# Citrus Tristeza Virus Field Isolates from Declined or Dwarfed Citrus Trees in Japan

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ABSTRACT. Various disorders such as yellowing, overabundant bloom, dieback and dwarfing were noted on 10 to 20 yr-old seedling trees of citrus in fields. Indexing for citrus tristeza virus (CTV) on citrus plants, coupled with ELISA, revealed the presence of severe strains of CTV. The most severe stem-pitting isolate was collected from a nucellar tree of Valencia orange. It caused marked dwarfing with severe stem-pitting of various citrus plants and some dwarfing of Satsuma mandarin with mild stem-pitting and beak-tipped leaves. Seedling yellows strains of CTV were frequently isolated from declined and healthy sweet orange (SW) trees. They did not cause dwarfing of SW on trifoliate orange trees. A yellowing component which caused stunting of SW on sour orange trees but mild symptoms on Mexican lime was collected from yellowed and declined Natsudaidai and pummelos. This component showed a low ELISA titer.
*Index words.* tristeza virus, indexing, stem pitting.

Preinoculation with a mild strain virus protective against citrus tristeza virus (CTV) is expected to control stem pitting disease in those areas where CTV and its effective vectors are established. Protection by mild CTV depends not only on the host (3, 7), but also on the severity of the challenge strains (8). In Japan, various complexes of severe strains of CTV have been collected from affected trees of old budlines or healthy trees (9).

Since 1973 several thousand seedlings of citrus and citrus hybrids have been observed at Kuchinotsu Branch, Fruit Tree Research Station. These were virus-free when planted in the field and were exposed to natural infection. Many trees showed various disorders such as yellowing, overabundant bloom, dieback, dwarfing, and stem pitting. These symptoms suggested that various severe strains of CTV were present in those affected trees.

This paper deals with symptoms observed in the field, collection of severe strains of CTV and the description of biological properties of those viruses.

## MATERIALS AND METHODS

**Observed trees.** Several thousand seedling trees have been planted in the field for breeding since 1967. These were self-rooted and virus-free when planted in the field. Old budline trees including Satsuma mandarin were also planted. Observation and bud collection were made in early July, 1982, 10-15 yr after planting. Twenty trees showing typical symptoms were selected and used for indexing. Collected buds were also grafted onto trifoliate orange (TRF) rootstocks and kept in a screenhouse free from aphids.

**CTV indexing.** Indexing was done on a set of four indicator hosts; seedlings of Mexican lime (ML), Marsh Seedless grapefruit (GFT), Eureka lemon (LM) and on a sweet orange grafted onto sour orange rootstock (SW/SO). Indicator plants were grown and maintained in a glasshouse. Graft-inoculations with collected tissues was done in early July, 1982. Two replications per host were used. Symptoms were read in March and October 1983. After reading, the indicator plants were cut back to about 20 cm to force development of new sprouts. Description of CTV strains was according to McClean (5).

**Virulence of stem pitting strains.** Six CTV sources were used to verify stem pitting disease in a set of six combination hosts; nucellar seedlings of Valencia orange (VL), GFT and Satsuma mandarin (SM) on TRF and rough lemon (RL) rootstocks. These

were grown in a glasshouse in a potting mix and graft-inoculated with the CTV sources when the trunk diameter was 5-8 mm in September 1985. Three replications per host and two buds for each tree were used. An additional graft inoculation was made due to negative reaction to CTV-ELISA. All trees were transplanted in larger pots and cut back to 30 cm in October 1986. Symptoms were read in May, 1988 and 1989. At these times five mature leaves per tree were collected from different twigs and used for ELISA. Total length of shoots including trunk was also measured.

**ELISA.** Double-antibody sandwich ELISA (1) was used to determine relative concentration of CTV in the plants. Petioles and midveins of leaves were macerated in a mortar with 10 volumes (V/W) of extraction buffer of Clark and Adams (1) plus 0.05% mercaptoacetic acid. As a standard for the virus concentration sweet orange leaves infected with CTV-SY were used.

