SSCP analysis of *Citrus tristeza virus* protectives isolates in Pêra sweet orange clones under northern Paraná state, Brazil conditions

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SUMMARY

Aiming to evaluate the performance of different clones of Pêra sweet orange (*Citrus sinensis* Osbeck cv. Pêra) constituting Paraná's orchards, in relation to different pathogens, leading to the installation of an experimental orchard in northern region. This study evaluated clones performance of Pêra Vacinada and Pêra Bianchi sweet oranges, in relation to the *citrus tristeza*, by analyzing the disease symptomatology. The stability of *Citrus tristeza virus* (CTV) mild and protective isolate present in these plants were studied over the years of the experiment using the SSCP (Single strand conformation polymorphism) technique. The *tristeza* symptoms were assessed by vegetative growth and stem pitting intensity in the trunks and branches. The molecular characterization by SSCP analysis, dsRNA (double-stranded RNA), was used to reverse transcription and CP (coat protein gene) by polymerase amplification chain reaction (PCR) and then subjected to electrophoresis on polyacrylamide gel. The *tristeza* symptoms assessment indicated that the clones did not differ in the intensity of stem pitting, size and canopy volume. Grades in stem pitting attributed to the trunks and branches ranged 0-2, indicating a severity of weak to moderate reaction in plants. For SSCP analysis, no difference was observed among cultivars, suggesting that mild isolates presence allowed normal plants development. There was a complex pattern of bands in all isolates, generating four to eight bands in the electrophoretic profiles, suggesting the presence of two to four haplotypes composing CTV isolates, some differing only by the absence of an intermediate band, which cannot have been transferred by propagation grafting. However, SSCP isolates clones patterns from Pêra Vacinada and Pêra Bianchi were not identical to any severe controls. The results suggested that the protective isolates which were inoculated in pre-immunization (IAC Pêra) and established by natural infection of the virus (Pêra Bianchi) contributed to plants good performance assessed by *tristeza* symptomatology and by SSCP analysis, showed stability of protective isolates in Pêra sweet orange, which has been maintained over years of experiment.

Index terms: Cross protection, pre-immunization, molecular characterization, symptomatology.

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RESUMO

Análise SSCP de isolados protetivos do *Citrus tristeza virus* em clones de laranja Pêra nas condições do norte do estado do Paraná, Brasil

A necessidade de avaliar o desempenho de diferentes clones de laranja Pêra (*Citrus sinensis* Osbeck cv. Pêra) que constituem os pomares paranaenses, em relação a vários patógenos, levou a instalação de um pomar experimental na região Norte do Estado. Este estudo visou verificar o desempenho de clones de laranja Pêra Vacinada e Pêra Bianchi, em relação à tristeza dos citros, por meio da análise sintomatológica. A estabilidade do isolado fraco protetivo do vírus da tristeza dos citros (*Citrus tristeza virus,* CTV), presente nessas plantas, foi estudado ao longo dos anos do experimento, por meio da técnica de SSCP (Single strand conformation polymorphism). A sintomatologia da tristeza foi avaliada através do desenvolvimento vegetativo e intensidade de caneluras apresentada pelas plantas. Para a caracterização molecular, através da análise SSCP, o dsRNA (RNA de fita dupla), foi utilizado para a transcrição reversa e amplificação do CP (gene da capa protéica) pela reação da polimerase em cadeia (PCR) e então submetidos à eletroforese em gel de poliacrilamida. A avaliação da sintomatologia da tristeza indicou que os clones não diferiram na intensidade de caneluras, tamanho e volume de copa. Notas de caneluras atribuídas aos troncos e ramos variaram de 0 a 2, indicando uma reação de severidade do isolado nas plantas de fraca a moderada. Pela análise por SSCP, nenhuma diferença foi observada entre as cultivares, sugerindo que a presença de isolados fracos possibilitou o desenvolvimento normal das plantas. Verificou-se um complexo padrão de bandas em todos os isolados, gerando de quatro a oito bandas nos perfis eletroforéticos, sugerindo a presença de dois a quarto haplótipos compondo os isolados de CTV, alguns diferindo somente pela ausência de uma banda intermediária, a qual pode não ter sido transferida pela enxertia na propagação. No entanto, os padrões de SSCP dos isolados dos clones de Pêra Vacinada e Bianchi não foram iguais a quaisquer dos controles severos. Os resultados sugeriram que os isolados protetivos inoculados na pré-imunização (Pêra IAC) e estabelecidos por infecção natural (Pêra Bianchi) contribuíram para o bom desempenho das plantas avaliadas em relação à sintomatologia da tristeza, e a análise molecular por SSCP apontou para uma estabilidade dos isolados protetivos em laranja Pêra, a qual tem sido mantida ao longo dos anos no experimento.

