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Authors

Nickel, O.
Santos Filho, H. P.
Vilarinhos, A. D.
et al.

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Segregation of Citrus Tristeza Virus Strains by Graft Propagation

O. Nickel, H. P. Santos Filho, and A. D. Vilarinhos

ABSTRACT. CTV isolates, obtained by natural field infection of Mexican lime seedlings were propagated by graft-inoculation in Mexican lime seedlings, and these plants analyzed for double-stranded (dsRNA) RNA patterns. Besides the full-length (M_r 13.3 \times 10⁶) replicative form of the CTV genome, some propagations had distinct subgenomic dsRNA bands that were not observed in other propagations. This finding suggested segregation of strains and that some propagations carried just a part of the initial CTV complex. Mild and severe subisolates were observed among propagations of severe and mild isolates, respectively. The possible implications of strain segregation in cross-protection breakdown are discussed.

Citrus tristeza virus (CTV) is the most economically important and widespread virus disease of citrus worldwide. In the late 1930's, it appeared as epidemic in South America and millions of trees grafted on sour orange were killed (2). The observation that plants grafted on sweet orange, trifoliolate orange, Rough lemon, mandarins, and Rangpur lime rootstocks were not affected (2, 4, 5) led to the wide use of these rootstocks to replace the intolerant sour orange rootstock. Later in the early 1960's, commercially important varieties such as the Pera sweet orange in Brazil, showed severe tristeza stem pitting independent of the rootstock used (23). The observation that some mild strains could interfere with symptom expression of severe strains (8, 9), and identification of mild CTV strains adequate to cross-protect the most sensitive varieties (17, 18) offered new hope. However, the protection provided by mild strains may break down in some instances in field trees (1).

CTV has diverse strains varying in biological properties (such as pathogenicity) and field plants are often infected by a mixture of strains (20). The predominating strains in an infected tree determine the symptomatology of the complex. Changes in this balance may alter important traits such as pathogenicity or aphid

transmissibility of an isolate. New isolates that cause damage to rootstocks previously considered tolerant have been reported (19) and new ones are likely to develop. The Capão Bonito isolate affects commercial varieties and rootstocks considered tolerant in other regions (19). In Bahia, Brazil, a new CTV strain was found causing severe stem pitting to the popular rootstock Rangpur lime (24). Bar-Joseph et al. (2) noted that tolerant rootstocks such as Troyer citrange, trifoliolate orange, Rangpur lime, Rough lemon, and others had been reportedly affected by one or more CTV strains.

Double stranded RNA (dsRNA) analysis has been used by several authors to diagnose viruses as well as to differentiate CTV isolates (6, 7, 10, 11, 14). The aim of this research was to study segregation of CTV strains using dsRNA analysis when isolates from naturally-infected plants of Mexican lime were propagated by graft-inoculation. The implications of strain segregation in the stability of cross-protection are discussed.

MATERIALS AND METHODS

Hosts and virus isolates.

Bahian CTV isolates were obtained by exposing Mexican lime seedlings to natural infection in the field by the brown citrus aphid *Toxoptera cit-*

ricida (Kirkaldy) as previously described (21). Isolates inducing mild to moderate (BA E21 and BA O27) or severe (BA B5) symptoms on Mexican lime were used in this study. The isolates were graft-transmitted to Mexican lime seedlings and these plants were maintained in an insect-proof screenhouse at average maximum and minimum ambient temperatures of 28.6°C and 20.2°C. Plants were fertilized by regular leaf sprays and treated with insecticides at 15-day intervals for pest control. In addition to graft transmission, BA B5 was transmitted by *T. citricida* to Mexican lime plants as previously described (21). These isolates were not indexed on other plants, therefore estimates of symptom intensity (vigor of plants, vein clearing, stem pitting, dwarfing), refer only to Mexican lime.

DsRNA analysis. DsRNA was extracted and purified from infected tissue by the procedures of Lee (11) and Morris and Dodds (16) with modifications. Total nucleic acids were phenol-extracted from fresh or frozen tender bark tissue and leaf mid ribs from young Mexican lime shoots ground in liquid nitrogen. After centrifugation for 15 min. at $10,000 \times g$ to separate phases, dsRNA was purified by CF-11 column chromatography (16). Separation of dsRNA was carried out by vertical electrophoresis in 5%, continuous polyacrylamide gels in electrophoresis buffer (0.04 M Tris, 0.02 M sodium acetate, 0.001 M EDTA, pH 7.2) (12). Nuclease treatments were performed on the gels with 1 µg/ml RNase A and 10 µg/ml DNase (3). DsRNA bands were visualized by silver staining according to Schumacher et al. (25) modified by doubling the developer and silver treatment to increase sensitivity. The size of the dsRNA bands was estimated by comparison with molecular markers (1 kb ladder, Bio-rad Laboratories, CA, USA) and plotting migration distance against

the logarithm of the molecular weights. Healthy Mexican lime plantlets were occasionally analyzed as controls.

