A Survey of Citrus Tristeza Virus in China by Enzyme-Linked Immunosorbent Assay

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ABSTRACT. Enzyme-linked immunosorbent assays (ELISA) confirmed that CTV is widely spread in citrus-growing regions of China. Fifty-four of 63 species and cultivars of citrus tested were infected. Of 382, 250, 140, 129, and 92 samples tested in Fujian, Guangdong, Jiangxi, Hunan, and Hubei Provinces, respectively, 61, 61, 66, 63, and 46% were positive for CTV infection. A high rate (97%) of CTV infection in symptomless commercial trees and lack of certified virus-free budwood contribute to the wide distribution of CTV. Presence of CTV-free mature trees in certain areas indicated that rapid natural spread is not universal in China. Most huanglungbin (greening)-infected trees tested were also infected with CTV. Ponkan mandarin and Mexican lime were good differential indicator plants for distinguishing dual infections of CTV and huanglungbin. Limited host range tests indicated that the predominant strain of CTV is a seedling yellows type; however, a mild, non-seedling-yellows isolate was found.
Index words. huanglungbin, virus transmission.

Citrus has been cultivated in China for many centuries. Currently there are over 50 species and cultivars grown in Guangdong, Guangxi, Fujian, Sichuan, Zhejian, Jiangxi, and Taiwan Provinces of south China (4, 5, 10).

For many years, citrus tristeza virus (CTV) was implicated as a cause of citrus huanglungbin (4, 11, 12), a disease similar to citrus greening in South Africa and India (7, 14), because huanglungbin-diseased citrus trees were usually infected with CTV and the huanglungbin agent (11, 17). Since this disease complex was first reported in the 1930's (6), it has caused damage in many citrus-growing areas of China, including Taiwan, where huanglungbin is called likubin.

The absence of tristeza-free orchards, the complexity of CTV strains, and the coexistence of the huanglungbin agent make a clear-cut evaluation of the impact of CTV on various citrus species and cultivars grown in China difficult. Extensive enzyme-linked immunosorbent assays (ELISA) and more limited indexing on indicator plants were used in this study to gain insight into CTV distribution in the citrus-growing areas of China.

MATERIALS AND METHODS

Locations and cultivars. The areas surveyed were in five provinces. In Fujian Province, they included Zhangzhou city, Yuanchun xian, Jianou xian, and Fuzhou city. In Guangdong Province, they included Guangzhou city, Xinhui xian, Yaoping xian, and Tinghai xian. In Jiangxi Province, they included Nanchang city, Dayu xian, and Xingguo xian. Changsa city in Hunan Province and Wuchang city in Hubei Province were also surveyed. Random samples were taken from various cultivars of citrus grown in these locations. The materials sampled in this study were sweet orange cvs. Lan-hua, Liu, Hamlin, Goose-egg, Fuzhou, Beikeng, Snow, Kai-xian, Jiang-jin, Peach leaf, Cadenera, Xian-fen, Xin-hui navel blood, Yin-zi, Valencia, Red-May Valencia, Italian blood, Xiang-shui, Large xin-hui, Xingguo, Xin-hua navel, Bing-tang, Ting-xian blood, Dai-hong, and Pushi; a sweet orange X manderin hybrid; mandarin cvs. Fuzhou, Ponkan, Succosa, Erythrusa, Tankan, Unshiu, Jiu-yue-huang, Unshiu-Miyagawa, Unshiu-Okitsu, Unshiu-New Miyagawa, Unshui-Ling, Unshiu-Yamada, Unshiu-Owari, Unshiu-Mucouyama, Unshiu-Lunghui,

Unshiu-Dungkou, Unshiu-Lianyuan, Unshiu-Shin-Jiang, Nan-jie, San-hu, Bai-nian, Nianki, August, Xin-hui, and Kinokuni; *C. natsudaidai; C. aurantifolia* cv. Decumana; Jin-xian pummelo; Ichang orange (*C. ichangensis*) ; Yuzu (*C. junos*) ; *Fortunella margarita*; *F. hindsii*; trifoliate orange; and citrange.

Tissue preparation and growing conditions. Sections of mature spring shoots about 10 to 15 cm long were removed from the field-grown trees. Occasionally, mature summer shoots were also tested. Bark tissues from the shoots were peeled, diced fresh, and dried under vacuum for 18 to 20 hours. The dried samples were individually packed in small, plastic vials and shipped to Florida for ELISA testing as coded samples. Tissues from greenhouse-grown, healthy Snow orange, Fuzhou tangerine, citron, and Eureka lemon were included as controls. The greenhouse was air-cooled and partially shaded with temperatures from 21 to 27°C in spring and fall, and 30 ± 2°C in summer. Previously tested CTV-positive dry samples from Florida were sent to China and returned to check for loss of serological CTV activity in transit.

