Evaluation of *Citrus psorosis virus* in Brazilian Citrus Germplasm Using DAS-ELISA

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ABSTRACT. Psorosis, caused by *Citrus psorosis virus* (CPsV), is one of the most important citrus diseases well-circulated. It has a long incubation period, causing bark scaling on branches and trunk of sweet oranges, mandarins and grapefruits. In Brazil, a survey conducted in the 1960's in São Paulo State indicated the presence of different psorosis types in several citrus cultivars. From the use of nucellar lines, and shoot-tip-grafting, psorosis is no longer a problem for the commercial citrus industry. However, "chlorotic leaf-flecking" and "oak-leaf pattern" have been found in biological tests of some old-line accessions of "Instituto Agronomico de Campians" (IAC) - Citrus Germplasm Bank (CGB), even though no typical psorosis trunk symptoms occur in the original plants. Application of a DAS-ELISA test with monoclonal antibody of the line PS29 for 43 sweet oranges, 4 tangelos, 4 mandarins, 2 lemons, 1 grapefruit and 1 accession of *Citrus bergamia*, that presented symptoms in biological tests, did not show a positive reaction. Those results, and the additional information of negative index for CPsV by ELISA with monoclonal antibodies (13C5 and 2A3) as well as RT-PCR, performed in samples of two of the same materials of IAC-CGB transferred to Embrapa CNPMF, suggest that the psorosis complex present in Brazil is not related to CPsV, probably belonging to the group of diseases that produce those symptoms in indicator plants, but are not considered psorosis.

Index words: *Citrus psorosis virus*, São Paulo State, biological indexing, RT-PCR.

Diseases of the psorosis complex (psorosis A, psorosis B, and citrus ringspot, caused by Citrus ringspot virus - CRSV) are widely distributed among species and varieties of citrus. Of viral etiology, psorosis has a known incubation period of up to 12 yr to express symptoms, which are characterized mainly by bark cracking and bark scaling of the trunk and branches of sweet orange, mandarins and grapefruit (10). In São Paulo, four decades ago, a survey conducted in five citrus regions indicated the presence of different psorosis types in several varieties (11). From the use of nucellar clones, and shoot-tip-grafting, psorosis is no longer a problem for the commercial citrus industry.

Two viral components of different morphology and coat protein capsid units of approximately 48 kDa, have been associated with different isolates of CRSV and psorosis (4). Because of the similarity of viral particles found in CRSV and other sources of psorosis (CPsAV Citrus psorosis-associated virus), it was proposed that it should be classified in a new genus, *Ophiovirus*, and *Citrus psorosis virus* (CPsV) for the name of the putative causal agent of psorosis (3, 6). Observations from Argentina, Texas and elsewhere suggested that there may be a vector of CPsV, since virus-free trees became infected in the field (9). According to the authors, CPsV could be detected in zoospores from roots infected with an *Olpidium*-like fungus associated with citrus roots, but further work is required to confirm that this fungus is able to transmit the virus to healthy citrus trees.

Until recently the only method of psorosis identification was through biological indexing. However, serological and molecular indexing involving the isolation and purification of virus for the production of specific antisera has been developed and used in several countries (3, 6, 12). The evaluation of these tools in the detection of pathogens present or not in Brazilian conditions is of great importance for mother trees registration and certification programs, as established

in São Paulo State in 1969 and restructured in 1998 (2).

The objective of this study was to evaluate the reaction of different accessions of Citrus Germplasm Bank (CGB) of the Instituto Agronômico de Campinas (IAC), previously diagnosed as psorosis infected by biological index, compared to the application of DAS-ELISA test with monoclonal antibody line PS29.

The research was conducted in the Laboratory of Biotechnology at the Centro APTA Citros Sylvio Moreira-IAC, in Cordeirópolis, SP. We evaluated 43 varieties of sweet oranges [*Citrus sinensis* (L.) Osbeck], 4 mandarins (*C. reticulata* Blanco, *C. clementina* Hort. ex. Tanaka, *C. unshiu* Marc), 1 grapefruit (*C. paradisi)*, 4 tangelos (*C. reticulata* x *C. paradisi)*, 2 lemons (*C. limon* Burm. f.) and 1 accessions of *C. bergamia* Risso & Poit (Table 1).

The presence of psorosis complex in the citrus accessions was previously detected by biological index using the "double grafting test" in 6-mo-old seedlings of Rangpur lime (*Citrus limonia* Osbeck) and 'Do Céu' sweet orange as indicator plants. Each test was performed in four replications, using as positive control a treatment inoculated with buds of Natal Sorose CN 482 sweet orange accession. For a negative control, we also included uninoculated 'Do Céu' sweet orange plants. The plants were kept in a greenhouse with 28^{o}C day and 16^{o}C night temperatures, and the readings of symptoms in young leaves were recorded twice a week, observing the occurrence of "oak leaf patterns" and/or "chlorotic leaf-flecking".

