

Screening for tolerance of citrus to *Xylella fastidiosa*, the causal agent of *citrus variegated chlorosis* CVC

Francisco F. Laranjeira^{a*}
Jorgino Pompeu Jr.^a
Antonio Garcia Jr.^b
Manoel Vieira^c
Ricardo Harakava^d
Maria Julia Gobbo Beretta^d

^a Centro de Citricultura Sylvio
Moreira, Instituto Agronômico de
Campinas, CP 04, Cordeirópolis,
SP 13490-970, Brazil

^b Casa da Agricultura de Neves
Paulista - CATI, av. Independência,
369, Neves Paulista,
SP 15200-000, Brazil

^c Bascitrus Agroindústria S/A, Rod.
Feliciano Salles Cunha, km 455,
Mirassol, SP 15130-000, Brazil

^d Instituto Biológico de São Paulo,
Seção de Bioquímica Fitopatológica,
av. Conselheiro Rodrigues Alves,
1252, São Paulo,
SP 04014-002, Brazil

* Correspondence and reprints

Received 28 January 1998
Accepted 16 April 1998

Fruits, 1998, vol. 53, p. 345–349
© Elsevier, Paris

RESUMEN ESPAÑOL, p. 349

Screening for tolerance of citrus to *Xylella fastidiosa*, the causal agent of *citrus variegated chlorosis* CVC.

Abstract — Introduction. Citrus variegated chlorosis (CVC), caused by *Xylella fastidiosa*, threatens seriously the Brazilian citrus industry, because it affects all sweet orange varieties. An intensive screening programme was launched in early 1990 to detect some degree of CVC tolerance among available citrus accessions. **Materials and methods.** Two large germplasm banks of 153 accessions each were established outdoor in the northwestern region of São Paulo state, Brazil, close to high inoculum pressure produced by adjacent affected groves of sweet orange. From March 1993, visual assessments were made bimonthly, and serological tests and polymerase chain reaction (PCR) detection were applied to samples of each variety. **Results and discussion.** CVC symptoms were observed on sweet oranges as early as September 1993. While, in early 1997, i.e. 48 months later, no symptoms were observed in most tangelo varieties, except Page and Swanee (slight leaf symptoms, but normal quality fruit and juice). The use of PCR diagnosis indicated that symptomless tangelos were carriers of *X. fastidiosa*. **Conclusion.** Because tangelo yield and fruit quality were not affected when established in high inoculum areas, tangelos could be an alternative crop for small plantation farmers of the northwestern region of São Paulo state. In January 1996 and 1997, 100 000 tangelo trees were planted in this region. Up to now not a single plant raised in outdoor nurseries with high inoculum pressure shows CVC symptoms nor has *X. fastidiosa* been detected by serological tests. (© Elsevier, Paris)

Brazil / Citrus / disease control / Bacteria / *Xylella fastidiosa* / pest resistances

Recherche d'une tolérance à *Xylella fastidiosa*, agent de la chlorose variégée des agrumes.

Résumé — Introduction. La chlorose variégée des agrumes, ou CVC, due à *Xylella fastidiosa*, menace sérieusement la citriculture brésilienne puisque cette maladie affecte toutes les variétés d'oranger. Un vaste programme de recherche a été lancé en 1990 pour détecter des tolérances parmi des variétés d'agrumes disponibles. **Matériel et méthodes.** Deux grandes collections de 153 accessions chacune ont été installées en plein air au nord-ouest de l'État de São Paulo (Brésil) à proximité de vergers d'orangers contaminés, à l'origine donc d'une forte pression d'inoculum. À partir de mars 1993, des observations bimensuelles ont été effectuées et des tests sérologiques, accompagnés de détection par PCR, ont été menés sur les échantillons de chacune des variétés. **Résultats et discussion.** Des symptômes de CVC ont été observés sur orangers dès septembre 1993, alors que, au début de l'année 1997, soit 48 mois plus tard, aucun symptôme n'était visible chez la plupart des variétés de tangelo, hormis Page and Swanee (légers symptômes sur les feuilles, mais fruits et jus de qualité normale). L'utilisation de la technique des PCR a montré que les tangelos sans symptômes étaient cependant hôtes de *X. fastidiosa*. **Conclusion.** Puisque la qualité des tangelos et leur rendement ne sont pas affectés en zone à forte pression d'inoculum, leur culture pourrait être conseillée à des exploitants de petites plantations du nord-ouest de l'État de São Paulo. En janvier 1996 et 1997, 100 000 arbres de ce groupe ont été plantés dans cette région. Jusqu'à présent, aucun plant parmi ceux des pépinières en plein air placées sous forte pression d'inoculum n'a montré de symptômes de CVC ni révélé, par tests sérologiques, la présence de *X. fastidiosa*. (© Elsevier, Paris)