## RESULTS

**Field symptoms and indexing results.** Large numbers of pummelo hybrid trees grew vigorously while young. They developed pitting on both trunk and stems, and became dwarfed gradually about 10 yr after planting (Table 1, No. 0137). The virus recovered from these plants was a stem pitting strain of CTV (CTV-SP) of intermediate virulence (INT). Tree No. 0001 and its neighbors showed foliage yellowing in 1981. The virus recovered from the plant caused mild vein-clearing of ML and mild yellowing of lemon and was CTV positive by ELISA.

Nucellar seedlings of GFT showed severe stem pitting, small leaves and short internodes 10 yr after planting (No. 2717). Tree No. 1417 had been markedly dwarfed earlier. The virus recovered from these plants was considered a tristeza (T) strain, because it caused severe symptoms in ML, GFT and SW/SO trees but no symptoms in lemon.

Large numbers of nucellar seedlings of SW were vigorous, even though they developed pitting on 2-3-yr-old twigs. Trees No. 1493, 1513 and 1672 were typical (Table 1). Tree No. 1130, 1522, 1552, and 1722 had been similar in appearance while they were young, however, they declined with pale colored leaves and abundant blooms. Those trees except for No. 1672 carried a seedling yellows strain of CTV (CTV- SY) which caused yellowing plus stunting of lemon and decline of SW/SO trees. The virus recovered from No. 1672 was CTV-SP. A marked dwarfing occurred in a nucellar seedling of Valencia orange, No. 1595. Severe pitting on both twigs and trunk and chlorotic leaves were observed. The twigs were easily broken off at the bases. The virus recovered was a severe strain of CTV-SP, because it did not cause decline of SW/SO tree as did strains of CTV-SY, but caused severe stem pitting, dwarfing and the bending of branches on inoculated RL seedlings.

Large numbers of nucellar seedlings of Natsudaidai were free of symptoms except for mild stem pitting (No. 2070). The virus recovered was INT-CTV-SP. A small number of these trees developed yellowing of leaves and overabundant blooms. Most of them died within a few years (No. 2048 and 2133). Detected virus content by ELISA was very low. Both field sources caused no symptoms in lemon but stunting of SW/SO. A marked dwarfing with severe stem pitting was observed in the trees No. 1990 and 2192. CTV-SP strains recovered from them were different each other in reaction on indicator plants.

From other hybrids or nucellar seedlings showing marked dwarf and severe stem pitting, CTV-SY was recovered (No. 0508 and 1215).

**Virulence for stem pitting disease.** Based on indexing, six field sources (FS) including CTV-SY, severe CTV-SP and intermediate CTV-SP, and T strain were used for the experiment. FS 0137 (INT-CTV-SP) caused dwarfing and stem pitting in

TABLE 1
VARIOUS SYMPTOMS IN FIELD CITRUS SEEDLING TREES AND INDEXING RESULTS FOR CITRUS TRISTEZA VIRUS (CTV)

| Code no. in field[z] | Symptoms in field[y] | | | | Relative Concn. CTV[x] | Results of indexing[w] | | | | Estimated strain of CTV[v] |
|---|---|---|---|---|---|---|---|---|---|---|
| | Y | Dc | Dw | Sp | | ML | GFT | LM | SW/SO | |
| (Pummelo hybrids) | | | | | | | | | | |
| 0001 | + + + | + + | — | — | –1.84 | VM | non | VM-Y | non | Unknown[u] |
| 0137 | — | + + | + + | + + + | –1.70 | IM | non | non | non | INT-SP |
| (Nuclellar seedlings of grapefruit) | | | | | | | | | | |
| 1417 | — | + | + + | + + + | –1.25 | VS | Sp | non | St | T |
| 2717 | — | + | + + + | + + + | –1.53 | VS | Sp | non | Us | (T) |
| (Nucellar seedlings of sweet orange) | | | | | | | | | | |
| 1130 (tr) | + | + + + | + | + + + | –0.88 | VS | St | ST, Y | VS-St | SY |
| 1493 (Ps) | — | — | — | + | –1.40 | IM | Us | Y | D | SY |
| 1513 (Ps) | — | — | — | + + | 0.00 | VS | St | St, Y | VS-St | SY |
| 1522 (Ps) | — | + + | + | + + + | –1.14 | VS | St | St, Y | St, Sp | SY |
| 1552 (Ps) | + | + + + | — | + + + | –1.20 | VS | St | St, Y | St | SY |
| 1595 (Vl) | + | — | + + + | + + + | | VS | St, Sp | VM-St | M-St, Sp | SP |
| 1656 (Ps) | — | + + + | — | — | –1.29 | VS | St | St, Y | St | SY |
| 1672 (Ps) | — | — | — | + + | –1.71 | IM | Sp | M-St | non | INT-SP |
| 1722 (Vl) | + + | + + + | — | — | –1.24 | IM | St | M-St | St | SY |
| (Nucellar seedlings of Natsudaidai) | | | | | | | | | | |
| 1990 | — | — | + | + + + | –1.63 | VS | Sp | M-St | non | INT-SP |
| 2048 | + + + | + + + | — | — | –3.16 | non | St | non | M-St | Unknown[u] |
| 2070 | — | — | — | + | –1.83 | IM | non | non | Us | (INT-SP) |
| 2133 | + + + | + + | + | — | –2.95 | VM | non | non | VS-St | Unknown[u] |
| 2192 | — | + + | — | + + + | –1.98 | VS | St | non | Sp | Sp |
| (Other hybrid) | | | | | | | | | | |
| 0508 (Hg·H) | + | — | + + | + + + | –0.43 | VS | St | St, Y | St | SY |
| (Tangor nucellar seedlings) | | | | | | | | | | |
| 1215 (Fn) | — | + + | + + | + + | –1.44 | VS | St | St, Y | VS-St | SY |

