Stability of *Citrus tristeza virus* protective isolates in field conditions

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Abstract – The objective of this work was to monitor the maintenance of *Citrus tristeza virus* (CTV) protective isolates stability in selected clones of 'Pêra' sweet orange (*Citrus sinensis*), preimmunized or naturally infected by the virus, after successive clonal propagations. The work was carried out in field conditions in the north of Paraná State, Brazil. Coat protein gene (CPG) analysis of 33 isolates collected from 16 clones of 'Pêra' sweet orange was performed using single strand conformational polymorphism (SSCP). Initially, the isolates were characterized by symptoms of stem pitting observed in clones. Then viral genome was extracted and used as template for the amplification of CPG by reverse transcription polimerase chain reaction (RTPCR). RTPCR products electrophoretic profiles were analyzed using the Jaccard coefficient and the UPGMA method. The majority of the clones had weak to moderate stem pitting symptoms and its CTV isolates showed alterations in the SSCP profiles. However, the stability of the protective complex has been maintained, except for isolates from two analised clones. Low genetic variability was observed within the isolates during the studied years.

Index terms: *Citrus sinensis*, cross-protection, preimmunization, single strand conformational polymorphism, stem pitting symptoms.

Estabilidade de isolados protetores contra *Citrus tristeza virus* em condições de campo

Resumo – O objetivo deste trabalho foi monitorar a manutenção da estabilidade de isolados protetores contra *Citrus tristeza virus* (CTV) em clones selecionados de laranja 'Pêra' (*Citrus sinensis*) pré-imunizados ou infectados naturalmente pelo vírus, após sucessivas propagações clonais. O trabalho foi realizado em condições de campo, no norte do Estado do Paraná. A análise do gene da capa protéica (GPC) de 33 isolados, coletados de 16 clones de laranjeira 'Pêra', foi realizada com o uso da técnica polimorfismo conformacional da fita simples (SSCP). Inicialmente, os isolados foram caracterizados por meio de sintomas de caneluras observados nos clones. Em seguida, o genoma viral foi extraído e utilizado como molde para a amplificação do GCP com uso da transcrição reversa da reação em cadeia da polimerase (RTPCR). Os perfis eletroforéticos dos produtos da RTPCR foram analisados com emprego do coeficiente de Jaccard e do método UPGMA. A maioria dos clones apresentou sintomas fracos a moderados de caneluras, bem como alterações nos perfis dos isolados de CTV. Contudo, a estabilidade dos complexos protetores foi mantida, com exceção dos isolados presentes em dois dos clones analisados. Foi observada baixa variabilidade genética nos isolados durante os anos avaliados.

Termos para indexação: *Citrus sinensis*, proteção cruzada, pré-imunização, polimorfismo conformacional da fita simples, sintomas de caneluras.

Introduction

Citriculture in Brazil is distributed over several states, and sweet orange [*Citrus sinensis* (L.) Osbeck], cultivars Pêra, Valencia and Natal are the most commonly cultivated. Pêra is considered the most important cultivar due to its excellent quality and its great acceptance both in domestic and international markets (Salibe et al., 2002). The plants of this variety have an intolerant tissue to some diseases, such as citrus tristeza caused by the *Citrus tristeza virus* (CTV).

Because of the susceptibility of sweet oranges to CTV, in the 1930's and 1940's, Brazilian citriculture was severely affected, resulting in the extermination of millions of trees grafted on sour orange [*Citrus aurantium* L.] (Müller & Costa, 1991). The recovery of the orchards was possible due to several control measures adopted. Among the measures adopted, the

substitution of the sour orange rootstock by the Rangpur lime (C*itrus limonia* Osbeck), the clonal cleaning, and the preimmunization or cross-protection with mild isolates of the virus can be mentioned (Müller & Costa, 1977; Müller et al., 1999).

The characteristic symptoms of citrus tristeza are reduced growth and vigor of the plant, reduced fruit size, leaves with zinc deficiency symptoms (chlorosis), small leaves, presence of stem-pitting, in which slots or grooves that are formed in the plant xylem can be found, in branches and the trunk (Müller et al., 1999; Salibe et al., 2002).