Brésil / Citrus / controle de maladie / Bacteria / *Xylella fastidiosa* / résistance aux organismes nuisibles

1. introduction

Citrus variegated chlorosis (CVC) is presently the most harmful disease for Brazilian citriculture. It was first detected in 1987, in sweet orange groves in the northwestern region of São Paulo state, Brazil [1], and is now widespread in this country [2] and some regions of Argentina [3]. The disease is caused by *Xylella fastidiosa*, a non-motile, xylem limited, gram-negative, fastidious bacterium [4, 5]. The disease epidemics occur through the transmission by 'sharpshooter' leafhoppers [6] or infected budwood [7]. This bacterium is also the etiological agent of a number of other diseases including Pierce's disease of grapevine or plum leaf scald [8].

The most typical symptom of CVC is a mottled leaf chlorosis on the upper surface of mature leaves and brown blisters on the other side corresponding to the chlorotic areas. As a result of xylem clogging, the trees exhibit foliar wilt symptoms [9] and the fruits are severely affected. Fruits become hard, very small, and the juice quality is seriously affected [10]. Therefore, the fruits are suitable neither for processing nor for fresh consumption. Fruit symptoms were mostly observed on sweet oranges [11]. Despite foliar symptoms noticed on mandarins [12] and Tahiti lime [13], only sweet oranges have exhibited fruit symptoms. The Brazilian citrus industry is primarily based on four sweet orange varieties: Pera, Natal, Valencia and

Hamlin, and all are very susceptible. Consequently, an intensive screening programme was launched in early 1990, with the intention of detecting some degree of CVC tolerance among 153 citrus accessions.

2. materials and methods

2.1. orchards

Two germplasm banks of 153 accessions belonging to the major citrus groups (table I) were established in the northwestern region of São Paulo state, Brazil (Mirassol and Barretos counties) in January 1993. Each 1.3 ha plot consisted of 5 individuals of a given accession randomly distributed and planted at a distance of 7.0 × 2.5 m (153 varieties × 5 trees/variety = 765 trees at each location). Both plots were adjacent to highly infected orange groves. Standard cultural practices were applied with the exception of insecticide treatment for enabling free sharpshooter contamination.

2.2. sampling

The evaluations (beginning in March, 1993) were made bimonthly and a sample of each variety was taken for serological tests. Each sample consisted of four to five leaves taken randomly around the trees. The sample processing was done accordingly to Lee et al. [14] using leaf petioles.

2.3. serological tests

2.3.1. dot immunobinding assay (DIBA)

The procedure described by Lee et al. [14], using *X. fastidiosa* (UF-26) specific antibodies in a 1:64 000 dilution, was adopted. As secondary antiserum, a goat anti-rabbit IgG conjugated to alkaline phosphatase enzyme (Sigma A-3 937) in a 1:30 000 dilution, was used. Nitroblue tetrazolium (NBT) and brome-chloride-indolyl-phosphate (BCIP) were the

Table I.

Summary of groups and number of citrus varieties present at the germplasm banks established in the northwestern region of São Paulo state, Brazil (total: 153 varieties).