The DAS-ELISA (double antibody sandwich indirect enzyme-linked immunosorbent assay) was applied according to Clark and Adams (1). The monoclonal antibody (MAb) 29 PS (1:1000 dilution) used was produced for the University of Bari (3) and distributed as a Kit from Agritest SRL, Italy. Young leaves were collected and prepared by grinding and homogenizing in 2 ml of phosphate buffer, pH 7.4, containing 0.5 ml/l tween 20 (PBST) and 20 g/l polyvinylpyrrolidone (PVP-40) at 1:20 (w/v) dilution. Extracts from healthy and symptomatic (oak leaf or flecking) plants were utilized as negative and positive controls, respectively. The assay had two repetitions per sample and was evaluated after 3 h of incubation. The plates were read with a BioRad ELISA Plate Reader at a 405 nm wavelength. The samples were considered positive if the optical density was higher three times greater than the mean of healthy controls. The results of biological indexing using sweet orange 'Do Céu' for detecting psorosis virus present in the materials of the CGB-IAC showed a large variability in expression of oak leaf pattern or chlorotic leaf-flecking symptoms, mainly due to variations in temperature in the greenhouse. However, all positive results presented in this study (Table 1) were obtained by a wide margin of safety, since the readings were taken weekly and the management of the plants was done with care to prevent leaf spot diseases that could interfere with the symptoms. No shock symptom, typical of psorosis - A (10), was observed in the indicator plants.

It is expected that a higher efficiency to detect psorosis virus can be obtained with the development of faster techniques for detection, such as those based on molecular tests (4) and serology (3, 6) and a more specific and sensitive such as the dot-blot hybridization assay for routine detection of CPsV (5). Differences in biological behavior of different isolates of CPsV suggest that different strains of the virus may exist, and the use of monoclonal antibodies indicated as a possible means for the serological identification (3). The

specificity of the detection kit tested in this study is based on research of those authors, who immunized mice with preparations of an Italian isolate of CPsV (AMI-191Xa) purified from *Chenopodium amaranticolor*. The authors

TABLE 1
RESULTS OF BIOLOGICAL AND SEROLOGICAL DETECTION OF *CITRUS PSOROSIS VIRUS* IN THE IAC CITRUS GERMPLASM BANK (IAC-CGB)

Variety/Clone	Cod. IAC-CGB	Origin	Biological Psorosis test	CPsV DAS-ELISA
Sweet oranges (*C. sinensis*)				
Abacaxi	CV 103	Brazil (?)	+	-
Baia Cabula	CV 25	Brazil (?)	+	-
Baia Tremenbe	CV 17	Brazil (?)	+	-
Baianinha 22	CV 22	Brazil (?)	+	-
Baianinha 33	CV 33	Brazil (?)	+	-
Baianinha Piracicaba	CV 23	Brazil/SP	+	-
Barão	CV 92	Brazil (?)	+	-
Barão Bocaiuva	CN 422	Brazil/SP	+	-
Blood Oval cv	CN 470	Brazil (?)	+	-
Cacau	CN 121	Brazil (?)	+	-
Caipira DAC	CV 137	Brazil (?)	+	-
Champagne	CN 15	Brazil (?)	+	-
Champagne	CV 03	Brazil (?)	+	-
Cipó	CV 96	Brazil (?)	+	-
Gardner cn (1440)	RG 035	Brazil/DF	+	-
Hamlin 73	CV 73	Brazil (?)	+	-
Homossassa	CV 83	Brazil (?)	+	-
Joao Nunes	CV 109	Brazil (?)	+	-
Lima	CV 09	Brazil (?)	+	-
Lima sem Sementes	CV 01	Brazil (?)	+	-
Lima Verde	CV 13	Brazil (?)	+	-
Mediterraneam (Xilop)	CN 427	Brazil/SP	+	-
Moro	CN 48	Brazil/RJ	+	-
Moro Acireale 2	CN 46	Brazil (?)	+	-
Navelina cv	CN 423	Brazil/SP	+	-
Ouro	CV 97	Brazil (?)	+	-
Paulista	CV 90	Brazil (?)	+	-
Pera	CV 155	Brazil (?)	+	-
Pera Caire	CV 157	Brazil (?)	+	-
Pera De Abril	CV 148	Brazil (?)	+	-
Pera Dibbern	Ensaio	Brazil (?)	+	-
Pera Mel	CV 153	Brazil (?)	+	-
Pera Perao	CV 150	Brazil (?)	+	-
Piralima	CN 411	Brazil/SP	+	-
Piralima	CV 02	Brazil (?)	+	-
Rosa	CV 95	Brazil (?)	+	-
Rubi 52	CV 52	Brazil (?)	+	-
Salustiana cv	CN 438	Brazil/SP	+	-
Seleta Amarela	CV 69	Brazil (?)	+	-
Valencia Murcha	B	Brazil/SP	+	-
Seleta Itaborai	CV 145	Brazil/RJ	+	-
Seleta Branca	CV 68	Brazil/RJ	+	-
Mandarins (*C. reticulata,C. clementina, C.unshiu)*				
Loose Jacket	CN 515	Israel	+	-
Cravo Tardia	CN 436	Brazil (?)	+	-
Clementina Nules	RG019 1742	Spain	+	-
Kara	CV 178	Brazil (?)	+	-
Tangelos *(C. reticulata x C. paradisi)*				
Minneola	CV 224	Brazil (?)	+	-
Orlando	CV 225	Brazil (?)	+	-
Sampson	CV 221	Brazil (?)	+	-
Simineole	CV 226	Brazil (?)	+	-
Lemons (*C. limon*)				
Americano	CV 264	Brazil (?)	+	-
Cowgill	CV 270	Brazil (?)	+	-
Grapefruit *(C. paradisi)*			+	-

