, Pera Roberto Gullo, Perola, Rosa, Serra D'água, Valência Olinda.
Assay II	Aziza, Baianinha, Besteiros, Jaffa, D. João Proc.49/97, Do Ceu, Gardner CV, Grada, Hamlin, Iapar 73, Laciniées SRA 571, Laranja A.R. H., China, Laranja Vale dos Besteiros, Mapo, Moro 12, Moro 16, Moro 17, Moro 22, Moro 28, Old Bud Line, Orange A Feuilles, Orange Rotuna SRA 511, Paulista, Salustiana SPA 11, Seleta do Rio, Seleta Tardia, Tarocco 11, Tarocco 15, Tarocco 14, Valencia Temprana IVIA, Valencia Campbell, Valencia Frost, Valencia Mutação, Valencia Olinda, Valencia Precoce, Valencia 1231, Valencia 1330, Vascaro Blood, Verde de Espanha, Westin.
Highly susceptible	
Assay I	Agridoce da Flórida, Agridoce Dummitt, Agridoce Stown, Agro Sevilhana, Bahia Bettin, Bahia IPEAL-14, Bahia Matatauba, Bahia Nevada, Bahia Sanguinea, Baianinha 23-4-60, Baianinha com Sementes, Baianinha IAC 31, Baianinha Piracicaba, Bebedouro, Blood Oval, Cacau Colcan, Caipira (gummosis-resistant), Clementina RS (Westin), Feijão Cru, Itacuruçá, Italiana, José Paulino, Lima, Limão, Melrose, Monjolo, Natal, Natal de Bebedouro, Parnazo de Goiás, Peito de Moça, Pera C–21.2, Pera CENA 2, Pera CENA 4, Pera CNPMF A 15.1, Pera CNPMF B 21.1, Pera CNPMF D 25.3, Pera G.S.2000, Pera Olímpia 1515, Perão do Rio (3), Perão do Rio2 (6), Peruchi, Seleta Abacaxi, Seleta Vermelha, Sweet Orange x Pomelo, Thompson Navel, Valência Campbell, Valência Variegada RG.034, Variegada.
Assay II	Baia Cabula, Baia Navelina, Biondo, Ciaculi 60/22A/2, Navelina SA, Clementina Nules, Cristalina, Moro 15, Moro 20, Natal, Orama, Prata Proc. 4997, Proc.435196, Sunstar, Tarocco 28, Valencia Campbell, Valencia Cutter, Valencia Folha Murcha, Valencia Precoce, Washington Navel.

Table 2. Response of mandarin, hybrids an other genotypes to infection by *Xanthomonas citri* subsp. *citri* strain, based on foliar area affected by canker lesions.

