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Biological Characterization of Different Citrus Tristeza Virus Isolates in Spain

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ABSTRACT. Sixty citrus tristeza virus (CTV) isolates representative of most Spanish citrus areas were collected and initially inoculated on Mexican lime and Duncan grapefruit. Ten of them were selected on the basis of origin, host and symptoms on those indicator plants. Selected isolates were separated from other virus and virus-like pathogens by transmission with *Aphis gossypii* from graft-inoculated sweet orange to Mexican lime seedlings. Purified isolates were inoculated into ten seedlings each of Duncan grapefruit and sour orange, 20 seedlings of sweet orange cvs. Pineapple and Comuna and 20 budlings of Washington navel orange grafted on sour orange. Inoculated Duncan grapefruit and sour orange seedlings were incubated in a temperature-controlled greenhouse maintained at 18-26°C. Sweet orange seedlings and navel orange budlings were split in two groups of ten plants each after inoculation. One of them was incubated under greenhouse conditions (18-26°C) and the other was kept in an insect-proof screenhouse.

Vein clearing in young flushes and stem pitting intensity inoculation were evaluated one year after by three observers and their scores averaged.

The following conclusions were drawn: 1) different pathotypes were found among the Spanish CTV isolates characterized; 2) none of the 60 CTV isolates initially inoculated on grapefruit gave a seedling yellows reaction; 3) none of the isolates studied induced stem pitting on Duncan grapefruit or sweet orange cvs. Pineapple or Comuna; 4) some differences in symptom expression were observed between plants incubated under greenhouse and screenhouse conditions.

The existence of strains of citrus tristeza virus (CTV) has been known for many years (13, 15, 16). CTV isolates differ in biological characteristics, such as symptoms severity in the field (2, 8, 9, 13, 21, 28), on several indicator citrus species (3, 4, 7, 18, 21), aphid transmissibility (6, 10, 17, 24, 25) or cross-protecting ability (4, 11, 22, 26, 28, 29).

In recent years there has been an increasing effort to characterize CTV strains to understand the variable performance of scion/rootstock combinations and to study cross-protection between mild and severe isolates (14, 19, 27).

In Spain, citrus trees of sweet orange, mandarin and grapefruit grafted on sour orange and infected by tristeza may show reactions ranging from quick decline to slight stunting with no foliar symptoms or even no symptoms at all (20). Those reactions may be affected by the variety, soil and cultural conditions, climatic variations, strains of the virus present in each citrus area and interactions of CTV with other virus and

virus-like pathogens that infect most of the Spanish citrus trees (23).

In order to establish and characterize a collection of pure CTV isolates representative of Spanish citrus plantings, a survey was initiated in 1980 including most of the citrus growing areas. Here we report preliminary results of the biological characterization carried out on a selected group of the isolates.

MATERIALS AND METHODS

Selection of CTV isolates. Sixty CTV isolates were collected from most citrus-growing areas in Spain, including the provinces of Tarragona, Castellón, Valencia, Alicante, Murcia, Almería, Málaga and Sevilla. Six isolates were kindly provided by Dr. Cuñat (Instituto de Agroquímica y Tecnología de Alimentos, Valencia). Most isolates were originally obtained from trees of different varieties on sour orange rootstock. All the isolates were graft-inoculated to Mexican lime, sour orange and Duncan grapefruit for a preliminary test for seed-

ling yellows and symptom intensity on lime.

Ten CTV isolates were selected according to the following criteria: a) geographical origin, b) variety of the source tree, c) visual aspect of the source tree and d) symptom intensity in the preliminary greenhouse tests. Table 1 shows the origin and scion/stock combination of the CTV isolates selected.

Purification of CTV isolates.

CTV isolates were separated from other virus and viruslike diseases infecting the source trees through transmission by *Aphis gossypii*. Groups of 400 aphids were given a 48-hr acquisition feed on sweet orange seedlings previously graft-inoculated with each isolate and then transferred for a 48-hr inoculation period to healthy Mexican lime seedlings. The appearance of vein clearing and the absence of vein enation symptoms was an indication that only tristeza had been transmitted. The presence of CTV was confirmed by enzyme-linked immunosorbent assay (ELISA).

Pure CTV isolates were graft-inoculated to Washington navel grafted on Troyer citrange rootstock growing in containers and maintained at the IVIA virus bank in an insect-proof screenhouse.

Indicator hosts assayed. Each CTV isolate was graft-inoculated to 10 seedlings each of Duncan grapefruit and sour orange, 20 seedlings of

Mexican lime, Pineapple and Comuna sweet orange, and to 20 plants of Washington navel grafted on sour orange.

Inoculated plants of grapefruit and sour orange were incubated in a temperature-controlled greenhouse kept at 18-26C, whereas inoculated plants of the other indicators were divided into two groups of ten plants each. One of them was incubated in the greenhouse and the other in an insect-proof screenhouse without temperature control. Ten self-inoculated plants of each species or combination were included in both groups as controls.