The CTV is transmitted by different aphid species, however, under Brazilian conditions, the most efficient means of transmission is through the brown citrus aphid (*Toxoptera citricida* Kirk). Limited to the phloem of the citric plants, CTV is considered the largest plants virus, and its genome consists of a single strand RNA (ssRNA) molecule (Bar-Joseph et al., 1989; Souza et al., 2002a). The complex population of CTV RNA and its characteristic pathogenicity may suffer alterations due to host changes or aphid transmission, which suggests great genetic variation (Albiach-Martí et al., 2000). The genomic structure of CTV populations, and their alterations have been detected by the single strand conformational polymorphism (SSCP) technique (Rubio et al., 2000; D'urso et al., 2003; Davino et al., 2005). SSCP is also used in cross-protection studies to monitor the protective isolate in preimmunized plants (Souza et al., 2000a; Corazza-Nunes et al., 2001; Kim et al., 2006), and for virus identification and characterization (Vives et al., 2005; Iglesias et al., 2008).

The objective of this work was to monitor the maintenance of the CTV isolates stability in selected clones of 'Pêra' sweet orange, preimmunized or naturally infected by the virus, after successive clonal propagations, in field conditions of the north of Paraná.

Materials and Methods

The CTV isolates analyzed were selected from clones of 'Pêra' orange, which were preimmunized or naturally infected. The clones from which the isolates were obtained were 'Bianchi 89C', 'D-6 111A', 'Vacinada 59B', 'Vimusa 66B' and 'Seleção 37'. They were grafted onto rangpur lime (*Citrus limonia* Osbeck.) and installed in an experimental orchard in the north of Paraná State. Originally, the 'Pêra Bianchi 89C' clone was selected from propagations of a naturally CTV infected tree growing in a commercial orchard in São Paulo state. 'Pêra Vacinada 59B' clones, however, were formed from clone propagations of 'Pêra IAC' preimmunized material, also from São Paulo (Salibe et al., 2002).

Other studied clones were originated from different clonal propagations of preimmunized mother trees from São Paulo ('Pêra IAC') and Bahia (D-6 clone) states, and from possible naturally infected trees with mild isolates from different origins of São Paulo and Paraná States, which were 'Pêra Vimusa 66B' and 'Pêra Seleção 37' (Salibe et al., 1992) (Table 1).

The isolates present in the original material from accesses of the Citrus Germplasm Active Bank (ABG) located in the experimental station of Instituto Agronômico do Paraná (Iapar, Londrina, PR), and the isolates supplied by Centro APTA Citros Sylvio Moreira (CCSM) (Cordeirópolis, SP) and by Embrapa Fruticultura Tropical (Cruz das Almas, BA) were also analysed (Table 1). These isolates were collected from plants kept in screenhouse with protection against aphids.

The assessments of stem pitting symptoms were performed in the trunks and branches, in March 2005 and March 2008. A trunk window about 3x5 cm was opened near the grafting point in order to assign stem pitting scores from 0 to 5, according to the following

Table 1. Origin of the *Citrus tristeza virus* isolates.

Clone	Procedence	Isolate
Pêra Bianchi 89C	Experimental orchard	1a, 1b, 1c, 1d and 1e
Pêra D-6 111A	Experimental orchard	2a, 2b, 2c, 2d and 2e
Pêra Vacinada 59B	Experimental orchard	3a, 3b, 3c, 3d and 3e
Pêra Vimusa 66B	Experimental orchard	4a, 4b, 4c, 4d and 4e
Pêra Seleção 37	Experimental orchard	5a, 5b, 5c, 5d and 5e
Pêra Bianchi 89C	Iapar	B-I
Pêra D-6	Iapar	D-6-I
Pêra Vacinada 59B	Iapar	PVac-I
Pêra Vimusa 66B	Iapar	V-I
Pêra Bianchi	CCSM	B-C
Pêra D-6	CCSM	D-6-C
Pêra Vimusa 66B	CCSM	V-C
Pêra D-6	Embrapa	D-6-B
Pêra	Mild control (CCSM)	Pêra IAC - PIAC
Pêra	Severe control (CCSM)	Capão Bonito - CB
Pêra	Severe control (Paraná State)	Rolândia - R

scale (Meissner Filho et al., 2002): 0, no stem-pitting; 1, rare superficial stem-pitting; 2, sparse number of stem-pittings; 3, moderate number of stem-pittings; 4, many superficial and some deep stem-pittings; and 5, surface entirely covered with superficial and deep stem-pitting. As to young branches, samplings were done around the tree canopy, using the same scale above.