Resistant
Assay I
Mandarin and hybrids: Cabrita, Clementina Caçula 3, Dieberger-14, King of Siam, Malvasio, Poncan Tuxpan, Poncan, Oleosa, Satsuma.
Tangor: Brinco, Sabará, São José, São Pedro.
Tangelo: Pina, Sampson Williams, Watt, Webber.
Sour orange: Bergamota, Paraguaia, Zancheta, Tunis.
Lemon, lime, and acid lime: Deba Ahmed, King, Lemons x Lime, Lisbon (thornless), Selvagem (lime), Taiti (Santa Cruz), Vermelha de Goiás (lime).
Grapefruit: Hart's, Marsh Seedless-11656, Red Mexican.
Pummelo: Doce, Melancia, Nakorn, Pink.
Others: Calamondin, *C. taiwanica*, *C. philiphine* hybrid, Murraya sp.
Moderately resistant
Assay I
Mandarin and hybrids: Branca, Cascalho, Chinotto, Clementina Caçula 4, Clementina, Cravo (seedless), Cravo Guidotti, Dancy, Dieberger-1, Dieberger-13, Dieberger-6, Guidotti-Variação, Ipanema, De Israel, Mel. Natsu Mikan, Osceola (Clem. x T. Orlando), Pau, Pectinífera, Poncan (1), Ponkan 197, Do Rio, Robinson, Romana (CN), Sto. Antonio da Posse, Swatow, Tankan (3), Willow (tetraplóide), WS.
Tangor: Baia x Cravo, Pera x Cravo, Reticulata, Sangue de Boi , Umatila.
Tangelo: Minneola, Page, Sampson, Sunshine, Swannee, Yalaha.
Sour orange: Algiers, Bigaradier, Catu, Egyptian, Oklawaha, Palmeiras, Rehovoth, São Paulo, US. Variegada.
Lemons, lime, and acid lime: Camargo, Cidra, Deodoro, Galego (Teffé), Kalpi, Kulu, Lemon x Citrus sp. Quatre Saisons Variegado, Sweet (lime), Tahiti CNPMF 02, Tahiti CNPMF 03, Taiti, Viradouro.
Grapefruit: Marsh Seedy, Red Blush, Imperial, Pernambuco, Royal, Mac Carthy, Foster.
Pummelo: Hawaiian, Inerme, Kao, Paune, Shatenayan 151-428, Siamesa, Singapura, Thong Dee, Vermelha, Zamboa.
Others: Cider Etrog, Cider of Commerce, *Citrus taiwanica* 150.924, Eremolemon Coachella, Limequat Lakeland *Microcitrus* sp., Satsumelo 10v-83.
Assay II
Mandarin and hybrids: Clementina Caçula, Clementina Oragrande, Clemenules SPA 17, Cravo Emperor, Hib. Cal. x Kang, Híbrido Nova SPA 3, Ortanique, Mexerica, Muscia, Nova, Ponkan, Rosehaugh Nartjee, Satsuma, Satsuma Hachimoto, Satsuma Okitsu, Satsuma Okitsu SPA 29, SPA 16, Span Americana, Szwuinkon, Vermelha, 114412.
Susceptible
Assay I
Mandarin and hybrids: Clementina, Clementina 2, Cravo, Montevideo, Oneco, Satsuma Anã no.3, Satsuma Unshiu Wase, Sunburst 1394.
Tangor: Tangerona.
Tangelo: São Jacinto, Williams.
Sour orange: Catu, Double Cálice, Spanish, Spanish Italian.
Lemon, lime, and acid lime: Americana (lime), Americano, Cravo Chapéu Vermelho, Dehra Dun, Lime x Tangelo, Tahiti (Vasconcelos), Tahiti CNPMF 2001.
Grapefruit: Thompson Pink.
Pummelo: Flemmings.
Others: *Atalantia ceylanica*, Citrumelo Tucson, *Citrus celebica*, Kunquat Meiwa, Kunquat Nippon, Limequat Eustis, Merope *C. longispina*, *Micromellum tephrocarpa*, Shekwasha 149-007.
Assay II
Mandarin and hybrids: Clemengold, Clementina Arrufatina, Clementina Caçula 4, Clementina Clemenules, Dekopon, Mexerica Miuda Proc., Ortanique, Tangerina Ladu.
Grapefruit: Marrs.
Lemon, lime, and acid lime: Monica.
Highly susceptible
Assay I
Mandarin and hybrids: Caçula 1, Campeona, Clementina, Clementina 1, Clementina 3, Clementina Caçula 2, Cravo, Dancy, Deodoro Putz, Dieberger, Dieberger-10, Dieberger-5, Do Para, Monselise (CN), Poncan 1400, Poncan Bicuda, Poncan variegada (viv.6), Portuguesa, Revero, Satsuma Anã, Satsuma Wase, Suen-kat, Sunburst 1376, Wilking, Willom cv. (tetraplóide).
Tangor: Alexandre Pereira, Mimosa, Mó (Maracujá), Murcott (feew seeds), São João Del Rei, Umatila, Umatila Verdadeiro.
Tangelo: Sampson, Thornton.
Sour orange: Azeda (thornless), Bergamota, Dummitt.
Lemons, lime, and acid lime: Armstrong Seedless, Cravo Mutação, Faustrine, Geórgia, De Goias, Indiano, Periforme, Siciliano, Tahiti CNPMF 2000, Tahiti Variegado, Vale do Paraiba (Sidnei-CPA), Woglum.
Grapefruit: Connores, Duncan, Hybrid Lemelo 150-926, Leonardy, Pomelo 325, Pomelo-do-Cabo, Retiro, Royal, Viçosa.

