# Differential Replication of a Mild and a Severe Citrus Tristeza Virus Isolate in Species and Varieties of Citrus

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ABSTRACT. The multiplication of a mild (Pera IAC) and a severe (Barão B) citrus tristeza virus (CTV) isolate was evaluated by Western blot analysis using polyclonal antiserum developed against CTV coat protein. Evaluation were done 10, 15, 20, 30, 60 and 90 d after graft inoculation (dai) in different species/varieties of citrus: Mexican lime, sweet orange varieties Pera, Baia and Hamlin, and Ponkan mandarin. The mild isolate replicated faster and at higher titer than the severe isolate in the tissues of all samples evaluated. The first detection of the mild isolate was 15 dai, mainly in Mexican lime and Pera sweet orange, both more susceptible to CTV. The severe Barão B isolate was detected 20 dai. In Ponkan mandarin, a more tolerant host for CTV, the virus replicated at lower rates and was first detected 30 d after grafting.

*Index words*. Western blot, CTV coat protein, CTV multiplication.

Tristeza is an important citrus disease caused by the phloem-limited citrus tristeza closterovirus (CTV) (1, 6). The virions consist of multiple copies of a 25 KDa coat protein (CP) and a genome of positive-sense single-stranded RNA of 19,296 nucleotides, encapsidated at one end of the CP homolog encoded by the p27 open reading frame (ORF) (3, 5). CTV has a number of biologically different isolates presenting replication patterns that changes according to the host species and varieties (8). In this paper, the replication of a mild and protective isolate, Pera IAC (11), and a very severe isolate, Barão B (4), in Mexican lime, sweet orange varieties Pera, Baia and Hamlin, and Ponkan mandarin was evaluated by Western blot analysis using polyclonal antiserum developed against CTV CP.

## MATERIAL AND METHODS

**Virus inoculation.** The CTV sources used were Pera IAC and Barão B sweet orange. Buds of shoot-tip grafted (virus free) Mexican Lime, sweet orange varieties Pera, Baia and Hamlin, and Ponkan mandarin trees were grafted on Rangpur lime rootstock. After approximately 40 d, two buds of each virus source were grafted into five virus free plants of each species/variety. Plants were kept in a greenhouse in Centro de Citricultura Sylvio Moreira. After 10, 15, 20, 30, 60 and 90 d after inoculation (dai), leaves from the growth flush of the scions were harvested and used for Western blot analysis.

**Protein extraction and Western blot.** Total proteins were extracted from 100 mg of dry tissue. Tissue was grounded in a cold mortar in liquid nitrogen and homogenized in 1 ml of extraction buffer (63 mM Tris-HCl pH 7.0; 5% mercaptoethanol; 2% SDS; 10% glycerol). After homogenization, the material was transferred to Eppendorf tubes, boiled 3 min, and then centrifuged 10 min at 12,000 rpm. The supernatant was transferred to a new Eppendorf tube and maintained at -20°C (12, 13).

Proteins were separated by 5% to 20% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) gradients (7) and transferred to nitrocelullose membranes (Hybond C, Amersham), according to the specifications of LKB (LKB, Sweden). Membranes were incubated 3 h in TBS (0.1 M Trizma;

0.9% NaCl, pH 7.6) containing 5% of nonfat dried milk. Membranes were then transferred to TBS containing 0.1% Tween 20 (TBS-T), 5% of nonfat dried milk, and polyclonal antibodies raised against CTV CP at a 1:10,000 dilution and incubated overnight. Membranes were then washed three times for 10 min in TBS-T, incubated 1 h in TBS-T containing 5% of nonfat dried milk and a 1:7,500 dilution of the goat anti-rabbit IgG alkaline phosphatase. Membranes were washed as described above and incubated in a color indicator solution containing 25 ml of buffer (0.1 M Tris-HCl, pH 9.5; 0.1 M NaCl; 5 mM MgCl2); 2 mg of BCIP (5-bromo 4-chloro 3-indolyl phosphate); 4 mg of NBT (nitro-blue tetrazolium). When the desired staining was achieved, the reaction was stopped by washing the membranes in distilled water. The intensity of the reaction was associated to the concentration of the virus in the tissues.

## RESULTS AND DISCUSSION

Figure 1 presents the result of the Western blot performed to confirm the presence of CTV isolates Pera IAC and Barão B in the sweet orange source trees before the graft inoculation of the healthy plants. Protein isolated from healthy tissue was used as a negative control (Fig. 1, lane 1). A strong reaction was observed when proteins isolated from Pera IAC (Fig. 1, lane 2) and Barão B were used (Fig. 1, lane 3).

Figure 2A presents the result of the Western blot using proteins extracted from Mexican lime infected with the mild Pera IAC isolate at 10, 15, 20, 30, 60 and 90 dai. After 10 dai, the virus was not detected (Fig. 2A, lane 1). The virus was detected after 15 and 20 dai (Fig. 2A, lanes 2 and 3). The reaction observed at 30, 60 and 90 dai was stronger, indicating a higher concentration of virions in the tissue (Fig. 2A, lanes 4, 5 and 6, respectively). The severe Barão B isolate was detected in the tissues of Mexican lime at 30 dai (Fig. 2A, lane 10), but not after 10, 15, and 20 dai (Fig. 2A, lanes 7, 8 and 9). The same pattern and intensity of reaction for CTV were observed at 60 and 90 dai (Fig. 2A, lanes 11 and 12). The intensity of the reaction observed in Mexican Lime with the severe Barão B isolate was weaker than the ones with the mild Pera IAC isolate.