Palavras-Chave: Proteção cruzada, pré-imunização, caracterização molecular, sintomatologia.

INTRODUCTION

The problem faced by Brazilian citriculture has been present since its early establishment, especially in phytopathological aspects. Among the diseases that affect the culture, the *Citrus tristeza virus* (CTV) is still considered highly destructive for those cultivars that are sensitive to this virus (Moreno et al., 2008).

Due to main aphid vector efficiency of the brown citrus aphid (*Toxoptera citricida* Kirk.), CTV became endemic under Brazilian conditions and was responsible for the death of millions trees that were grafted on Sour orange rootstocks (*Citrus aurantium* L.), prior and during the 1930s and 40s. The citrus industry was recovered, at first, with pre-existing clones propagation on CTV tolerant rootstocks like the Rangpur lime (*Citrus limonia* Osbeck) (Baptista & Maia, 1997). However, the change of the rootstock did not solve CTV varieties problem like grapefruits (*Citrus paradisi* Macfad.), acid limes (*Citrus aurantifolia* Swingle) and some sweet oranges (*Citrus sinensis* Osbeck). Amongst the sweet oranges highly affected by virus severe isolates, the Pêra sweet orange (*Citrus sinensis* Osbeck cv. Pêra) the main commercial variety, can be highlighted. It

has contributed to put the country in the first place in worldwide exportation ranking of processed and fresh citrus fruits (FAO, 2005). Pêra orange tree has a high juice production characteristic, which explains its huge use in commercial orchards, towards to concentrated juice and fresh fruits exportation. Pêra orange tree is not so high, with branches sort of straight and acuminated leaves, when compared to other varieties, that are considered high, with round canopy and abundant leaves (Figueiredo, 1991). The CTV control on this intolerant variety was obtained through pre-immunization with mild protective isolates of the virus (Müller et al, 1999).

Although CTV still represents a threat to country's citriculture, due to its endemic nature and severe variants existence, it doesn´t make part of quarantine diseases, which movement is restrained. Therefore, in the past and still today, in some commercialization areas and nursery trees transport have no rules. This fact has allowed nursery trees dissemination, many times infected by severe virus strains, in several countries' citrus regions, including Paraná state.

The citriculture was only reestablished in Paraná's north and northwest regions in 1982, right after release areas that had been interdicted during 30 years due to endemic citric canker occurrence (*Xanthomonas citri* subsp. *citri*) (Morimoto et al., 1991). Pêra sweet orange orchards in these regions came from São Paulo state and from Bahia nurseries of pre-immunized clones so called IAC Pêra (D-6 and D-9 pre-immunized clones).

Besides these, other regions trees possibly naturally infected by CTV mild isolates, and also carrying severe strains, were most of the times, illegally introduced, without phytopathogenic control (Salibe et al., 2002; Zanineli–Ré, 2004).

Aiming to evaluate the performance and behavior of different Pêra sweet orange clones, in 2000 the Cooperativa Agroindustrial de Rolândia (COROL) installed an experimental orchard of this variety, which was constituted of orchards bulk from Arapongas in northen part of Parana´s state. Scions originated from pre-immunized mother trees with different clonals propagations from São Paulo state (IAC Pêra), Bahia (D-6 clone) and from possible naturally infected trees with mild isolates from different origins of São Paulo and Paraná state (Pêra Bianchi, Pêra Morretes, Pêra Gullo, Pêra Vimusa and Pêra selection).

MATERIAL AND METHODS

Plant materials and CTV isolates origin: CTV isolates that were part of this study were obtained from plants originated from two different Pêra orange clones (Table 1), established on Rangpur lime rootstock and belonging to the experiment above. Pêra Bianchi 89 C clone came from propagations of a naturally CTV infected tree growing in a commercial São Paulo State's orchard. Pêra Vacinada 58B and 59B clones, however, were formed from clone propagations of IAC Pêra pre-immunized material, from São Paulo, as well.