Imperial	CV 320	Brazil (?)	+	-
Bergamia *(Citrus bergamia)*				
Citrus bergamia	CN 702	Brazil (?)	+	-
Positive control = Natal Sorose CN 482 sweet orange accession		Brazil (?)	+	-
Negative control = Healthy 'Do Céu' sweet orange, not inoculated.			-	-

** Positive (+) or negative (-) results for psorosis virus complex, as tests performed in 'Do Céu' sweet orange indicator.

selected, multiplied and tested individually 24 virus-specific Mabs by ELISA against psorosis from 40 sources in different geographical regions (Italy, Lebanon, Spain and USA), and 16 different epitopes were identified and a large variability of serological reactions, apparently related to the geographical origin was identified.

In the present study, the evaluation of leaf samples of 55 different accessions showed no positive reaction (Table 1). Whereas all of them showed symptoms of "oak leaf" patterns or chlorotic leaf-flecking in bioassays, these results could indicate the apparent lack of specificity of the antiserum to the psorosis virus strain present in Brazilian conditions, as the results of (3) who found that none of the 24 evaluated Mabs was able to recognize all the 40 sources of CPsV. The specificity in the detection of different isolates of CPsV can be greatly affected by the concentration of virus in tissue (3). In this study, however, due to environmental control, the high genetic variability (43 different varieties of sweet oranges, 4 tangelos, 4 mandarins, 1 lemon 2 grapefruits and 1 *Citrus bergamia*) and diversity of geographical origin (São Paulo, Rio de Janeiro and the Federal District in Brazil, Spain and Israel) of the materials evaluated, it is believed that little or no possibility that the concentration of virus in the tissue was so low that it does not provide significant value in absorbance.

Psorosis is reported to be of great importance in the past in the State of São Paulo, infecting old lines of sweet oranges and grapefruits with variable symptom expressions (7). These same authors reported that, after the use of nucellar lines, the only cultivar of commercial importance infected by the disease was the 'Folha Murcha' sweet orange, but without major damage. This cultivar was also evaluated in this study and the results indicate that, like the other materials evaluated, the infectious agent present is probably not the same as that which occurs in Italy, Lebanon, Spain and USA, and possibly also in Argentina, where the disease may be associated with a vector and is considered of importance (12). Additional information arising from ELISA with monoclonal antibodies (13C5 and 2A3) as well as RT-PCR, performed in Spain in samples of 'Folha Murcha' sweet orange leaves, sent to Dr. Pedro Moreno of the Instituto Valenciano de Investigaciones Agropecuarias - IVIA (Barbosa, pers. comm., 2006) reinforce this hypothesis. This analysis, carried out in IVIA also showed negative results for samples of other sweet oranges of IAC-Citrus Germplasm Bank, transferred to Embrapa CNPMF, as Pera Caire CV 157 and Natal Sorose CN 482, also evaluated in this study. The field evaluation did not show any typical psorosis symptoms in IAC-Citrus Germplasm Bank plants.

The absence of typical psorosis - A shock symptoms was observed in the indicator plants, indicating that the psorosis complex present in our conditions probably belongs to a group of other diseases that cause other leaf symptoms in indicator plants, but are not classified as psorosis, as described by (10). Thus, the previous report of its occurrence in the São Paulo State (11)

should have been associated with damage on the trunk caused by other anomalies such as "popcorn", characterized according the same author to formation of small pustules, with or without gum exudation, or even leprosis. Considering also that the form described as "Psorosis type Bahia", in Bahia and Sergipe States is now considered a distinct disease (8), it is possible that the CPsV does not occur in Brazilian conditions.

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