RESULTS AND DISCUSSION

Citrus plants infected with Bahia CTV isolates usually displayed an M_r 13.3×10^6 band, absent in healthy plants, corresponding to the reported full length replicative form (RF) of the virus genome (6). A variable number of subgenomic bands ranging from M_r 7×10^6 to 0.5×10^6 was observed. These bands were resistant to DNase and RNase under high ionic strength, but sensitive to RNase at low ionic strength, which proves that they are dsRNA (3).

Propagation of isolates by graft-inoculation resulted in individual CTV subisolates that sometimes showed characteristic, distinct dsRNA patterns. For instance, subisolates BA O27-18, -10, and -2 show characteristic major bands of 3.35×10^6 , 2.7×10^6 and 4.3×10^6 (M_r) respectively, not present in other subisolates (Fig. 1). Several authors have reported dsRNA patterns that were unique for certain isolates (11, 14).

Several dsRNA bands seemed to be common to many isolates. For example, most subisolates from BA E21 (Fig. 2) and from BA B5 (Fig. 3) shared at least 6 bands (cb1 through cb6), and band cb7 was present in BA E21-1 and -6 (Fig. 2) and in several subisolates from BA O27 (Fig. 1). These bands had an estimated M_r of 1.5, 1.47, 1.40, 1.20, 1.12, 0.8 and 0.5×10^6 , respectively. The 0.8×10^6 band, that was common to most subisolates (Fig. 2 and 3, cb6), could correspond to the dsRNA band of the same size reported to be common to Spanish isolates (14). The fast migrating band, about 0.5×10^6 (cb7), found in some subisolates from BA O27 and BA E21 (Fig. 1 and 2) may correspond to a similar band associated by Dodds et al. (7) to

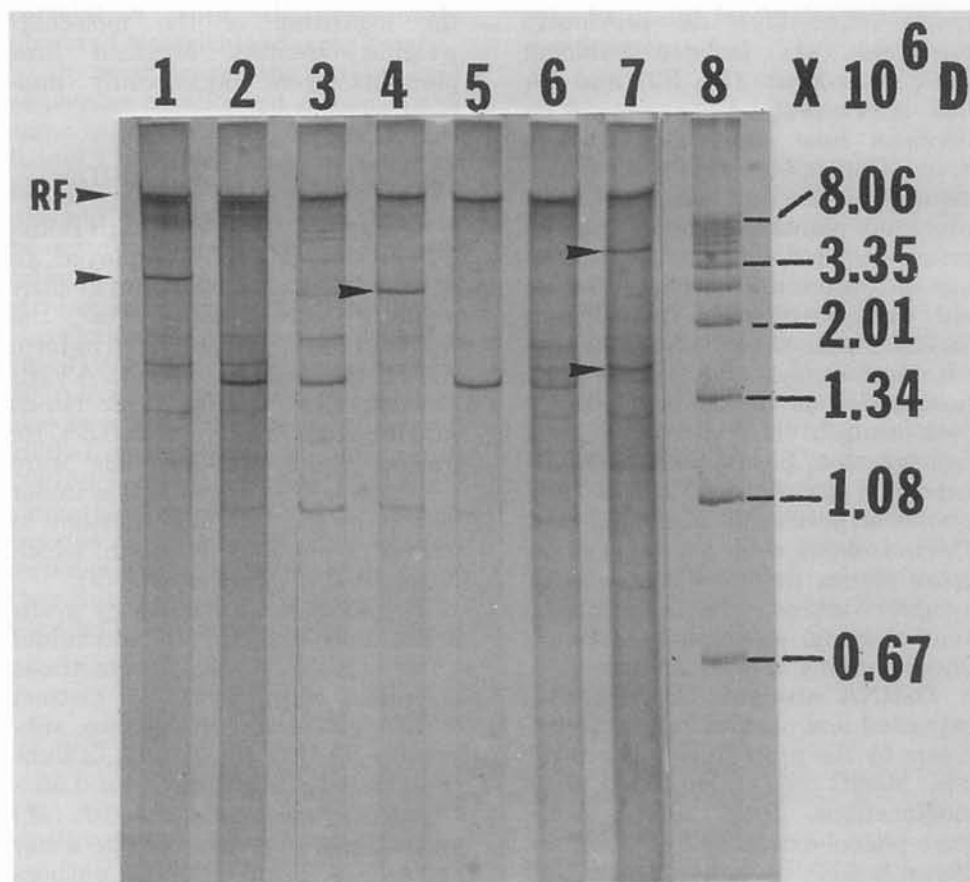


Fig. 1. DsRNA profiles obtained from Mexican lime plants inoculated with buds infected with CTV isolates BA 027. Lanes 1-7: Subisolates BA 027-18(1), -12(3), -10(4), -9(5) -5(6) and -2(7). Lane 8, molecular markers. Arrows mark isolate-specific bands. RF: full-length replicative form of CTV genome.

severe isolates inducing seedling yellows and/or stem pitting in grapefruit or sweet orange.