As mild controls, IAC Pêra and Pêra Bianchi isolates were used. They came from Centro APTA Citrus Sylvio Moreira, Cordeirópolis County, São Paulo state mother trees. As for severe control, were used Barão B and Capão Bonito isolates from the above mentioned research center collection. A third severe isolate was also used to check, named Rolândia, it came from a CTV severely affected tree located in a commercial orchard from Northern Paraná state.

A total amount of 16 CTV isolates were analyzed. Each clone was evaluated in five CTV symptomatology repetitions, and for molecular analysis, three Pêra Bianchi repetitions, four Pêra Vacinada 58B repetitions and four repetitions of Pêra Vacinada 59B.

CTV evaluation: The *tristeza* symptomatologies presented by the plants were evaluated through vegetative development and stem pitting intensity. The vegetative development was evaluated by using trees height and diameter measurements to calculate canopy volume.

To evaluate stem pitting intensity, four young branches segments 20cm length were cut around from the canopy of each tree then put in paper bags and kept in a dry place. After the bark removal, grades were attributed to stem pitting present in the branches: 0 = no stem pitting; 1 = rare superficial stem pitting; 2 = moderate number of stem pitting; 3 = an intermediate number of stem pitting between 1 and 5; 4 = lots of superficial and some deep stem pitting, and 5 = surface full of deep and superficial stem pitting, according to Meissner-Filho et al. (2002).

A stem pitting intensity complementary evaluation was made by opening a window on the bark at the bud union level, allowing stem pitting scoring degree at that point (Müller et al., 1996). The obtained data underwent statistical analysis to determine

differences among the clones. The data was processed using SISVAR software, 4.3version, developed by "Departmento de Ciências Exatas, Universidade Federal de Lavras (UFLA) Minas Gerais". The test used to compare vegetative development amount average and stem pitting grades was the Scott & Knott at 0,05 significance level.

Molecular analysis: For CTV coat protein gene molecular analysis, young shoots and leaves were collected. Afterwards, the shoot bark and the leaves main ribs were cut in small slices lyophilized and kept at -20 ºC. The molecular analysis was carried out at "Núcleo de Pesquisa em Biotecnologia Aplicada" (NBA), at "Centro de Ciências Agrárias da Universidade Estadual de Maringá."

dsRNA extraction: The dsRNA were extracted from lyophilized tissues by 2 chromatography cycles in cellulose column CF-11 (cellulose fibrous – Whatman) following the procedure proposed by Valverde et al. (1990).

Complementary DNA (cDNA) synthesis by reverse transcriptase: Quantified dsRNA served as a model for the first cDNA strand synthesis, according to the procedure described by Sambrook et al. (1989). The synthesis was conducted in a thermocycler at 37 ºC for 2 hours.

Coat protein gene amplification through PCR: The CP was isolated and amplified through polymerase chain reaction (PCR) with specific primers: CN 119 (5'AGA TCT ACC ATG GAC GAC GAA ACA AAG 3') and CN 120 (5'GAA TTC GCG GCT CAA CGT GTG TTA AAT TTC C 3') Florida T36 strain derivatives (Cevik et al., 1996). The PCR reactions were carried out in a thermocycler programmed for 36 cycles: 1 minute denaturation at 94ºC, 1 minute annealing at 50ºC and 2 minutes DNA synthesis at 72ºC, followed by 5 minutes extension period at 72ºC. The amplification reactions products were analyzed through electrophoresis in agarose gel at 1% containing etidium bromide and photographed under UV light.

SSCP analysis: The SSCP analysis was made according to Corazza-Nunes et al. (2001) methodology. From purified CP amplification product, aliquots were used, later, mixed with the same denaturing solution volume amount. Denatured CP was submitted to electrophoresis in non-denatured polyacrylamide gel at 10%, colored with silver nitrate.

RESULTS AND DISCUSSIONS

Symptomatology: No statistical differences were observed among IAC Pêra (pre-immunized) and Pêra Bianchi clones in stem pitting intensity, size and canopy volume. As observed in table 1, the stem pitting intensity values attributed to the trunks and twigs varied from 0 to 2, indicating that the analyzed plants are infected by a mixture of mild to moderate CTV isolates. Although the severe isolate Rolândia, which was identified in commercial orchards in the region (Zaninelli–Ré, 2004), near this experimental orchard, severe symptoms of stem pitting were not observed in this study.