Measurements of plant height, diameter and canopy volume were accomplished in order to evaluate plant vegetative development. The equation $V = ⅔ (\pi r^2 H)$ (Mendel, 1956) was used to calculate the canopy volume. These data were transformed using $(X + 1)^{0.5}$, for normality of errors. The means were compared using Scott-Knott test, at 5% probability. The analyses were performed using Sisvar 4.3 software (Ferreira, 2008).

The dsRNA was extracted from leaves and young branches from the clone's quadrant cup, collected in 2005 and 2008, using the Trizol reagent, in agreement with the manufacturer's instructions (Invitrogen Carlsbad, CA, USA).

RNA extracted from the CTV isolates was used as a template for reverse transcription (RT) reactions (M-MLV reverse transcriptase, RNaseOUT recombinant ribonuclease inhibitor and random primer (Invitrogen, Carlsbad, CA, USA). Aliquots of the first strand cDNA synthesis were used to amplify the CTV coat protein gene (CPG) by polymerase chain reaction by Platinum PCR Supermix, (Invitrogen, Carlsbad, CA, USA) using the primers CN-119 (5' AGA TCT ACC ATG GAC GAC GAA ACA AAG 3') and CN-120 (5' GAA TTC GCG GCC GCT CAA CGT GTG TTA AAT TTC C 3'). The products of the amplification reactions, fragments of approximately 670 bp that correspond to the virus CPG, were analyzed in 1% agarose gel, according to the procedure described by Sambrook et al. (1989).

The SSCP analyses were performed according to modifications on the methodology described by Corazza-Nunes et al. (2001). Aliquots from 4 to 10 µL of the amplicons were mixed with an equal volume of denaturing solution (95% formamide, 2 mM EDTA and 0,05% bromophenol blue). Samples were denatured at 95ºC for 10 min, immediately put on ice, and then submitted to electrophoresis in 8% non-denaturing polyacrylamide gel containing TBE buffer (45 mM Tris-Boric, 1 mM of EDTA, pH 8,5), at 200 volts, for 15 hours at 25ºC. The gels were stained with silver nitrate, according to the procedure described by Beidler et al. (1982).

Electrophoretic profiles obtained were initially compared among themselves. Afterwards, they were compared with the profiles of the isolates from the original materials of Iapar, CCSM and Embrapa. The segments were converted into a binary matrix of presence and absence of strands. The analyses were performed using Genes Program (Cruz, 1997), and the genetic distances between isolates were estimated based on the Jaccard coefficient. Genetic distance matrixes were used to make the grouping analyses, using as criterion the unweighted pair-group method with arithmetic averages (UPGMA).

Results and Discussion

There were significant differences in the mean values of stem-pitting in the trunk and branches and in plant height (Table 2). The highest averages of the stem-pitting incidence were observed in 'Pêra Vimusa 66B' and 'Pêra Seleção 37', which showed severe to moderate symptoms of tristeza, respectively.

Table 2. Mean values of branches and trunk stem-pitting, plant height, crown diameter and volume of 'Pêra' orange clones grafted on Rangpur lime[(1)].

Clone	Stem-pitting[(2)]		Plant height (m)	Crown volume (m^3)	Crown diameter (m)
	Branches	Trunk			
Pêra Bianchi 89 C	1.0a	1.0b	2.47b	9.55a	2.65a
Pêra D-6 111 A	1.3b	1.1b	2.22a	7.75a	2.52a
Pêra Vacinada 59 B	1.3b	0.7a	2.49b	10.22a	2.67a
Pêra Vimusa 66 B	1.7b	3.1c	2.32a	8.77a	2.66a
Pêra Seleção 37	2.1b	1.6b	2.51b	9.44a	2.58a

[(1)]Means followed by equal letters do not differ in the columns by Scott Knott test, at 5% probability. [(2)]According to Meissner Filho et al. (2002).

Variability in the number and the position of strands in each electrophoretic profile, among the analyzed clone protective isolates were verified (Figure 1). The profiles obtained revealed the occurrence of four to ten bands, suggesting the presence of two to five haplotypes of population variants genetically related in each CTV isolate. As described by Gillings et al. (1996), Corazza-Nunes et al. (2001) and Souza et al. (2002a), plants are usually infected by isolates constituted of haplotypes mixture. Moreover, most of the plants of the same cultivar presented the same electrophoretic profile.