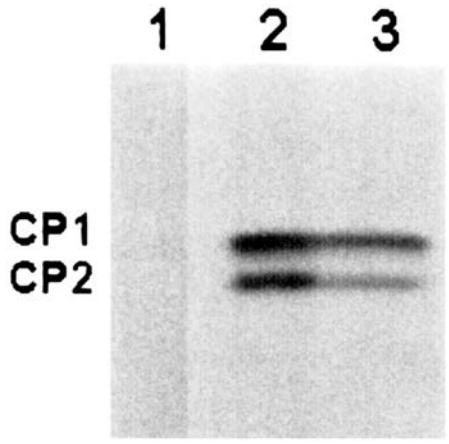


**Fig. 1. Western blot of proteins extracted from plants used as source of CTV. 1. Healthy Pera sweet orange; 2. Pera IAC infected sweet orange; and 3. Barão B infected sweet orange.**

Figure 2B (lanes 1 to 6) presents the results obtained using proteins extracted from Pera sweet orange infected with the mild isolate. After 15 dai a weak reaction was observed (Fig. 2B, lane 2). Stronger reactions were observed at 20, 30, 60 and 90 dai (Fig. 2B, lanes 3 to 6, respectively). When Pera sweet orange was inoculated with the severe Barão B isolate, the virus was not detected at 10 and 15 dai (Fig. 2B, lanes 7 and 8). The virus was detected at 20 dai (Fig. 2B, lane 9). Stronger reactions were observed at 30, 60 and 90 dai (Fig. 2B, lanes 10, 11 and 12).

Figure 2C (lanes 1 to 6) presents the result of the Western blot using

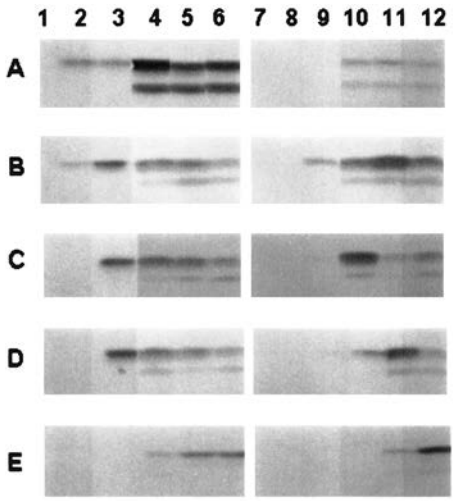


**Fig. 2. Western blot of CTV coat protein extracted from tissues of indicated host at 10, 15, 20, 30, 60 and 90 d after the graft of infected buds of the mild Pera IAC (lanes 1 to 6) and the severe Barão B CTV isolates (lanes 7 to 12). Hosts are: A) Mexican lime, B) Pera sweet orange, C) Baia sweet orange, D) Hamlin sweet orange, and E) Ponkan mandarin.**

proteins extracted from Baia sweet orange infected with the mild isolate. The virus was not detected at 10 and 15 dai (Fig. 2C, lanes 1 and 2). The virus was detectable at 20 dai (Fig. 2C, lane 3 to 12). The severe Barão B isolate was detected in Baia sweet orange 30 dai (Fig. 2C, lanes 10 to 12). Similar results were obtained when Hamlin sweet orange inoculated with both isolates were analyzed (Fig. 2D, lanes 1 to 12).

Pera IAC CTV isolate was detected in the tissues of Ponkan mandarim at 30, 60 and 90 dai (Fig. 2E, lanes 4, 5 and 6). In this case, the reaction was not as strong as the ones observed for the sweet orange varieties. At 10, 15 and 20 dai the virus was not detected, suggesting a slower replication rate in such tissues (Fig. 2E, lanes 1, 2 and 3). The severe Barão B isolate was detected at 60 dai (Fig. 2E, lane 11), and the intensity of the reaction was weaker than those observed for the other samples. At 90 dai the reaction was stronger (Fig. 2E, lane 12). At 10, 15, 20 and 30 dai the virus was not detected (Fig. 2E, lanes 7 to 10).

The results obtained in this work demonstrated that the minimum period to detect CTV in infected tissues varied according to two factors: i) the infected species/variety of citrus; and ii) the CTV isolate used. The concentration of the mild CTV isolate Pera IAC in Mexican lime tissues was higher than the ones observed in the tissues of the other species/varieties tested. This characteristic of Mexican lime to allow a high concentration of virus was already observed, and probably it was due to this that this citrus species was chosen to be an indicator of CTV (2). The severe isolate Barão B replicated in all species/varieties used, but the titer was lower than the titer of the mild isolate. In tolerant varieties, like Ponkan mandarin, CTV seems to have reduced replication capacity. The tolerance of mandarins to CTV in canopies and rootstocks is known (2, 9). According to Moreira et al. (9), some mandarins varieties have great value as rootstocks due to their tolerance to CTV.

According to Lee et al. (8), one of the characteristics of mild CTV isolates selected empirically for cross-protection is the high concentration of virus in the plant tissues but inducing mild symptoms in all hosts. The Pera IAC isolate is considered mild and has been used for over 30 yrs in the cross-protection program of Pera sweet orange in São Paulo State, Brazil (10). We have demonstrated that this Pera IAC isolate has a higher capacity of replication and occurs at high concentration in the tissues of the species/varieties tested, which confirms the observation of Lee et al. (8) on mild isolates. Based on the results obtained in this work, a preliminary selection of mild isolates may be done using this strategy.

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