Spread of Citrus Tristeza Virus in a Heavily Infested Citrus Area in Spain

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ABSTRACT. Spread of Citrus Tristeza Virus (CTV) in a heavily infested citrus area in Southern Valencia (Spain) has been monitored since 1981. Two adjacent plots with 400 trees each were selected and tested yearly by ELISA (enzyme-linked immunosorbent assay). One of them was planted to 4-yr-old Newhall navel orange on Troyer citrange and the other to 8-yr-old Marsh seedless grapefruit on the same rootstock. Both had been established using virus-free budwood. In 1981, 98.7% of the Newhall navel plants indexed CTV-positive and by 1984 all of them were infected, whereas only 17.8% of the Marsh grapefruit indexed CTV-positive in 1981, and 42.5% were infected in 1986. This is an indication that grapefruit is less susceptible than navel orange to tristeza infection under the Spanish field conditions. Wild plants of 66 species collected in the same heavily tristeza-infested area were also tested by ELISA to find a possible alternate non-citrus host. CTV was not found in any of the more than 450 plants analyzed.

Index words. virus spread, ELISA, noncitrus hosts.

Tristeza was first detected in Spain in 1957 and since then has caused the death of about 10 million trees grafted on sour orange and the progressive decline of an additional several thousand hectares of citrus on this rootstock. The disease was initially observed at the Ribera Alta area (Southern Valencia), but new foci, probably originating through uncontrolled movement of infected budwood, successively appeared in other spots. At present, tristeza can be found with variable incidence in most of the citrus areas (2).

Three aphid species (*Aphis gossypii* Glover, *Aphis citricola* Van der Goot and *Toxoptera aurantii* (Boyer de Fonscolombe)) have enabled transmission of CTV in Spain, with variable efficiency depending on virus isolate and vector species (4). These aphids, particularly *A. gossypii* and *A. citricola*, are thought to be responsible for natural spread of tristeza (4) from the new foci established through infected budwood.

Decline of trees in tristeza-infested orchards has been occurring at a variable rate depending on years and citrus areas (6). Nevertheless, no data on the actual rate of virus spread under field conditions in Spain are available.

In 1981 several plots were selected in different citrus areas to monitor virus diffusion under various environmental conditions. In this paper we present data on CTV spread in a heavily infested area. A survey among wild plants growing in the same citrus area was also undertaken to search for some noncitrus natural hosts of CTV.

MATERIALS AND METHODS

In 1981, two adjacent plots with 400 trees each were selected at El Realengo, a citrus farm located at the Ribera Alta (Southern Valencia). This farm is within the area where tristeza was first detected. One of them was planted to 4-yr-old Newhall navel orange on Troyer citrange and the other to 8-yr-old Marsh seedless grapefruit on the same rootstock. Both of them had been established using virus-free budwood.

Tristeza spread was monitored by yearly indexing all trees by ELISA. Each tree was sampled taking two young shoots of the last flush from opposite sides of the tree. Shoots were trimmed, the clippings placed into plastic tubes and homogenized in ca. 10 volumes of an extraction buffer using a Polytron Kinematica homogenizer. Extracts were tested by a standard ELISA double antibody sandwich procedure (1, 11) using CTV antisera (879 or R4, kindly provided

by Dr. S. M. Garnsey and Dr. D. J. Gumpf, respectively) or monoclonal antibodies specific to CTV obtained as described elsewhere (11, 12).

The rate of CTV increase was analyzed using the expressions Ln (x/1-x) or Ln (1/1-x) (10), where x is the per unit incidence of the disease at a given time.

Tristeza incidence around the experimental plots was estimated by random sampling and testing by ELISA 2.5% of the citrus trees grown within a band 150 m wide around the plots (120 trees in each case). CTV incidence in citrus plantings of different age or scion-rootstock combination was also estimated separately.

Wild plants of a number of species growing around the experimental plots were also sampled and tested by ELISA in the same conditions as citrus trees. In some cases, extracts of wild plants were also examined by immunoelectron microscopy (IEM) as described by Garnsey *et al.* (3).

RESULTS

The amount and distribution of inoculum around the monitored plots is shown in figure 1. At the beginning of the experiment, the proportion of CTV-infected trees in the 150 m band surrounding the grapefruit plot was 69 ± 9% ($P \leq 0.05$). Corresponding values for the navel plot were 70 ± 9% respectively.

