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Letter to the Editor

A historical note on two unreported obstacles for cross-protecting mature citrus trees against severe *Citrus tristeza virus* isolates.

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During the years 1970 to 1986 the Israeli citrus industry had undertaken a costly and ambitious program for suppressing the outbreak of a *Citrus tristeza virus* (CTV) epidemic. For comprehensive reviews of CTV and the tristeza disease see Dawson et al. (2013), Moreno et al. (2008), and Bar-Joseph et al. (1989). The program, which involved millions of ELISA tests, was a coordinated effort of virus research, extension, and regulatory agencies funded by the local citrus marketing board and the Ministry of Agriculture. These major commitments of CTV control by a "search and destroy" strategy were based on statistical analyses indicating that CTV infection rates throughout citrus areas were low (Bar-Joseph et al. 1989). Because of the absence of reliable diagnostic methods to differentiate between mild and severe CTV isolates at early stages of infection, the program policy was to eliminate every tree that showed a positive ELISA reaction.

An increase in the incidence of CTV detection during 1983 to 1985 and grower reluctance to cooperate with timely removal of symptomless infected trees, pointed to the need for reevaluating CTV infection rates. Results in 1986 suggested that despite the suppression efforts, which for economic reasons had been already reduced a few years earlier, there were about 50,000 undetected infected trees, spread over 5000 hectares (Bar-Joseph et al. 1989). It was also apparent from the high ratio of symptomless to declining trees that the majority of these sweet orange on sour orange rootstock trees were carriers of "mild" CTV isolates. Trees infected with such isolates remained symptomless for 5 to 10 years, even when the isolates were infecting a decline sensitive combination of sweet orange scions on sour orange rootstocks. Cost-benefit analyses indicated that locating these symptomless trees among the millions of citrus trees cultivated at that time in Israel would have involved testing and compensation costs far beyond the industry's funding resources.

Once the CTV suppression program came to a halt, testing the mild isolates from symptomless trees for their ability to protect trees in plots with severe decline causing isolates became an option. In 1988 a cross protection experiment to prevent decline was established in a mature (around 20 years old) Valencia orange grove grafted on sour orange. The experimental plot was part of the Yachin Company Morasha plantation located east of Tel Aviv. In this area an extremely severe isolate Mor-T (Ben-Zeev et al. 1988), belonging to the VT strain (Mawassi et al. 1993; Shalitin et al. 1994) was causing rapid decline of Minneola tangelo, Valencia, and Shamouti orange trees at early stages of natural infection. Trees of sensitive combinations infected by Mor-T were showing quick decline long before the virions pervaded the canopies to allow routine detection by ELISA (Ben-Zeev et al. 1988).

For mild strain cross protection we used several CTV isolates belonging to the VT strain, including Ach-T, Miqveh-T, and Miqveh-127K (Mawassi et al. 1993; Shalitin et al. 1994), obtained from mature Shamouti orange trees on sour orange stocks that had been infected for several years with CTV, and did not show observable differences when compared with uninfected Shamouti orange trees. Screen house tests showed that, unlike the original VT and Mor-T isolates, these mild VT isolates did not induce seedling yellows when graft inoculated to sour orange seedlings. In addition, simultaneous placing of a Valencia bud and CTV inoculum buds on 1-year-old sour orange seedlings resulted in severe chlorosis of the sprouting Valencia shoot when the buds were infected with Mor-T, while similar Valencia buds on plants simultaneously grafted with the Ach-T and Miqveh-T inocula allowed the development of apparently normal shoots, similar to mock inoculated control plants.

The cross protection experiment was conducted on a 2 ha plot, essentially still free of CTV infected trees, located west of the Morasha citrus planted area. The experimental design was simple: each of the protective isolates maintained in *Citrus macrophylla*, was graft-inoculated along entire rows of about 50 to 60 trees each, with at least 2 replications. Rows on the orchard edges and in the middle of the plot were left as unprotected controls. Trees were graft inoculated on about 1 cm lateral branches at a height of about 60 to 80 cm on 2 sides of each tree. The

choice of inoculum placement was based on the grafter's convenience.

Graft take was low, with a large proportion of grafted trees having only a single bud-take and about 10% to 20% of trees with both inoculated buds failing. Since the trees were closely spaced within the row forming a continuous wall, we expected that natural aphid transmission would lead to movement and spread of the mild CTV isolates from trees with successful grafts to nearby uninoculated trees. Surprisingly however, ELISA tests conducted in this plot on trees with at least a single successful graft failed to detect CTV infection in more than two-thirds of the sampled trees after about 1 year from grafting, and approximately one-third of these trees were still ELISA negative 2 years after grafting. It should be noted that graft-take of similar budwood on experimental sour orange seedling or on sweet orange plants grafted on sour orange rootstocks were normally successful in the range of 95% or above.

Thus, the limited systemic spread of the virus could not be associated with the inoculum quality and was apparently due to some differences in the receptive host or graft position. Among the possible causes was the horizontally positioned grafting on lateral and mostly bent stems. Such positioning might have limited the systemic spread of the virus from the inoculation site, probably turning the graft site into a photosynthate sink rather than source, thus preventing virus movement to roots and subsequently from the roots apically throughout the canopy (Bar-Joseph and Nitsan 1991; Zhou et al. 2002).