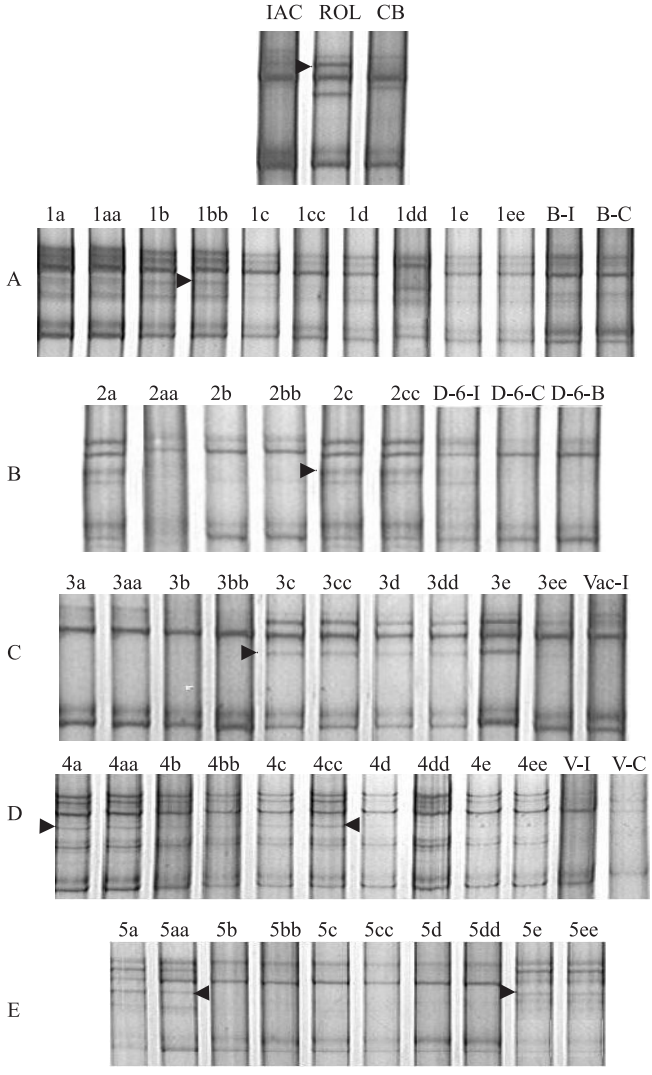

Figure 1. SSCP patterns of coat protein gene of different CTV isolates. A, 'Pêra Bianchi 89C' clones; B, 'Pêra D-6 111A' clones; C, 'Pêra Vacinada 59B' clones; D, 'Pêra Vimusa 66B' clones; E, 'Pêra Seleção 37' clones. Mild Control, 'Pêra IAC' (IAC); Severe controls = Rolândia (ROL) and Capão Bonito (CB). Samples with one letter, collection year 2005; samples with two letters, collection year 2008.

As to 'Pêra Bianchi 89C' clones (Figure 1 A), electrophoretic patterns of isolates were very similar in all replications in the assessed years, except for the clone 1dd, which is different from all. The profiles found in the isolates from Iapar (B-I) and from São Paulo (B-C) were identical amongst themselves, and similar to almost all isolates from 'Pêra Bianchi 89C' clones.

As to the isolates from D-6 111A clones, the electrophoretic profiles of isolates 2a and 2c, assessed in 2005, were identical (Figure 1 B). As for the isolate 2b, the electrophoretic pattern was identical to the pattern found in the original material from Iapar (D-6-I). Identical pattern was also observed among the isolates obtained from the material from São Paulo (D-6-C) and Bahia (D-6-B). However, the patterns were different from those found in the Iapar isolates, and in the other isolates from 'D-6 111A'. In the evaluation of 2008, no alteration was observed in those patterns.

In the case of isolates from 'Pêra Vacinada 59B' clones, isolates 3a and 3b were similar to the isolates from the Iapar collection (Vac-I) in both evaluations (Figure 1 C). However, in both evaluations, the 3c, 3d and 3e isolates showed patterns which were very similar to the one found in the Rolândia isolate, used as a severe control. 'Pêra Vacinada 59B' clones showed good field resistance regarding tristeza symptoms. Therefore, they are probably tolerant to CTV, since, in spite of allowing virus multiplication, they are not significantly affected by the disease.