Groups	Number of varieties
Sweet oranges	81
Mandarins	35
Tangors	16
Tangelos	10
Others	11

enzyme substrates used according to Sigma Co. directions. The positive samples were distinguished by a contrasting purple color.

2.3.2. Western-blotting

Ten ml of each sample were subjected to electrophoresis (200 V, 42 min) in a polyacrylamide mini-gel with SDS (Mini-Protean II, Bio Rad). A discontinuous gel system was used, with a 4% concentrating gel and a 12% separation gel. The separated proteins were transferred to a nitrocellulose membrane using a semi-dry apparatus (TransBlot SD, BioRad) at 15 V for 30 min. For filter paper and nitrocellulose membrane wetting, a buffer consisting of Tris 48 mM pH 9.2, glycine 39 mM, SDS 1.3 mM and methanol 20% was used. The membrane was immersed in TTBS buffer (Tris-HCl 50 mM, NaCl 0.15 M, Tween 20 0.05%, pH 9.5), boiled in microwave oven for 1 min and blocked with non-fat milk 5% p/v in TTBS for 30 min. The membrane was washed twice with distilled water (15 min each) and twice with TTBS (15 min each). The membrane was incubated at room temperature for 6 h with shaking in UF-26 antiserum diluted 1:64 000 in TTBS with bovine albumine serum (SAB) 0.5%. The membrane was washed again as formerly described, then incubated overnight with shaking at room temperature in a solution containing goat anti-rabbit IgG conjugated to alkaline phosphatase enzyme (Sigma A-9919) diluted 1:15 000 in TTBS with SAB 0.5%. The enzyme substrates NBT (0.083 mg·mL) and BCIP (0.167 mg·mL) were diluted in a buffer of Tris-HCl 0.1 M pH 9.5, NaCl 0.1 M, MgCl₂ 5 mM.

2.4. polymerase chain reaction (PCR)

Detection by PCR was run according to the procedure described by Minsavage et al. [15] and Harakava et al. [16], using specific primer to CVC-*X. fastidiosa*.

3. results and discussion

The two germplasm screening blocks of Mirassol and Barretos gave similar results in terms of CVC sensitivity. Typical CVC symptoms showed up on orange leaves as early as September 1993. It was not until late 1994 that scattered leaf symptoms were found on some mandarins and on the Page tangelo. The presence of *X. fastidiosa* in the above groups of citrus was confirmed by DIBA and Western blotting. After 48 months scattered leaf symptoms appeared on Swanee tangelo, and CVC was confirmed by positive DIBA and Western blotting (table II). To date not a single leaf symptom has been detected on 2 560, Fairchild, Minneola, Nova, Orlando, Sampson, Thornton and Weber tangelos. DIBA and Western blotting failed to detect *X. fastidiosa* in symptomless tissues of such varieties. However, in June 1996, plants were subjected to a PCR detection test and *X. fastidiosa* DNA was found in all assayed tangelo varieties with the exception of 2 560, Fairchild and Sampson. However, the degree of host colonization by the bacterium was not determined. These results show that at least some tangelo varieties are hosts of this bacterium. Moreover, the presence of *X. fastidiosa* in their tissues suggests that the bacterium is naturally

Table II.
Results of visual assessments, serological tests and PCR for tangelos.

Tangelo variety	Symptoms	DIBA	Western-blotting	PCR
2560	-	-	-	-
Minneola	-	-	-	+
Nova	-	-	-	+
Orlando	-	-	-	+
Page	+	+	+	+
Sampson	-	-	-	-
Swanee	+	+	+	+
Thornton	-	-	-	+
Weber	-	-	-	+
Fairchild	-	-	-	-

DIBA: dot immunobinding assay; PCR: polymerase chain reaction.

transmitted to tangelos in non-protected environments; that is, tangelos have no resistance against bacterium vectors. However, the multiplication of bacteria in tangelos does not seem to be very fast or efficient, since their presence was detected only by PCR. *X. fastidiosa* detection by serological tests was possible in southeastern São Paulo [17]. In this region, only tangelo Swanee were reported as being infected by *X. fastidiosa* and showing symptoms [17].