Pummelo: Chinesa, Cuban, Indian Red, Indochina 151.427, LauTau, Periforme, Sunshine, Toranja.
Others: *Atalantia ceylanica*, Citrumelo Tucson, *Citrus celebica*, Kunquat Meiwa (*F. crassifolia*), Kunquat Nippon, Limequat Eustis, Merope Citrus longispina, *Micromellum tephrocarpa*, Shekwasha 149-007, Yuzu (*C. ichangensis* x *C. reticulata*).

Assay II

Mandarin and hybrids: Clementina Comune, Mexerica Mogi das Cruzes, Sunburst.

be susceptible to *Xcc* 306 (Tables 1 and 2, Fig. 1). Remarkably, moderate resistance to citrus canker was represented in the highest number of accessions. As expected, the lowest number of accessions was that with high resistance to the disease. Approximately 55% of all varieties, in a certain degree, were found to be resistant against citrus canker (13% of all varieties were found to be resistant, and 42% moderately resistant), 20% susceptible, and 25% highly susceptible.

Interestingly, some varieties exhibited a wide range of responses according to their mutations, such as Valencia sweet orange, which showed accessions with high resistance (i.e. 'Valencia Taquari'), moderate resistance ('Valencia Berry'), susceptibility ('Valencia Mutação'), and high susceptibility ('Valencia Precoce'). Remarkably, the identification of such accessions of citrus that are resistant to canker is helpful in indicating mutant varieties that could replace, with minor modifications in the fruit, those traditional cultivars that are susceptible to canker. On the other hand, the fact has to be taken into account that many of the varieties included in germplasm collections worldwide are provided by growers, who name such plants (basically by resemblance) despite its origin. Although a classification refinement of such varieties is performed by specialists during their introduction in the germplasm collection, it is not rare to find that the same genotype shows various names. In summary, there is a chance that more than one accession represent the same genotype.

Moreover, there might be a relationship between the strain of *Xcc* and the variety of a few species of citrus, such as seen in pummelo (*Citrus grandis*), which shows a differential response to *Xcc* strains on its cultivars Otachibana, Banpeiyu and Anseikan, probably due to the limited genomic variation of the bacterium (Shiotani *et al*., 2000).

A point to be taken into consideration would be whether the continued resistance would be real, because of the short period between infection and evaluation, since all leaf samples were collected 30 days after inoculation. However, it seems unlikely that the resistance reaction should have become modified after that period. Lesion expansion, which is used to describe resistance of citrus plants to canker in greenhouse (Gottwald *et al.*, 1993), has been shown to cease at nearly 20 days after inoculation and the bacterial population was substantially decreased after 40 days (Graham *et al*., 1990; Koizumi, 1981).

In some citrus species, the production of certain bacterial inhibitors by a citrus variety was found to be associated with prevailing weather conditions, like some areas of Argentina and Japan where a slow decrease of *Xcc* population density associated with leaf lesions may occur over time, which may be the a result of defense reactions, such as accumulation of phenolic compounds, developed by the host at late stages of disease development (Pruvost *et al*., 2002).

In conclusion, this study provides an additional and reliable approach for evaluation of citrus response to canker infection. The technique promotes no mechanical damages to leaves and, thus, mimics well the field conditions where canker occurs, but where citrus leafminer is controlled or is not present. Moreover, this study indicates good candidates, such as calamondin, 'Pera mutação' and Satsuma mandarin, among others, to be included in breeding programs as well as in gene cloning or even field introductions.

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