Plants were grown in a steam-sterilized artificial potting mix (50% sand and 50% peat moss) and fertilized according to the standard procedures described elsewhere (1).

Symptom observation. Inoculated plants were observed four times for vein clearing during a 12-month period and symptom intensity rated on a 0 to 5 scale, using 0 for no vein clearing and 5 for a very severe vein clearing accompanied by severe chlorosis, leaf corking and small leaves. Stem pitting was also rated on a 0 to 5 scale by three different observers, using 0 for the absence of pitting and 5 for the most severely pitted stems. Total growth of the navel orange on sour orange budlings was also measured at the end of the experiment. Results were statistically

TABLE 1
GEOGRAPHICAL ORIGIN AND SCION/ROOTSTOCK COMBINATION OF THE
SOURCE TREES OF SELECTED CITRUS TRISTEZA VIRUS (CTV) ISOLATES

CTV isolate no.	Geographical origin	Source tree (scion/rootstock)
T-300	Puebla Larga (Valencia)	Mexican lime
T-307	Benegida (Valencia)	Cadenera sweet orange/sour orange
T-308	Burjasot (Valencia)	Calamondin/unknown
T-309	Almenara (Castellón)	Calamondin/sour orange
T-311	Jeresa (Valencia)	Clementina/sour orange
T-315	Huerca (Almería)	Castellana sweet orange/sour orange
T-332	Villarreal (Castellón)	Navelate sweet orange/sour orange
T-344	Orihuela (Alicante)	Macetera sweet orange/sour orange
T-346	Sevilla	Satsuma/sour orange
T-354	Rinconada (Sevilla)	Navelina sweet orange/sour orange

analyzed by the mean separation test of Tukey.

Aphid transmissibility. Aphid transmissibility of CTV isolates was estimated by feeding 100 individuals of *A. gossypii* on sweet orange donor plants and then to eight to 10 Mexican lime seedlings according to the procedure previously described (17).

RESULTS

None of the CTV isolates assayed induced any symptoms (including vein clearing, stem pitting, growth reduction or leaf yellowing) on Duncan grapefruit, sour orange and Pineapple and Comuna sweet orange seedlings. No pitting or vein clearing was observed on inoculated Washington navel on sour orange rootstock.

Table 2 shows the vein clearing and stem pitting intensity induced by the different CTV isolates on Mexican lime. CTV isolates could be placed in four groups according to the vein clearing intensity induced on Mexican lime leaves. One of them would include isolates inducing only mild vein clearing (rated less than 2.5), this group being represented by the isolate T-311. A second group, represented by isolates T-307 and T-344,

would include isolates inducing moderate vein clearing (rated 2.5 to 3). The third group, including isolates that induce severe vein clearing (rated 3 to 4), is represented by T-309 or T-346. And finally the fourth group, represented by T-308, would include those isolates inducing very severe vein clearing (rated 4 or more). Incubation under greenhouse or screenhouse conditions produced only minor variations on the mean values calculated for vein clearing intensity and CTV isolates would generally be included in the same group under both incubation conditions. The widest variations occurred with isolates T-308 and T-315 which induced milder vein clearing in the screenhouse than in the greenhouse. However, in both cases, T-308 was the most severe and T-315 the second mildest of the isolates assayed.

CTV isolates could be placed in three groups according to the intensity of stem pitting induced on Mexican lime. The group inducing mild pitting (rated below 1.8) on Mexican lime was represented by T-315 or T-311, but would include most of the isolates studied. The group inducing moderate pitting (rated from 1.8 to 3) could be represented by T-309. The only

TABLE 2
SYMPTOM INTENSITY INDUCED BY TEN SPANISH CITRUS TRISTEZA VIRUS (CTV) ISOLATES ON MEXICAN LIME SEEDLINGS UNDER GREENHOUSE AND SCREENHOUSE CONDITIONS.

CTV isolates	Leaf symptoms		Stem pitting	
	Greenhouse ^z	Screenhouse ^z	Greenhouse ^y	Screenhouse ^y
T-308	4.5a ^x	4.0a	4.0a	3.9a
T-309	3.6b	3.6ab	2.7b	1.9b
T-346	3.3bc	3.0bc	1.8cd	0.7f
T-300	2.8cd	3.0bc	1.6d	1.8bc
T-307	2.8cd	2.8c	2.3bc	1.0de
T-332	2.3de	2.6c	1.8cd	1.2cde
T-344	2.5d	2.6c	1.7d	1.5bcd
T-354	2.9bcd	2.5c	1.6d	1.3bede
T-315	2.3de	1.5d	1.4d	0.8e
T-311	1.7e	1.3d	1.6d	1.0de
Control	0.0f	0.0f	0.0f	0.0f

^zAverage values of ten plants rated four times using a scale from 0 (no vein clearing) to 5 (very severe vein clearing).