Occasionally, the RF band of some subisolates could not be recovered (Fig. 2 and 3) or were barely visible. Recently, it has been reported that some subgenomic dsRNA are the replicative form of defective RNAs (D-RNAs) (13). These RNAs tend to accumulate in infected tissues and may impair replication of genomic RNA. This phenomenon might be the cause of the low concentration of the RF band observed in some subisolates.

The dsRNA pattern of isolate BA B5 was more stable than the other two isolates assayed and little varia-

tion was observed among subisolates (Fig. 3). Subisolates obtained by graft or by aphid inoculation behaved similarly. Most of these subisolates induced severe symptoms on Mexican lime similar to those caused by BA B5, however, one of them (B5-5) was milder and induced only weak vein clearing. Conversely, most subisolates obtained from BA E21 and BA O27 remained mild on Mexican lime as the source isolates, but a few of them (E21-9 from BA E21, and O27-1 from BA O27) caused stunting, small cupped leaves with strong vein clearing, and stem pitting on this indicator.

Differences in dsRNA pattern were not always associated with

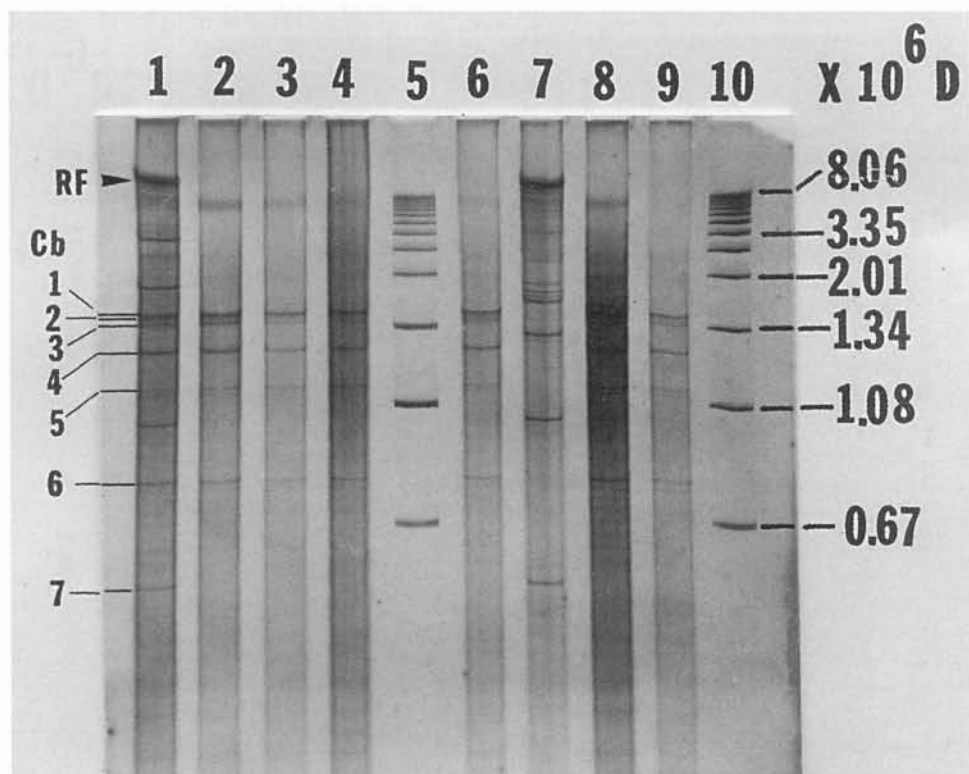


Fig. 2. DsRNA profiles obtained from Mexican lime plants inoculated with buds infected with CTV isolates BA E21. Lanes 1-4: Subisolates BA E21-1 through 4. Lanes 5 and 10, molecular markers. Lanes 6-9: Subisolates BA E21-5 through 8. RF: full-length replicative form of CTV genome. Cb1 through 7: common bands to several subisolates.

changes in pathogenicity, which confirms previous observations that these two traits are not necessarily correlated (14). Symptom expression of these subisolates in other indicator species are being studied. CTV exists in plants as a mixture of strains that may differ in aphid transmissibility (22). The acquisition by the aphids of only a part of the complex may cause changes in pathogenicity (26). In our experiment, *T. citricida* did not act as a segregating agent. Work is now underway to check the behavior of other isolates following aphid transmission. It seems less likely that a bud would carry only part of the strain mixture, particularly of systemic viruses such as CTV; however, our results indicate that separation

of strains by graft inoculation is indeed possible.