The statistical tests by Scott & Knott showed no differences among Bianchi's cultivar clones, as well as among Pêra Vacinada 58B and Pêra Vacinada 59B. No difference was noticed among the cultivars, suggesting that mild strains presence allowed a normal plant development of these cultivars in this experimental orchard.

Analysis of the SSCP results: In every PCR electrophoretic profiles a segment of 670 base pairs (bp) (Figure 1) CP correspondent was observed (Sekya et al., 1991). The electrophoretic profiles of SSCP analysis were conducted by observing the number and position of the bands (Sambade et al., 2002). Comparisons were established amongst CTV isolates obtained from experimental orchard plants, with their respective controls (Table 1) and with the severe isolates used as controls: Rolândia, Capão Bonito and Barão B.

SSCP analysis of the CP showed complex bands pattern in each electrophoretic profile, as observed in other studies (Leonel, 2004; Corazza-Nunes et al., 2001; 2006). Approximately four to eight bands were observed, suggesting the presence of two to four strains in each CTV isolate. Most of the same cultivar plants showed the same electrophoretic profile (Figure 2).

CTV isolates obtained from the clones 2B of Pêra Vacinada 59B (Figure 2, lane 6) and 3A, 3B, 3C and 3D of Pêra Vacinada 58B (Figure 2, lanes 10, 11, 12 and 13), showed the same SSCP electrophoretic profile. These isolates profiles were also identical to those from IAC Pêra control (Figure 2, lane 9), suggesting that the mild protective isolate, inoculated at the mother plant pre-immunization, has been kept throughout the

Table 1. Height, diameter, canopy volume values and stem pitting degree attributed to Pêra Bianchi and Pêra Vacinada clones of north Paraná's experimental orchard

Clones	Cultivar/Access	Canopy height	Canopy diameter	Canopy volume	Stem pitting degree
1 A	Pêra Bianchi 89 C	2.15 ^{ns}	2.50 ^{ns}	7.03 ^{ns}	1 ^{ns}
1 B	Pêra Bianchi 89 C	2.25 ^{ns}	2.15 ^{ns}	5.44 ^{ns}	1 ^{ns}
1 C	Pêra Bianchi 89 C	2.35 ^{ns}	2.50 ^{ns}	7.69 ^{ns}	1 ^{ns}
2 A	Pêra Vacinada 59 B	2.30 ^{ns}	2.70 ^{ns}	8.77 ^{ns}	0 ^{ns}
2 B	Pêra Vacinada 59 B	2.35 ^{ns}	2.50 ^{ns}	7.69 ^{ns}	0 ^{ns}
2 C	Pêra Vacinada 59 B	2.15 ^{ns}	2.35 ^{ns}	6,21 ^{ns}	0 ^{ns}
2 D	Pêra Vacinada 59 B	2.25 ^{ns}	2.70 ^{ns}	8.58 ^{ns}	0 ^{ns}
3 A	Pêra Vacinada 58 B	2.15 ^{ns}	2.10 ^{ns}	4.96 ^{ns}	2 ^{ns}
3 B	Pêra Vacinada 58 B	2.25 ^{ns}	2.20 ^{ns}	5.70 ^{ns}	2 ^{ns}
3 C	Pêra Vacinada 58 B	2.25 ^{ns}	2.25 ^{ns}	5.96 ^{ns}	2 ^{ns}
3 D	Pêra Vacinada 58 B	2.20 ^{ns}	2.25 ^{ns}	5.83 ^{ns}	2 ^{ns}

^{ns} = not significant by Scott & Knott test (P<0,05)