Souza et al. (2002b), in a similar study with 'Pêra' sweet orange plants preimmunized with 'Pêra IAC' isolate, verified that their electrophoretic profiles were identical to the original protective isolate, even after a year of field exposure, in a region with high incidence of the severe complex "Capão Bonito". The alteration in the profiles of isolates 3c, 3d and 3e of that cultivar clones, making them similar to the ones found in Rolândia isolates, may have occurred, due to the high inoculum pressure exercised by the Rolândia complex. The alteration in the viral complex and the good viral multiplication within the plants may result in a protection break down, with consequent emergence of severe symptoms of the disease. According to Moreno et al. (1996), independently of the isolate mixture in the coinoculated plants, it is necessary a detectable

multiplication of the severe isolate for the symptoms to appear.

Isolates from 'Pêra Vimusa 66B' clones showed the largest number of bands and, consequently, haplotypes, when compared to the others (Figure 1 D). In 2005, identical electrophoretic profiles were found between the isolates 4b and 4c and between the isolates 4a and 4e. The isolates 4d differed from the others. None of the isolates presented patterns similar to those from the Iapar (V-I) and São Paulo (V-C) collections (Figure1 D). A significant difference when the isolate profiles of those materials were compared amongst themselves was observed. In 2008, considerable alteration was only verified in the profile of isolate 4dd.

In the isolates from 'Pêra Seleção 37' clones, the profiles of the isolates 5b, 5c and 5d were identical in 2005, which was maintained in 2008 (isolates 5bb, 5cc and 5dd) (Figure 1 E). Different patterns were found for the isolates 5a and 5e. None of the electrophoretic patterns were similar to the mild or severe controls.

The variability of the CTV coat protein gene was also evaluated through SSCP in an experiment using 'Pêra' sweet orange clones installed in the town of Capão Bonito, São Paulo State, a place with high inoculum pressure of "Capão Bonito" severe complex (Muller et al., 2000). In that study, the results indicated that, although the occurrence of an isolate common to all plants often occurs, there are variations between isolates, possibly associated to cross inoculation by aphids. According to these authors, patterns of isolates considered protective have already been mixed with other components.

Vyver et al. (2002), working with grapefruit selections, preimmunized with two CTV protective isolates from South Africa, observed the presence of additional haplotypes of the virus in some preimmunized trees in field, when compared to the original isolates kept as control in the greenhouse.

Changes in the electrophoretic profiles were also observed in the isolates from Iapar collection (D-6-I and V-I) (Figure 1 B D). The changes may have occurred due to the segregation of the protective isolate after successive propagations, or by the contamination on the nursery plants. The production of rootstocks and seedlings of citric plants in the nurseries became protected with anti aphid screens in Brazil only recently. This fact may have led to contamination of these plants in the nursery – or even before grafting –, with isolates of protective virus.

An intermediate band, characteristic of the Rolândia isolate, used as severe control, was present in great part of the clone isolates analyzed in both years. This fact suggests that the mixture between these isolates occurred, probably, in the first five years of the clone exposure in the field in a region of high pressure of the severe complex (Figure 1).

The electrophoretic profiles observed in the isolate from 'Pêra Vimusa 66B' and 'Pêra Selection 37' clones demonstrate the occurrence of several haplotypes of the virus in the plants. The symptomatology evaluation revealed severe and moderate symptoms of tristeza disease in these plants. In this case, the protection breakdown is probably related to the mixture between the isolates, associated to the sensitivity of the plants tissues to the virus.

The occurrence of stem-pitting in plants of preimmunized 'Pêra' orange, as well as the alteration in the SSCP pattern of CTV CPG obtained from the plants, compared to the pattern found in the mild isolate used for the preimmunization, suggests a protection breakdown (Souza et al., 2000b, 2002a). According to Souza et al. (2002a), the coexistence of different haplotypes does not necessarily implicate the development of disease symptoms. However, the development of symptoms in preimmunized plants should be considered a protection breakdown. The monitoring of the stability maintenance of the CTV protective isolates by SSCP analysis of the coat protein gene showed that larger complexity of isolates is verified in the evaluation of 2005, compared to 2008.

Similar result was observed by Souza et al. (2000a), as they characterized CTV isolates initially established in sweet orange varieties after three years of field exposure. The SSCP analysis revealed that the electrophoretic patterns of the isolates collected from three-year-old plants were less complex compared to those observed in two-year-old plants. This lower complexity suggests that there is a competition between certain isolates.