[z]Trees of No. 1-508, 1130-1215, 1417-2192, and 2717 were planted in the field in 1967, 1970, 1971, and 1972, respectively. Tr = Trovita, PS = Parson Brown, Vl = Valencia, Hg = Hyuganatsu, H = Hassaku, Fn = Funadoko.
[y]Y = yellowing, Dc = decline, Dw = dwarfing, Sp = stem pitting; — to + + +, none to severe, observed in 1982.
[x]Logarithm of the dilution to compare with a standard sample of CTV in ELISA. Serial dilutions of the standard were arranged on each plate.
[w]ML = Mexican lime, GFT = grapefruit, LM = lemon, SW/SO = sweet orange grafted on sour orange; VM = very mild, M = mild, IM = intermediate, VS = very severe; St = stunting, Sp = stem pitting, Y = yellowing; Us = unsuccessful inoculation, D = dead because of inoculation.
[v]T, Tristeza; SY, seedling yellows; SP, severe strain of stem pitting; INT-SP, SP with intermediate virulence.
[u]Probably related to yellowing components.

TABLE 2
VIRULENCE OF CITRUS TRISTEZA VIRUS FIELD SOURCES FOR STEM PITTING DISEASE

| Inoculum source[z] (strain) | Root stock[y] | Scion variety: Grapefruit Growth[x] | Grapefruit Symptoms[w] | Grapefruit EIA[v] | Valencia orange Growth[x] | Valencia orange Symptoms[w] | Valencia orange EIA[v] | Satsuma mandarin Growth[x] | Satsuma mandarin Symptoms[w] | Satsuma mandarin EIA[v] |
|---|---|---|---|---|---|---|---|---|---|---|
| 0137 (INT) | TRF | 367 | — | 1.13 | 306 | — | 1.06 | 326 | — | 0.36 |
| 1215 (SY) | TRF | 434 | P++ | 0.85 | 417 | P+ | 1.19 | 328 | B | 1.51 |
| 1417 (T) | TRF | 332 | P+, D+ | 1.47 | 276 | P+ | 1.17 | 321 | — | 0.33 |
| 1513 (SY) | TRF | 399 | P+ | 1.18 | 358 | P++ | 0.74 | 427 | B | 1.02 |
| 1595 (SP) | TRF | 281 | P+++, D++ | 1.57 | 196 | P+++ | 1.20 | 281 | P+, B | 1.05 |
| 2192 (SP) | TRF | 312 | P+ | 0.84 | 293 | P++ | 0.65 | 323 | B | 0.73 |
| Control | TRF | 408 | — | 0.00 | 329 | — | 0.00 | 365 | — | 0.03 |
| 0137 (INT) | RL | 354 | P+, D+ | 0.50 | 322 | — | 0.71 | 538 | — | 0.08 |
| 1215 (SY) | RL | 451 | P++ | 1.12 | 392 | P+ | 1.36 | 558 | B | 1.13 |
| 1417 (T) | RL | 409 | P++, D+ | 1.24 | 355 | P+ | 0.56 | 486 | — | 0.25 |
| 1513 (SY) | RL | 409 | P+ | 1.32 | 391 | P++ | 1.06 | 514 | B | 1.80 |
| 1595 (SP) | RL | 421 | P++ | 1.06 | 314 | P+++ | 0.98 | 436 | P+, B | 1.10 |
| 2192 (SP) | RL | 372 | P+ | 0.75 | 397 | P++ | 0.81 | 474 | B | 0.94 |
| Control | RL | 513 | — | 0.00 | 360 | — | 0.00 | 566 | — | 0.02 |