Variation of CTV incidence with time in both plots is summarized in

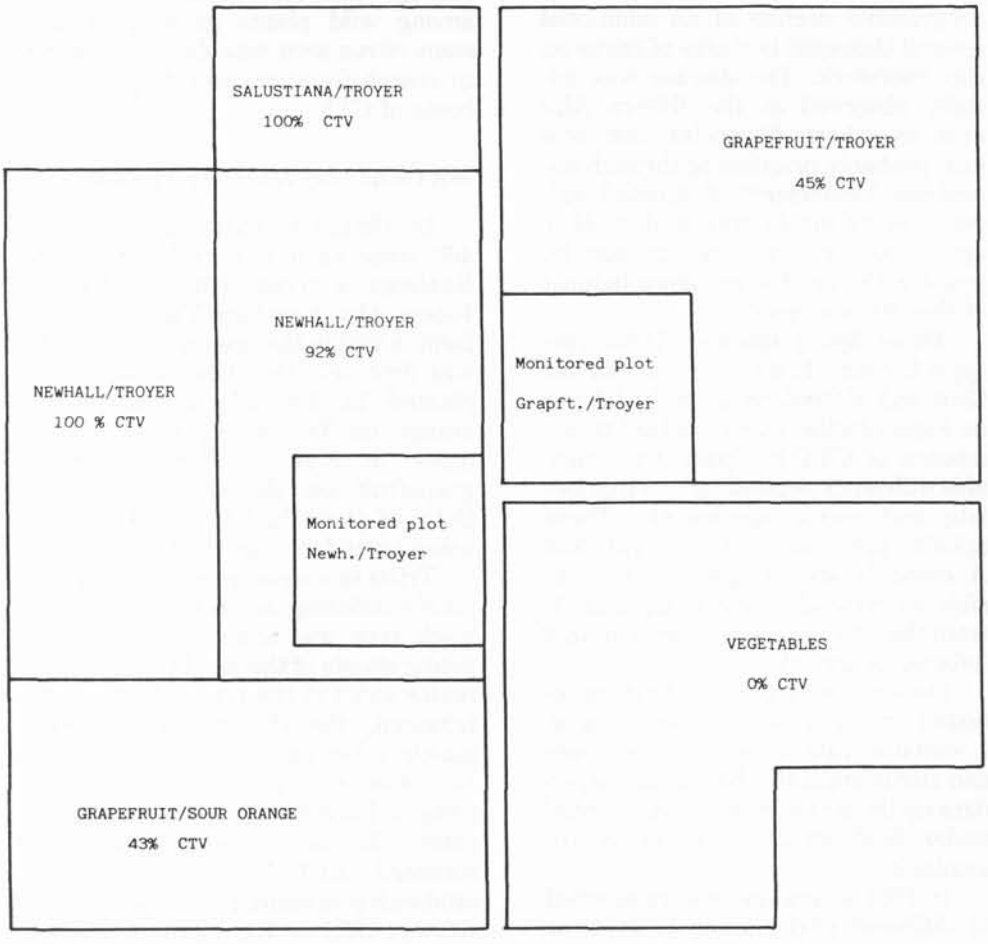

Fig. 1. Scion-rootstock combinations and tristeza incidence in citrus plantings around the experimental plots.

TABLE 1
RATE OF CITRUS TRISTEZA VIRUS SPREAD IN SOUTHERN VALENCIA (SPAIN)

Years	Newhall navel oranges[z] (% infected)[y]	Marsh grapefruit[z] (% infected)[y]
1981	98.7	17.8
1982	99.2	26.0
1983	99.7	27.8
1984	100.0	29.8
1985	—	39.3
1986	—	42.5

[z]Each plot contained 400 trees.
[y]As determined by ELISA.

table 1. All navel plants were infected by tristeza seven years after being planted in the field and more than 98% were already infected in the fourth year. In contrast, less than 18% of the grapefruit trees were infected in the eighth year and more than a half of the trees were still healthy 13 yr after planting.

Figure 2 shows the tristeza increase in the grapefruit plot (data from table 1), using two logarithmic transformations of the per unit incidence. Our data can be reasonably well fitted to a straight line with either of the two transformations with a correlation coefficient of 0.97 in both cases. Thus, natural spread of tristeza in grapefruit, in our conditions, may be explained by the exponential or compound interest model (transformation Ln (x/1-x)) as well as by the linear or single interest model (transformation Ln (1/1-x) (10).

CTV incidence in the Newhall navel plot was too high from the beginning as to make any mathematical consideration about the rate of disease increase in the following years.