ELISA procedures. Dry samples were rehydrated in buffer for at least 1 hour before grinding. The extracts were prepared by homogenizing 0.12 g dry tissue in 5.0 ml of phosphate-buffered saline containing 0.5% Tween 20 and 2% polyvinylpyrrolidone with a SDT Tissumizer® homogenizer for 15 seconds. Filtered extracts were tested in a replicated pattern in polystyrene Microtiter® plates (Dynatech Lab. Inc.) using a standard double sandwich ELISA procedure (1, 2). Procedures for purifying CTV, preparing CTV antiserum, and preparing labeled antibodies, and test conditions were basically as described earlier (1, 2). Test samples were not considered positive unless OD_{405} values were at least twice the value for healthy control samples. In some cases, we rated samples near the threshold level as questionable pending further confirmation.

Graft inoculation tests. Seventeen CTV isolates were collected from infected Fuzhou tangerine, Ponkan, Tankan, and Succosa, and Washington navel trees in Fujian and Guangdong Provinces, and were grafted (single bud) on Mexican lime, Eureka lemon, Fuzhou tangerine, and Ponkan seedlings. Each treatment was replicated three times. Test plants were kept in the insect-proof greenhouses for up to 2 years for symptom development.

RESULTS

Detection of CTV in field collections. Extracts from 24, 15, 13, 16, and 11 cultivars of citrus in Fujian, Guangdong, Jiangxi, Hunan and Hubei Provinces, respectively, were assayed by ELISA (table 1). Of the 382 samples from Fujian Province, 61% tested positive and 11 more were questionable. Of 250 samples from Guangdong Province, 61% tested positively and six more were questionable. In Jiangxi Province, 69% of the 140 samples tested positive while 67% of 129 samples from Hunan were positive. Forty-one of 92 trees from Hubei Province tested positive for CTV and one more was questionable. The results are summarized by area and variety in table 1.

The procedures for preparing vacuum-dry tissue for intercontinental transport worked well for most samples, and no appreciable decrease in OD_{405} values was noted in infected control samples after 2 months and a round trip between Florida and China. Thirty-two

TABLE 1
SURVEY OF CITRUS TRISTEZA VIRUS (CTV) BY ELISA IN CITRUS CULTIVARS IN FUJIAN, GUANGDONG, JIANGXI, HUNAN AND HUBEI PROVINCES IN CHINA

	No. cultivars tested*	Fujian	Guandong	Jiangxi	Hunan	Hubei
C. sinensis	28	99/156†	73/107	5/10	48/48	—
C. reticulata	27	131/206	79/138	83/104	20/39	41/81
C. aurantifolia	1	—	—	6/6	—	—
C. grandis	2	—	0/5	0/10	0/2	—
C. ichangesis	1	—	—	—	0/10	0/1
C. junos	1	—	—	—	10/10	—
Fortunella margarita	1	—	—	2/10	4/10	0/10
F. hindsii	1	—	—	—	1/5	—
Poncirus trifoliata	1	2/20	—	—	—	—
P. trifoliata X *C. sinensis*	1	—	—	—	0/5	—

*Names of the cultivars appear in the text.
†Numerator = no. of plants infected; denominator = no. of plants tested.

samples stored 14 months at –20°C gave similar but somewhat weaker reactions than when first received.

Age of citrus tree in relation to CTV infection. One experiment was conducted to determine whether the age of citrus affected the percent of CTV infection. Trees of different ages (table 2) were randomly selected from orchards in Fujian and Guangdong Provinces for comparison. In the 1-year-old trees, 57% of the 81 samples of sweet orange and 40% of the 71 samples of mandarins were positive. In the trees 2 to 15 years of age, CTV infection in sweet orange ranged from 68 to 82% and from 75 to 93% in mandarins (table 2). All 24 samples of healthy, glasshouse-reared plants of orange, mandarin, lemon and citron were negative.

Comparison between grafted and nongrafted mature trees. We also compared CTV infections in grafted and nongrafted citrus trees planted in the same areas. Of the 20 nongrafted mandarin trees between the ages of 8 to 18 years tested, only one tested positively for CTV, whereas 77.2% of the 101 grafted mandarin trees between the ages of 15 to 20 years tested positive for CTV.

Comparison between symptomless and huanglungbin-infected trees. Sixty-eight apparently healthy trees of sweet orange and mandarin 2 to 7 years old were compared with a similar group of

TABLE 2
RELATION OF TRISTEZA VIRUS INFECTION TO TREE AGE AS DETERMINED BY ELISA

Tree age (years)	*Citrus sinensis*		*C. reticulata*	
	No. tested	% CTV +	No. tested	% CTV +
1	81	57	71	40
2	95	83	43	93
3	39	80	—	—
6	44	73	45	89
15	17	68	92	75

83 huanglungbin-infected trees from the same areas. Ninety-seven percent of the symptomless trees and the 93% of the huanglungbin-infected trees were CTV-infected.

Graft inoculation. Seventeen CTV-infected Fuzhou tangerine, Ponkan, Tankan, Succosa, and Washington navel trees were indexed on Mexican lime, Eureka lemon, and Fuzhou and Ponkan tangerines.

After more than 2 years in the greenhouse, none of the 102 inoculated Ponkan and Fuzhou tangerine trees developed symptoms, although ELISA tests revealed they were infected with CTV.