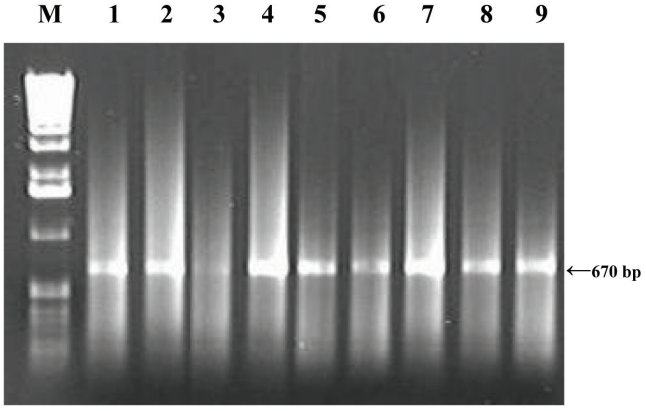

Figure 1. The CTV isolates capsid protein gene amplification products by PCR, reveled in agarose gel at 1%, from North of Paraná. M = 1 kb DNA marker; 1, 2 and 3 clones of Pêra Bianchi; 4 and 5 clones of Pêra Vacinada 59 B; 6, 7 and 8 clones of Pêra Vacinada 58 B; 9 clones of IAC Pêra.

years, without severe isolates or protection breakdown occurrence. According to Müller et al. (1999), IAC Pêra pre-immunized material has conferred protection against severe virus strains over 35 years in many Brazilian states. These plants performance in relation to symptomatology just highlight this isolate protective value (Table 1).

The clones 2A, 2C and 2D of Pêra Vacinada 59B (Figure 2, lanes 5, 7 and 8) showed the same electrophoretic profile among themselves and a standard band similar to other of this cultivar clones and their respective control, differing just for the absence of an intermediate band, what may be a non transferred strain during propagation. However, these plants performance in relation to symptomatology did not allow the suggestion of a protection breakdown by severe isolates as observed in other studies (Broadbent et al., 1995; Gillings et al., 1996).

All of the CTV isolates of Pêra Bianchi plants showed the same electrophoretic patterns (Figure 2, lanes 1, 2, 3 and 4), also showing the protective complex maintenance, that was naturally established. CTV isolates of Pêra Vacinada and IAC Pêra control showed SSCP patterns different from the Bianchi isolates. Great differences were observed among Rolândia, Capão Bonito and Barão B showed severe controls patterns (Figure 2, lanes 14, 15 and 16). However, the SSCP isolates patterns of Pêra Vacinada and Bianchi clones were not equal to any of the severe controls.

The UPGMA dendrogram (Figure 3) shows that the Capão Bonito and Rolândia severe isolates are distantly related, presenting a 40 and 50% similarity coefficient, respectively, in relation to other isolates and 40 % amongst themselves. In the biggest group, formed by the rest of the CTV isolates, we notice two correlated subgroups with a similarity index of approximately 68%.