The species tested in the survey of CTV among vegetation in the citrus orchards are listed below with the number of plants sampled in brackets. AMARANTHACEAE: *Amaranthus* sp. [2], *A. blitoides* [2], *A. gracilis* [29], *A. hybridus* [1]; APOCYNACEAE: *Nerium oleander* [3]; ARACEAE: *Arisarum vulgare* [70]; CARIOPHYLLACEAE: *Dianthus valentinus* [4], *Stellaria media* [1]; CHENOPODIACEAE: *Beta* sp. [22], *Chenopodium album* [7], *Vibo spinosum* [7]; COMPOSITAE: *Andryala integrifolia* [2], *Calendula arvensis* [5], Centaurea aspera [8], Chondrilla juncea [2], *Conyza ambigua* [2], *Cychorium intibus* [2], *Inula viscosa* [6], *Phagnalon rupestre* [3], *Senecio vulgaris* [3], *Sonchus oleraceus* [4], *S. tenerrimus*

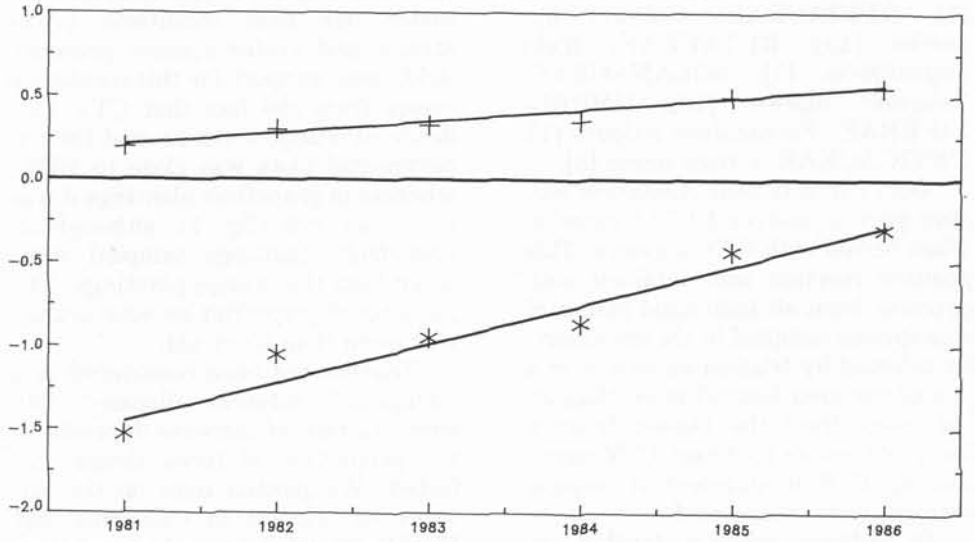

Fig. 2. Progress of tristeza in a grapefruit plot in Southern Valencia (Spain). Per unit incidence of tristeza (x) plotted as Ln x/1-x (*) and as Ln 1/1-x (+).

[7]; CONVOLVULACEAE: *Convolvulus althaeoides* [1], *C. arvensis* [14]; CRUCIFERAE: *Diplotaxis erucoides* [18], *Lepidium draba* [5], *L. graminifolium* [5], *Lobularia maritima* [10], *Sinapis nigra* [3]; DIPSACACEAE: *Cephalaria leucantha* [1], *Scabiosa maritima* [1]; SCROPHULARIACEAE: *Linaria viscosa* [1], *Verbascum sinnuatum* [7], *Veronica persica* [1]; EUPHORBIACEAE: *Euphorbia* sp. [3], *Euphorbia segetalis* [1]; GERANIACEAE: *Erodium cicutarium* [1], *E. malacoides* [6], *E. moschatum* [4]; GRAMINEAE: *Cynodon dactylon* [1], *Poa annua* [1], *Setaria* sp [23]; HYPERICACEAE: *Hypericum perforatum* [1]; LABIATAE: *Sideritis angustifolia* [1], *Thymus vulgaris* [1]; LEGUMINOSAE: *Glycirhiza glabra* [11], *Ononis minutissima* [1], *Psoralea bituminosa* [36]; LILIACEAE: *Allium* sp. [2], *Allium ampeloprasum* [6], *Asparagus* sp. [2]; MALVACEAE: *Malva* sp. [10], *M. sylvestris* [17]; OXALICACEAE: *Oxalis corniculata* [3]; POLYGONACEAE: *Polygonum aviculare* [2], *Rumex crispus* [9]; PORTULACACEAE: *Portulaca oleracea* [6]; RANUNCULACEAE: *Clematis flammula* [14]; ROSACEAE: *Rubus ulmifolius* [10], *Sanguisorba minor* [5]; RUBIACEAE: *Gallium apparine* [10]; RUTACEAE: *Ruta angustifolia* [1]; SOLANACEAE: *Solanum nigrum* [10]; UMBELLIFERAE: F*oeniculum vulgare* [1]; URTICACEAE: *Urtica urens* [3].