Limited grafting tests on mature trees were conducted following the disappointing systemic spread of the potential cross-protecting inoculum. The procedure included changing the inoculation site from the lateral approximately 1 cm branches, to 1 to 3 year-old vertically growing high water content shoots of 2 to 3 cm in diameter. The inoculum was positioned on 2 to 3 such branches per tree, at heights of about 1.5 meter and, 2 to 3 months after graft take, the grafted branches and most of the other branches on these trees were topped to a height of about 50 cm above the grafting sites. In these trees the inoculated CTV isolate was detected regularly by ELISA about 1 year following graft inoculation. These results, which we obtained on a far smaller scale, involved far more labor in the inoculation procedure which limited the pace of inoculation, and also involved a certain amount of fruit loss due to the topping process. Yet it is clear that in mature field grown trees graft-take is not sufficient to allow regular and rapid systemic movement, even of a phloem associated virus like CTV, if the inoculum was not positioned at a site with active carbohydrate flow toward the root system.

During this time, to provide a rapid practical control method for sensitive trees and reduce the severe damage of the rapidly spreading Mor-T isolate, all the CTV-free trees of the Yachin Company at the Morasha area were either replanted with new trees grafted on CTV tolerant rootstocks, or top grafted with disease-free Oroblanco budwood. This rather unconventional control practice was based on our observations and ELISA results which indicated that within the Morasha area an approximately 10-year-old Oroblanco plot remained disease free, while numerous instances of declining trees were present among the surrounding groves planted with Shamouti, Valencia oranges, and Minneola tangelo. It should be noted that top-grafting of Oroblanco on Mor-T infected trees showing decline did not cure the disease or prevent further decline of the infected trees. Yet the absence of diseased trees in the solid Oroblanco block originally planted with CTV-free budwood suggested that the Oroblanco scion was less susceptible to natural infestation by the local vector, *Aphis gossypii* (Bar-Joseph and Loebenstein 1973; Raccah et al. 1976), than the sweet orange and Minneola trees in this area.

Unfortunately the cross protection experiment had to be discontinued less than 3 years from its inception. This was due to the finding of CTV induced stem-pitting symptoms on Star Ruby grapefruit and Oroblanco trees at a few separate locations. Symptoms of infection included severe dwarfing with typical stem pitting, deformed branches, and poor production of mainly small fruits. The most common denominator of these stem-pitted trees was their earlier top grafting of clean budwood of Star Ruby and/or Oroblanco on symptomless sweet or sour orange combinations infected with mild VT.

The unexpected stem-pitting reaction from what had been considered mild VT isolates was supported by 2 different types of evidence. First were the results of experimental inoculation of several “mild” isolates on container grown acid-less pummelo (*Citrus grandis*) plants grafted on *Citrus volcameriana*. These “mild” isolates were originally collected from symptomless Shamouti orange groves grafted on sour orange (Ach-T, Miqveh-T, and 127-T) or from samples of screen house grown sour orange leaves recovered from seedling yellows symptoms of VT. Both types of “VT-mild” isolates gave typical stem pitting (SP) symptoms in less than a year following inoculation of the pummelo indicators. These results were further confirmed by independent experiments in South Africa, where isolates Miqveh-T and 127T were included in an experiment to identify a mild CTV isolate to replace the South African GFMS12 isolate that had provided protection to Marsh seedless grapefruits but was ineffective for protecting the Star Ruby grapefruit trees (van Vuuren and van der Vyver 2000). The SP symptoms of Miqveh-T infected Marsh seedless grapefruit trees did not differ from that of trees planted virus-free. However Star Ruby grapefruit trees infected with Miqveh-T had significantly more SP than trees that were planted virus-free.

With the realization that VT isolates considered as mild for decline of trees on the sour orange rootstock caused severe stem pitting symptoms on Star Ruby grapefruit and Oroblanco, and with the widely adopted practice in the Morasha area of scion replacement to Oroblanco, it became clear that the Valencia orange trees inoculated with the VT mild isolates in our large cross protection experimental plot posed a threat to the recently

top grafted companies' groves. This necessitated an immediate uprooting of the entire cross protection experiment plot. Apparently the uprooting preceded the natural spread of the mild VT isolates from the cross protection experiment plot as indicated by the long survival of the Oroblanco top grafted replacement trees, which are still producing acceptable yields in the Morasha groves 25 years later.

Uniform distribution within the tree of a pre-immunizing CTV isolate is important for the success of cross protection (Zhou et al. 2002). However, despite the extensive use of cross protection for stem-pitting control, the question of inoculum bud-take was rarely discussed, although both situations of high rates of protective isolate bud-take (Zhou et al. 2002) and low rates (Broadbent et al. 1995) were noticed in experiments reported from Australia. Naturally, the problem is of less importance in places like Brazil, where the protective isolates are present in the budwood of the grafted variety. With the renewed interest in cross protection and the better understanding of the underlying limits (Folimonova 2012, 2013; Folimonova et al. 2010), as well as with the use of a CTV vector for possible treatment of huanglongbing infected trees (Dawson and Folimonova 2013; Hajeri et al. 2014), the inoculum position seems to become an important issue especially when considering inoculation on large trees. Thus, the proper site positioning of the CTV inoculum still holds considerable practical importance.

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