[z]See Table 1.
[y]TRF = trifoliate orange, RL = rough lemon.
[x]Total length (in cm) of shoots and trunk per tree.
[w]P = stem pitting, D = dwarfing, B = beak-tipped leaves; — to +++ = none to severe.
[v]Ratio of ELISA value (A415nm) to that of standard sample. ELISA value for CTV standard sample = 0.468-0.544, that for healthy control = 0.003-0.016. Data were obtained in May 1989, 4 yr after inoculation.

GFT but no symptoms in VL and SM (Table 2). FS 1215 and 1513 (CTV-SY) caused mild dwarfing of GFT/TRF and no dwarfing of VL and SM, even if stem pitting developed on GFT and SW trees.

Severe CTV-SP (FS 1595 and 2192) and T strain (FS 1417) caused not only severe stem pitting but also marked dwarfing in six combination plants with FS 1595 being the most severe. Clear stem-pitting and dwarfing were observed in SM inoculated with the FS. The SM inoculated with CTV-SY and severe CTV-SP developed beak-tip leaves similar to those caused by satsuma dwarf virus, especially in early spring.

CTV concentration in affected tissues, detected by ELISA, varied markedly among the inoculum sources, scion varieties and rootstocks. Inoculation with FS 1215, 1513 and 1595 usually provided high ELISA values for CTV in all six combination plants (Table 2). FS 2192 was similar, but with somewhat lower concentrations. FS 0137 and 1497 provided a high concentration of CTV in GFT and SW but a very low concentration in SM, and also produced higher ELISA values in the trees on TRF rootstock than in those on RL rootstock.

## DISCUSSION

It is certain that various strains of CTV are present in complexes in host plants (2, 5), especially in old budlines. When those viruses were indexed on some indicator plants, induced symptoms are determined by the multiple strains or by the most severe strain in the complex. This investigation was made on various seedling trees which had been virus-free when planted in fields and exposed to natural transmission for 10-15 yr. This is based on an assumption that segregation of individual strains may occur due to the small amount of virus transmission by aphid, and that a single component may be present in a tree for some time after infection. Due to host reactions a specific strain could be screened from the complex.

Indexing revealed the presence of various CTV-SP, CTV-SY, T strains and unknown components which caused yellowing. The latter components were recovered from pommelo and Natsudaidai, which are closely related to sour orange in CTV-SY reaction. These components may be yellows factors which have been considered difficult in isolation from the CTV-SY complex.

To induce stem pitting disease in citrus grafted on CTV-tolerant rootstocks, CTV-SP is the most virulent, followed by the T strain. CTV-SY did not cause severe symptoms even if it was usually accompanied with stem pitting components and had high titer of CTV in the tissues. Intermediate CTV-SP caused stem pitting only in GFT and then with mild symptoms. Among CTV-SP sources No. 1595 was the most severe, causing dwarfing on Valencia orange and Satsuma mandarin as well as GFT. Valencia orange has been considered higly resistant to stem pitting disease where severe CTV such as the Capão Bonito strain is prevalent (7). SM has been also believed highly resistant to stem pitting and a symptomless host. However, recent studies revealed that an early maturing variety of Satsuma mandarin became dwarfed when it was affected with severe strain of CTV (4). This caused more attention to be paid the control of severe strains of CTV-SP.

Collected field sources except for No. 1130, 1493, 1552 and 1656 are maintained in the screenhouse at Okitsu Branch, FTRS.

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