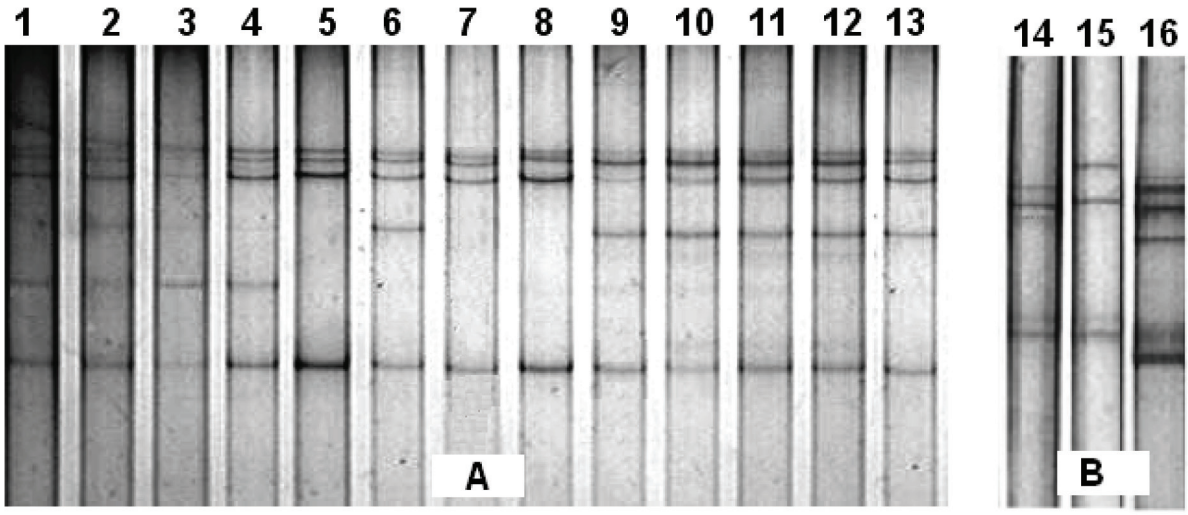

Figure 2. (A) SSCP patterns of CTV isolates, in 10% poliacrilamide gel. Pêra Bianchi clones: 1, 2 and 3 (1A, 1B and 1C clones); Pêra Bianchi control: 4; Pêra Vacinada 59B clones: 5, 6, 7 and 8 (2A, 2B, 2C and 2D clones); IAC Pêra control: 9; Pêra Vacinada 58B clones: 10, 11, 12 and 13 (3A, 3B, 3C and 3D clones); (B) severe controls, Capão Bonito: 14, Barão B: 15 and Rolândia: 16.

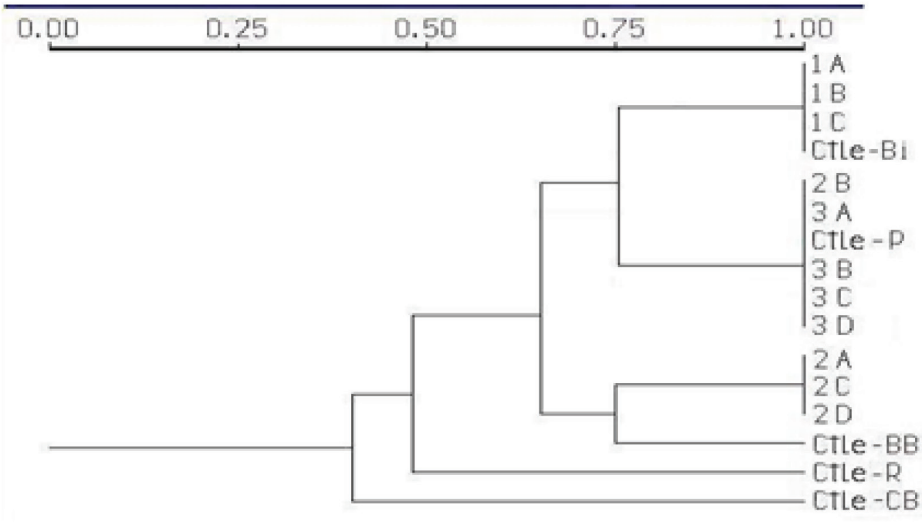

Figure 3. Dendrogram showing genetic similarity amongst Citrus tristeza virus (CTV) isolates of, determined by coat protein gene (Jaccard coefficient) SSCP. Pêra Bianchi clones: 1A, 1B and 1C; Pêra Vacinada 59B clones: 2A, 2B, 2C and 2D; Pêra Vacinada 58B clones: 3A, 3B, 3C and 3D; Pêra Bianchi control: Ctle-Bi, IAC Pêra control: Ctle-P, severe controls, Barão B: Ctle-BB, Capão Bonito: Ctle-CB and Rolândia: Ctle-R.

The first subgroup, which was divided into two branches, was constituted by isolates of Bianchi 1A, 1B and 1C clones and respective control with 100% similarity in the first branch. The second branch included Pêra Vacinada 59B, 2B clone, Pêra Vacinada 58B, 3A, 3B, 3C and 3D clones and IAC Pêra control with 100% similarity are also part of this subgroup. These two branches were related with 75% similarity coefficient. The second subgroup is constituted by the isolates of Pêra Vacinada 59B, 2A, 2C and 2D clones that showed 100% similarity, relating with Barão B isolate severe control by a similarity coefficient means of 75% approximately.

CONCLUSIONS

The vegetative growth data and stem pitting degree indicated that the protective isolates inoculated by the pre-immunization technique in IAC Pêra and naturally infecting Pêra Bianchi were stable under North Paraná's conditions and contributed to a good performance of evaluated plants in relation to *tristeza* symptomatology.

The molecular results analysis by SSCP technique means suggested that the mild isolates that are infecting the IAC Pêra and Bianchi clones are similar, since they grouped with those of IAC Pêra and Bianchi controls at the SSCP dendrogram analysis.

The symptoms and SSCP analysis also suggested that the protective complex stability in Pêra orange has been kept throughout years of experiment.

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