The genetic distances were small between the CTV isolates analyzed throughout the two years in which the evaluations were carried out. The values observed ranged between 0.08 and 0.54, in 2005, and between

0.09 and 0.46, in 2008, indicating the occurrence of low genetic variability between the isolates.

In the 2005 UPGMA dendrogram (Figure 2), the formation of three well-defined groups was observed. Group III was divided into most subgroups. In this group, the isolates from 'Pêra Bianchi 89C', 'Pêra Vimusa 66B' clones, and only two isolates from 'Pêra Seleção 37' clones, in addition to the isolates from the Iapar (B-I) and São Paulo (B-C) collections were present. The group II was formed exclusively by the isolates from 'Pêra D-6 111A' clones, and the isolate from 'Pêra D-6-I' presented greater similarity with the isolates 2b (2005).

Group I consisted of other isolates from 'Pêra Seleção 37' and 'Pêra Vacinada 59B' clones, of the isolates from Iapar (V-II and Vac-I), São Paulo (D-6-C and V-C) and

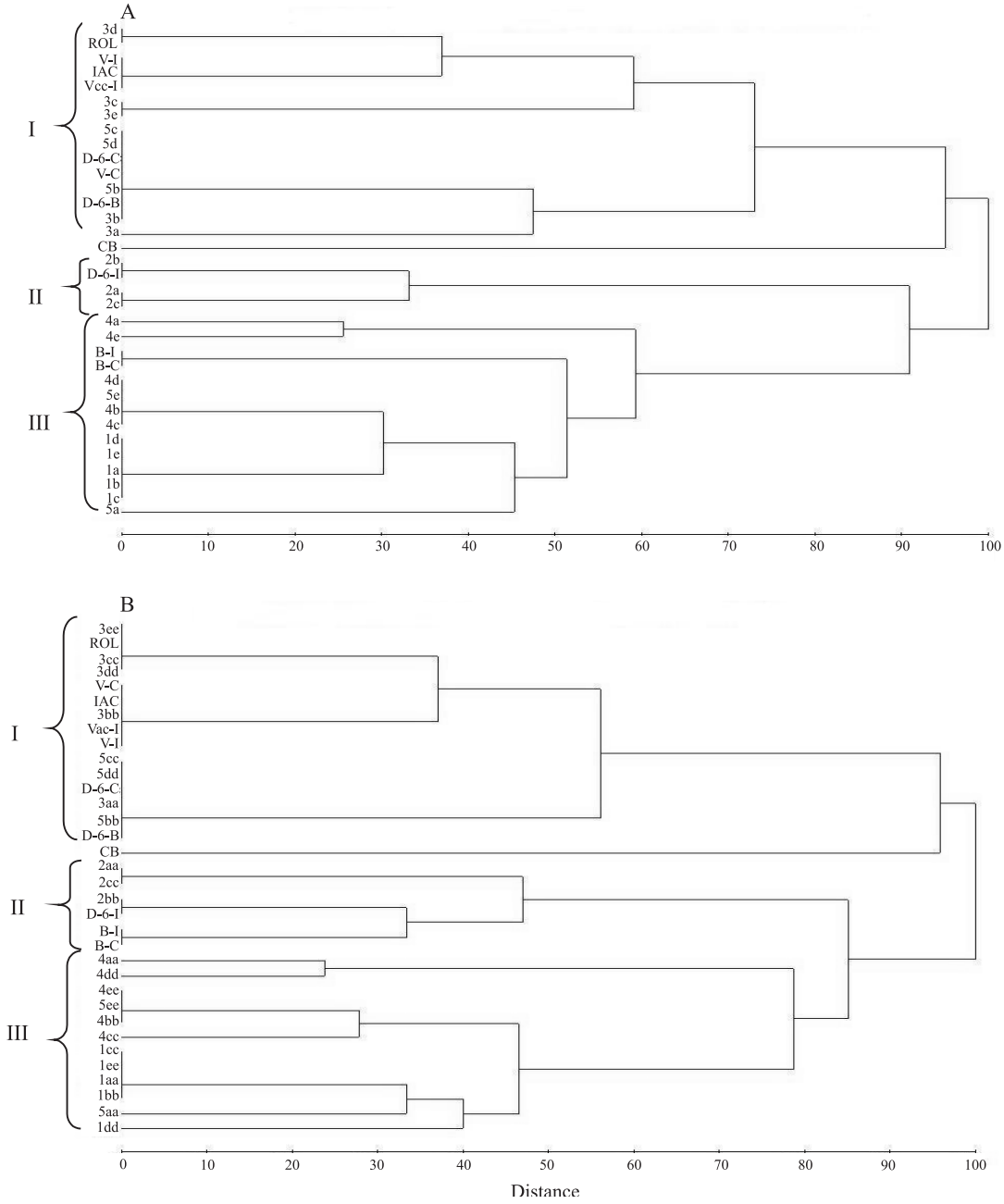

Figure 2. Grouping of CTV isolates from different clones of 'Pêra' sweet orange, determined by SSCP of the coat protein gene. A, collection year 2005; B, collection year 2008.

by Bahia (D-6-B) collections, and also of the isolate from 'Pêra IAC', used as mild control (Figure 2). The isolate 3d from 'Pêra Vacinada 59B' clone presented greater similarity with the Rolândia severe isolate.

In the 2008 UPGMA dendrogram (Figure 2), the maintenance of the three groups previously formed was observed. However, some alterations in the groupings were verified. In this dendrogram, the isolates B-I and B-C appeared in group II with the isolates from 'Pêra D-6 111A' clones. In addition to the isolate 3d from 'Pêra Vacinada 59B' clone, two other isolates from that same clone (3cc and 3ee) presented similarity with the Rolândia severe isolate.