Twelve of 17 isolates caused strong symptoms in lime and lemon indicators 3-6 months after inoculation. The Mexican lime plants showed stunting, vein clearing, strong stem pitting, and some dieback (after 6 months). The symptoms on Eureka lemon included stunting, yellowing, leaf drop, and small leaves in new flushes.

Four of seventeen isolates produced strong symptoms in lime including stunting, vein clearing, and stem pitting. The inoculated Eureka lemons developed some chlorosis and leaf drop, but were not stunted.

One isolate caused only vein-clearing symptoms without stunting in Mexican lime and no symptoms in Eureka lemon.

DISCUSSION

CTV infection in China was efficiently surveyed by ELISA, and we demonstrated that CTV is widely distributed. These findings confirm earlier suggestions that citrus tristeza is endemic in China (16).

It was actually surprising that 30% of the orange trees and 38% of the mandarin trees tested negative for CTV. This suggests that natural spread of CTV does not occur rapidly in some areas of China. Some negative results could be due to low titer in the test sample and/or some loss of serological activity during sample processing, shipping, and storage. However, lower rates of infection in young trees and in seedlings suggest that negative test results were not spurious.

Presence of serologically distinct CTV isolates in China could have caused some negative results; however, serological differences among CTV isolates have not been demonstrated previously in either extensive tests against numerous isolates, which probably all originated in Asia, or in extensive field indexing (1, 2, 8). In addition, the 17 CTV isolates tested on indicator plants showed three types of symptom response and all reacted serologically. Testing of additional ELISA-negative trees by grafting or microscopy will be required to conclusively answer this question.

Negative results were expected from some plants. For example, trifoliate orange is normally resistant to CTV infection, and was expected to test negative for CTV infection. Variable rates of CTV infection have also been found previously in pummelo and grapefruit, along with erratic test results from known CTV-positive trees of these varieties (8, Garnsey *et al.*, unpublished). It is interesting that two trifoliate orange plants did test positive (table 1). These two plants could be unrecognized hybrids which retained trifoliate orange phenotypic characteristics, or were infected with a strain of CTV capable of infecting trifoliate orange. Attempts will be made to index these two sources to the indicator plants and to perform more serological tests.

Under field conditions, CTV is transmitted by grafting and aphids. Propagation or introduction of infected budwood is usually the source of primary infections.

Aphid spread has been largely responsible for subsequent epidemic spread of CTV into mature plantings in many citrus areas, i.e. South America, Spain, southern California, and parts of Florida. Where the virus is endemic, deducing the source of infection is often complex. In some instances, aphid transmission has accounted for 50% of the CTV spread in the field (3). The brown citrus aphid, *Toxoptera citricida* (Kirk), is the most efficient vector of CTV, followed by the melon aphid, *Aphis gossypii* Glov. (15). These two aphid species have been occasionally found in Fujian and Guangdong Provinces, but may not be the major factor in spreading CTV in China. We believe that absence of virus-free orchards and use of infected budwood has been important to the wide distribution of CTV in China. We found 40-95% of the 1- to 3-year-old grafted field trees were infected with CTV, whereas only 5% of the mature mandarin seedling trees tested were infected (versus 77.2% in grafted trees). Spread of CTV into new areas by vegetative propagation could be prevented or slowed by a budwood registration program. Gradual replacement of infected trees with healthy ones in existing old orchards may also eventually reduce the overall level of CTV infection.

We found most huanglungbin-infected trees were doubly infected with CTV and, although huanglungbin has existed since at least the 1930's (6), its biology remains little studied. The effects of single and mixed infections of the two pathogens on the growth and production of citrus are complex and should be considered as top priority for the future study. Many commercial citrus cultivars such as Snow orange, Liu orange, Goose-egg orange, sweet orange, Fuzhou tangerine, Kinokumi, Lianki, Succosa, Ponkan, Tankan, and Satsuma orange are symptomless carriers of CTV, but severely affected by huanglungbin. This would indicate that CTV alone does not cause huanglungbin. It is helpful that Ponkan and Mexican lime can be used as differential indicator hosts for indexing CTV and huanglungbin infections.

Commonly used rootstocks such as trifoliate orange, Sunki, and Fuzhou tangerine are resistant or tolerant to CTV, but we do not know with certainty that CTV has no harmful effects on orange and mandarin commercial cultivars budded to these stocks, because there have been no comparisons with tristeza-free trees. To resolve this issue, future experiments will have to be conducted with infected and virus-free plants in areas with little natural spread.

Chao *et al.* (4) proposed that CTV in China is a seedling yellows type based on yellowing and stunting symptoms in the seedlings of Eureka lemon, grapefruit, and sour orange, and vein clearing and stem-pitting symptoms in seedlings of Mexican lime and *C. hystrix*. Seedling yellows strains also are predominant in other Asian countries such as the Philippines (18). We also found that 12 of 17 isolates produced strong seedling yellows symptoms, but the milder symptoms produced by five other sources indicated that a mixture of CTV strains exists in China as has been described elsewhere (9, 13, 19).

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