Only extracts from *Arisarum vulgare* gave a positive ELISA reaction when tested with CTV antisera. This positive reaction was obtained with extracts from all individual plants of this species sampled in the area heavily infested by tristeza as well as in a non-citrus area located more than 25 km away from the closest tristeza focus. Attempts to detect CTV particles by IEM in different *Arisarum* extracts were unsuccessful.

In addition, when a clarified extract from this plant species was added to the substrate solution (0.1% p-nitrophenyl phosphate in diethanolamine buffer, pH 9.6) the solution turned yellow in a few minutes, suggesting that the positive ELISA reaction observed was probably due to unspecific binding of enzyme present in *Arisarum* extracts that converted the substrate to nitrophenol and caused a false positive reaction.

DISCUSSION AND CONCLUSIONS

The estimated incidence of tristeza in the citrus plantings close to the monitored plots is similar to that obtained for this area in a general survey throughout the Valencian Community (2). About 95% of citrus trees (mainly oranges and mandarins) were estimated to be infected in the Rafelguaraf district, where our experimental plots are located. At El Realengo farm almost all orange or mandarin trees are CTV infected. In addition, the aphid species vectoring tristeza feed mainly on these species and much less on grapefruit. Thus, the inoculum potential of CTV around the experimental plots was very high.

The different rates of CTV spread measured suggest that navel trees (and probably any sweet orange) are much more susceptible than grapefruit trees to CTV infection, at least under our field conditions (virus strains and vector species present). Additional support for this conclusion comes from the fact that CTV incidence in orange trees around the experimental plots was close to 100%, whereas in grapefruit plantings it was less than 50% (fig. 1), although all grapefruit plantings sampled were older than the orange plantings. The planting of grapefruit on sour orange was more than 35 yr old.

Tristeza has been considered as a "compound interest disease" (10) since its rate of increase depends on the proportion of trees already infected. We plotted data on the advance of tristeza in California and Florida (8) using Ln (x/1-x) and they fitted straight lines with correlation coefficients of about 0.98.

In our grapefruit plot, the rate of increase of CTV incidence with time fitted a straight line whether the exponential or the linear model was used. The key for a biological explanation of our data is that the exponential model for disease spread is based on the assumption that the only inoculum available is the infected plants present at a given time. This assumption might not be true in this case, since the inoculum infecting new grapefruit trees might come from orange or mandarin trees around the plot where the inoculum potential was very high. In addition, CTV transmission from grapefruit to grapefruit occurs at much lower efficiency than from orange to grapefruit (9). Thus, it seems more realistic to assume that most of the new grapefruit infection in the monitored plot occurred with inoculum coming from outside rather than from previously infected grapefruit trees within the plot, at least during the initial years when the proportion of infected trees was still relatively low.

The linear model of disease spread assumes that the rate of disease increase depends on the amount of inoculum initially available. Considering that in our situation most of the inoculum could come from orange and mandarin plantings around the grapefruit plot, where the inoculum potential was very high and stable, the linear model might be adequate to explain tristeza increase in the grapefruit plot for a number of years. Later, when the proportion of infected trees grew higher, new infections may have taken place with inoculum coming from outside as well as from inside. This particular situation may explain why our data can be fitted to both mathematical models.

Data on tristeza spread within the navel plot cannot be fitted to any particular model since the disease incidence was already too high the first year of this study. Nevertheless, since the peak aphid feeding on citrus in the area occurs in late spring with a secondary peak in early autumn (5) and inoculated trees have a latent period of several months before becoming systemically infected, aphid-inoculated trees probably only become an inoculum source in the year after infection. Since 98.7% of the trees in the Newhall plot were already infected four years after planting, we can safely assume that a high proportion of them may have been infected with from outside inoculum and, consequently, that the disease increase model may have been similar to that in the grapefruit plot. In this hypothetical situation, the average number of new infections per year in the navel plot would have been close to 25% of trees, in contrast to less than 5% in the grapefruit plot.

More than 450 plants of 66 species sampled from wild vegetation in the heavily infested CVT area were found free of the virus. This confirms that CTV has a very restricted host range. Negative results were also obtained by Müller and Garnsey (7) who tried to slash-inoculate nonrutaceous plants of 55 different species. The positive ELISA reaction obtained with extracts of *Arisarum vulgare* proved not to be due to CTV infection but rather to the presence of some enzyme inducing a yellow reaction in the substrate solution. This emphasizes the importance of using more than one method to confirm diagnosis of virus diseases, particularly when assaying new hosts.

Summarizing, tristeza spread in heavily infested areas of Spain occurs at a very high rate in orange trees and at slower rate in grapefruit and the virus seems to be restricted to citrus trees.

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