4. conclusion

The CVC symptoms that developed on Page and Swanee tangelos in the north-western region suggest that caution should be taken when selecting tangelo varieties for breeding programs of CVC tolerance, even if fruit quality is not affected. Small fruits were not observed in any of the tested tangelos, including Page and Swanee. Orlando tangelo, used in Brazil as a rootstock, must receive special attention in terms of CVC sensitivity at the different stages of development i.e. from the young seedling to the adult stages.

Considering the plants were established in high inoculum areas, the above results are promising. Because yields and fruit quality were not affected, tangelos could be an alternative crop for small plantation farmers of the northwestern region of São Paulo state [18].

These results prompted the planting of 20 000 tangelo trees in São Paulo in January 1996 and an additional 80 000 in January 1997. Up to now not a single plant raised in outdoor nurseries with high inoculum pressure shows CVC symptoms nor has *X. fastidiosa* been detected by serological tests. Nevertheless our results show that tangelos can be asymptomatic hosts and therefore must be considered as inoculum carriers to adjacent sweet orange groves.

references

- [1] Rosseti V., Garnier M., Bové J.M., Beretta M.J.G., Teixeira A.R.R., Quaggio J.A., De Negri J.D., Présence de bactéries dans le xylème d'oranges atteint de chlorose variégée, une nouvelle maladie des agrumes au Brésil, Cr. Acad. Sci. III-Vie 310 (1990) 345–349.
- [2] Tubelis A., Barros J.C.S.M., Leite R.M.V.B.C., Difusão da clorose variegada dos citros em pomares comerciais de laranja no Brasil, Laranja 14 (1) (1993) 239–254.
- [3] Contreras J., Pecosita ou 'Falsa mancha grascienta' na Argentina, Laranja and CIA 31 (1992) 6.
- [4] Hartung J.S., Beretta M.J.G., Brlansky R.H., Spisso J., Lee R.F., Citrus variegated chlorosis bacterium: axenic culture, pathogenicity, and serological relationships with other strains of *Xylella fastidiosa*, Phytopathology 84 (1993) 591–597.
- [5] Chang C.J., Garnier M., Zreik L., Rosseti V., Bové J.M., Citrus variegated chlorosis: cultivation of the causal bacterium and experimental reproduction of the disease, Proc. 12th IOCV (1993) 294–300.
- [6] Lopes J.R.S., Beretta M.J.G., Harakava R., Almeida R.P.P., Krügner R., Garcia Jr A., Confirmação da transmissão por cigarrinhas do agente causal da clorose variegada dos citros, *Xylella fastidiosa*, Fitopatologia Brasileira 21 (Suplemento) (1996) 343.
- [7] Rosseti V., Carvalho M.L.V., Chagas C.M., Ensaio de transmissão de clorose variegada dos citros (CVC), em campo, Fitopatologia Brasileira 20 (Suplemento) (1995) 351.
- [8] Purcell A.H., Hopkins D.L., Fastidious xylem-limited bacterial plant pathogens, Ann. Rev. Phytopathol. 34 (1996) 131–151.
- [9] Machado E.C., Quaggio J.A., Lagoa A.M.M.A., Ticelli M., Furlani P.R., Trocas gasosas e relações hídricas em laranjeiras com clorose variegada dos citros, Rev. Bras. Fisiol. Veg. 6 (1) (1994) 53–57.
- [10] Laranjeira F.F., Palazzo D., Determinação preliminar dos efeitos da Clorose Variiegada dos Citros em características físico-químicas de frutos de laranja Natal, Fitopatologia Brasileira 19 (Suplemento) (1994) 309.
- [11] Laranjeira F.F., Pompeu Jr J., Harakava R., Seleção de variedades de citros tolerantes e/ou resistentes à clorose variegada dos citros (CVC), Fitopatologia Brasileira 20 (Suplemento) (1995) 324.