^yAverage values of ten plants rated by three different observers using a scale from 0 (no stem pitting) to 5 (very severe stem pitting).

^xMeans with a common letter are not significantly different at $P \leq 0.05$.

isolate that induced severe pitting (rated over 3) was T-308. Incubation in the screenhouse generally resulted in reduced stem pitting intensity when compared with values obtained under greenhouse conditions. The most noticeable reductions occurred with isolate T-307, which produced a moderate pitting in the greenhouse but only mild pitting in the screenhouse, and isolate T-346, which induced the most pitting within the mild group in the greenhouse, but had the least pitting in the screenhouse.

Total growth of Washington navel on sour orange budlings was not significantly affected by any of the ten CTV isolates under any of the incubation conditions. The aspect of the plants was generally good and the only difference with uninoculated controls was the tendency of some inoculated plants to bloom. Total growth of plants in the screenhouse was slightly superior to those in the greenhouse.

Table 3 shows transmissibility of the CTV isolates by *A. gossypii*. There was a highly transmissible group of isolates represented by T-346 and T-354, a poorly transmissible group of isolates including T-344 and T-308, and an intermediate group transmitted with variable efficiency.

DISCUSSION AND CONCLUSIONS

CTV strains differing in symptom intensity induced on Mexican lime and in aphid transmissibility have been found among virus isolates collected from different Spanish citrus areas and from several varieties. Although a continuous range of symptom intensity was obtained, the isolates could be placed in four groups regarding vein clearing or into three groups regarding stem pitting induced on Mexican lime. Incubation in the screenhouse produced only minor differences in vein clearing intensity in comparison with plants incubated in the greenhouse, whereas stem pitting intensity was generally lower in the screenhouse. Some isolates could be

TABLE 3
TRANSMISSIBILITY OF TEN SPANISH
CITRUS TRISTEZA VIRUS (CTV)
ISOLATES BY APHIS GOSSYPHII

CTV isolates	Transmission (%) ^z
T-344	30.0
T-308	40.0
T-311	50.0
T-307	62.5
T-309	66.7
T-332	77.8
T-315	80.0
T-300	82.2
T-346	100.0
T-354	100.0

^zPercent of infected plants using 100 aphids, a 48-hr acquisition feeding on inoculated sweet orange and a 48-hr inoculation feeding on eight to ten healthy Mexican lime seedlings.

equally included in two groups when considering only plants incubated in screenhouse or in the greenhouse, but when considering data from both conditions the groups of isolates were better defined. The group inducing mild stem pitting on Mexican lime basically included the isolates inducing mild or moderate vein clearing in this indicator, whereas the isolates inducing moderate (T-309) or severe (T-308) pitting included severe or very severe vein clearing, respectively.

Differences in aphid transmissibility were also observed between isolates. Although a higher number of receptor plants would probably be necessary to more precisely define transmission efficiency of the different isolates by *A. gossypii*, the percentages obtained in this experiment with the isolates T-300 (82.2%) and T-308 (40%) match reasonably well with those obtained in previous experiments (17) using 50 receptor plants (80% and 28%, respectively). Table 3 basically shows three groups of isolates. One of them being readily transmissible, a second one with very low transmission efficiency, and a third with an intermediate behaviour.

There was no obvious correlation between severity of CTV isolates and aphid transmissibility. Thus, trans-

mission efficiency of T-308, the most severe isolate, was very close to that of T-311, one of the mildest isolates.

Most of the CTV isolates were selected in the field on the basis of visual symptoms shown by trees with sour orange rootstock. Nevertheless, none of them caused significant growth reduction or any decline symptoms on navel on sour orange budlings under greenhouse or screenhouse conditions. The relatively short period of observation (12 months after inoculation), as well as the growing conditions, might be responsible for the symptomless infection obtained with those CTV isolates on the susceptible scion/stock combination. Cuñat *et al.* (12) reported the presence of mild stem pitting, mild to severe vein clearing and reduction of growth and root weight of sweet orange on sour orange budlings inoculated with three CTV isolates from the Valencia citrus area. Those isolates had not been purified by aphid transmission and, consequently, the symptoms observed on the sweet-on-

sour combination might be due to the presence of other virus and viruslike pathogens infecting most Spanish field trees.

None of the 60 isolates initially collected induced a seedling yellows reaction on sour orange or grapefruit and none of the 10 isolates selected for this study induced stem pitting on grapefruit, navel orange and Pineapple and Comuna sweet oranges. This is a confirmation of data obtained from field trees. In Spain, tristeza damage is restricted to trees grafted on sour orange rootstock and stem pitting has not been observed in sweet oranges or grapefruit. Under these conditions, tristeza damage is easily controlled in new plantations by using tristeza-tolerant rootstocks. The recent detection of a CTV strain, illegally introduced with a satsuma variety from Japan, that induces seedling yellows and stem pitting on sweet orange and grapefruit (5) is a great threat to the Spanish citrus industry although efforts are being made to eradicate infected trees.

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