Pre-immunization has been achieved by bud propagation of protective isolates (17, 18), however, there is concern about breakdown of cross-protection. This may have several causes, including changes in the virus complex as a consequence of some physiological influence, such as aging (1). An alternative explanation is that a sub-isolate of the protecting isolate with differing protective abilities was introduced into the plant at the pre-immunization step. Data presented here indicate that variations in the strain mixture can occur through bud propagation of an isolate. The strains present in different subisolates may differ in their invasiveness. This trait may be critical

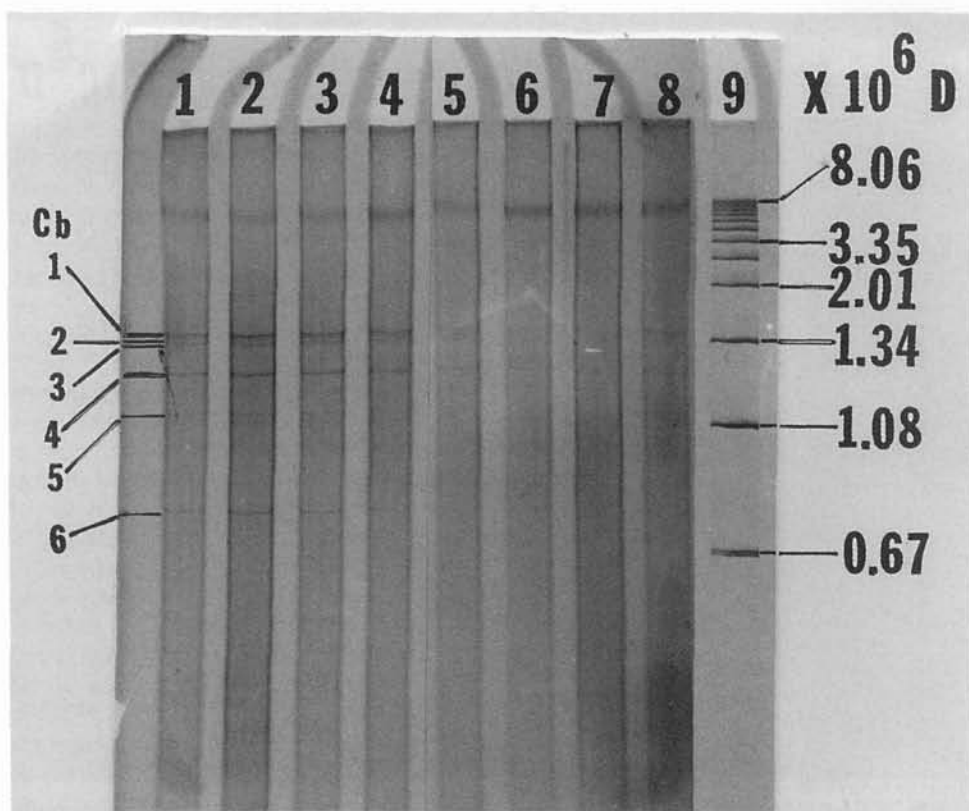


Fig. 3. DsRNA profiles obtained from Mexican lime plants inoculated with buds infected with CTV isolate BA B5. Lanes 1-4: Subisolates BA B5-1 through 4 (aphid inoculated). Lanes 5-8, subisolates BA B5-6 through 9 (graft-inoculated). Lane 9, molecular markers. Cb, common bands to several subisolates.

for their protective capacity (17). Isolate segregation accompanied by variations in pathogenicity has been reported previously (14, 15). These authors concluded that some host species may favor multiplication of certain strains or cause disappearance of others. In this study, we found that graft-transmission to the same host can also induce segregation of strains. This indicates that, in addition to the possibility that each host species alters the balance of strains in an infected tree, these strains may not be uniformly distributed within citrus plant tissues.

These findings may explain at least some cases of cross-protection breakdown. These could be due to

plant pre-immunization with segregants, i.e., subisolates with just a fraction of the whole strain mixture that might have lower protecting capacity, lower invasiveness, or even be more pathogenic itself. Strain stability should be a factor to look upon when selecting protective strains.

Work to determine if some of the dsRNA bands characteristic of certain isolates are, in fact, D-RNAs (13) and how they may interfere with pathogenicity, are underway.

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