- [12] Pompeu Jr J., Laranjeira F.F., Harakava R., Beretta M.J.G., Primeira constatação de sintomas foliares de clorose variegada dos citros (CVC) em tangerinas e híbridos de tangerina. *Fitopatologia Brasileira* 19 (Suplemento) (1994) 318.
- [13] Beretta M.J.G., Coelho R.C.S., Leal A.M.B., Gama T.T., Lee R.F., Derrick K.S., Tahiti lime is tolerant to Citrus Variegated Chlorosis, *Fitopatologia Brasileira* 18 (Suplemento) (1993) 277.
- [14] Lee R.F., Beretta M.J.G., Derrick K.S., Hooker M.E., Development of a serological assay for citrus variegated chlorosis: a new disease of citrus in Brazil, *Proc. Fla. State Hort. Soc.* 105 (1992) 32–35.
- [15] Minsavage G.V., Thompson C.M., Hopkins D.L., Leite R.M.V.B.C., Stall R.E., Development of a polymerase chain reaction protocol for detection of *Xylella fastidiosa* in plant tissue, *Phytopathology* 84 (1994) 456–461.
- [16] Harakava R., Beretta M.J.G., Derrick K.S., Lee R.F., Use of a polymerase chain reaction to routinely detect *Xylella fastidiosa* in citrus variegated chlorosis affected trees, *Fitopatologia Brasileira* 20 (Suplemento) (1995) 341.
- [17] Laranjeira F.F., Harakava R., Carvalho S.A., Pompeu Jr J., Figueiredo J.O., Avaliação serológica e sintomatológica da ocorrência de *Xylella fastidiosa* e clorose variegada dos citros (CVC) no Banco Ativo de Germoplasma do IAC, *Fitopatologia Brasileira* 21 (Suplemento) (1996) 335.
- [18] Anonymous, Tangelos resistem à CVC e têm bom aproveitamento industrial, *Informativo Coopercitrus X* (119) (1996) 16–17.

Búsqueda de una tolerancia a *Xylella fastidiosa*, agente de la clorosis variegada de los agrios.

Resumen — Introducción. La clorosis variegada de los agrios, o CVC, causada por *Xylella fastidiosa*, amenaza seriamente el citricultivo brasilero dado que esta enfermedad afecta todas las variedades de naranjo. Un amplio programa de búsqueda fue lanzado en 1990 para detectar tolerancias entre variedades de agrios disponibles. **Material y métodos.** Dos grandes colecciones de 153 accesiones cada una fueron instaladas al aire libre al noroeste del Estado de São Paulo (Brasil) a proximidad de huertos de naranjos contaminados, por lo tanto, al origen de una fuerte presión de inoculo. A partir de marzo de 1993, se realizaron observaciones bimensuales y se llevaron a cabo pruebas serológicas, acompañadas con detección por PCR, en las muestras de cada una de las variedades. **Resultados y discusión.** Se observaron síntomas de CVC en naranjos a partir de septiembre de 1993, mientras que, a principios del año 1997, o sea 48 meses más tarde, ningún síntoma era visible en la mayoría de las variedades de tangelo, excepto Page and Swanee (ligeros síntomas en las hojas, pero frutos y jugos de calidad normal). El empleo de la técnica de los PCR mostró que los tangelos sin síntoma eran no obstante huéspedes de *X. fastidiosa*. **Conclusión.** Dado que la calidad de los tangelos y su rendimiento no se hallan afectados en zona de fuerte presión de inoculo, su cultivo podría aconsejarse a explotadores de pequeñas plantaciones del noroeste del Estado de São Paulo. En enero de 1996 y 1997, se sembraron 100.000 árboles de este grupo en esta región. Hasta el momento, ninguna planta entre las de los viveros al aire libre, colocados bajo fuerte presión de inoculo, mostraron síntomas de CVC, ni revelaron, mediante pruebas serológicas, la presencia de *X. fastidiosa*. (© Elsevier, Paris)

Brasil / Citrus / control de enfermedades / Bacteria / *Xylella fastidiosa* / resistencia a las plagas