Regarding the Capão Bonito severe control, it differed from all the other isolates analyzed by not being grouped with them (Figure 2). The correlation between isolated genetic distance matrixes (Figure 3) allows a better visualization of the genetic proximity between the CTV isolates present in the evaluated clones, indicating 81% of proximity between them.

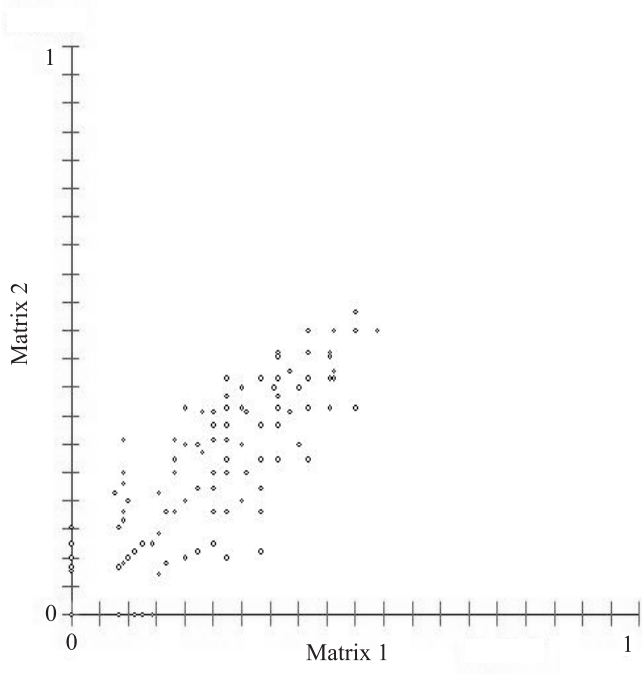

Figure 3. Representative graph of the correlation between the genetic distance matrixes.

Conclusions

1. There are alterations in the electrophoretic profiles of the *Citrus tristeza virus* coat protein gene in the isolates from the majority of evaluated clones, when compared amongst themselves and to the materials from Iapar collection and from States of São Paulo and Bahia, but these alterations do not avoid the protective effect of the isolates.

2. A probable occurrence of tristeza disease protection breakdown was observed in clones of 'Pêra Vimusa 66B' and 'Pêra Seleção 37'.

3. There are several haplotypes of *Citrus tristeza virus* in the same plant.

4. Low genetic variability is observed between isolates from analyzed clones during the studied period.

Acknowledgements

To Instituto Agronômico do Paraná, Centro APTA Citros “Sylvio Moreira” and Embrapa Mandioca e Fruticultura Tropical, for providing the material; to Coordenadoria de Aperfeiçoamento de Pessoal de Nível Superior, for the financial support.

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Received on September 10, 2009 and accepted on June 4, 2010