Latency of Systemic Infection in Young Field-Grown Sweet Orange Trees Following Graft-Inoculation with *Citrus tristeza virus*

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ABSTRACT. Experiments were conducted to determine the time required for *Citrus tristeza virus* (CTV) to begin migration from the site of inoculation, and the subsequent incubation period required for systemic infection to occur. Young CTV-free sweet orange trees propagated on *Citrus macrophylla* rootstocks were grown in the open in an area where commercial plantings are rare. They were bud-inoculated with CTV during the fall 1997 and spring 1998. Four branches were labeled on each tree; one received inoculum buds and three were uninoculated. Following inoculation, trees were pruned 15 cm below bud insertion at 3 or 8 weeks or left unpruned. Control trees grafted with healthy buds were included to monitor potential natural CTV infections. Progress of infection was monitored by ELISA and tissue immunoblots. Inoculated branches were sampled at the time of pruning, and bark patches were taken from unpruned branches. Leaf petioles were collected periodically from uninoculated branches. All trees in the plot were sampled periodically following flushing to monitor the occurrence of systemic infections. When trees were inoculated in the fall of 1997 and left unpruned, four of five trees tested positive the following spring and all tested positive by the fall of 1999. Trees pruned three weeks post-inoculation remained CTV-free during the course of the test, while four of five trees pruned 8 weeks post-inoculation were CTV-positive by the fall of 1998 and all were positive by the fall of 1999. Trees inoculated in spring 1998 showed no systemic infection during the rest of that growing season, but all of five unpruned trees, three of five pruned 3 weeks post-inoculation, and three of five pruned 8 weeks post-inoculation tested positive in spring 1999. The results indicate that initial movement is more rapid following spring inoculation and that an overwintering period is required before systemic infections become easily detected. Considerable variation in incubation period was also noted among groups of trees inoculated at the same time; this can affect analyses of rates of spread and the timing of detection assays employed to control the spread of CTV.

Index words. Latency, inoculation, serological assay.

The latent period between the time that *Citrus tristeza virus* (CTV) infection occurs and when systemic infections are readily detectable under field conditions remains relatively uninvestigated even though this information is important for understanding of CTV epidemiology, and for control programs. Rates of tree loss in various areas affected by tristeza have been measured (13) and temporal increases in CTV infections have also been analyzed (6, 8), but latent periods between infection and detectable infection were not determined. Some information has been generated under glasshouse conditions with small container-grown plants (12). Under field conditions in Florida and South Africa, CTV detected by ELISA was found to be fairly evenly distributed in individual sweet orange trees but was often unevenly distributed in grapefruit in Florida and variable at times in new flushes (10). Tolba et al. (14) found that CTV required a minimum of 8 days to move out of infected leaf pieces used as inoculum into surrounding healthy tissues and was eventually found in leaves stem bark and roots by indexing one year later. More recently, a CTV decline isolate in Israel causing a severe seedling yellows reaction was found to be extremely variably in distribution throughout the canopies of several citrus varieties on sour orange rootstock. In sour orange seedlings this isolate was found to remain for 51 days in the basal parts of the plants inoculated but found systemically throughout the plant by 58 days post inoculation (1).

Field experiments to address the question of latency require an area where the probability of natural infection by aphid vectors is low.

Experimental inoculation of trees must be permissible, and resources to do repeated testing must be available. In the present study we have used such conditions and attempted to more accurately determine the time elapsed before CTV begins to migrate through the scaffold limbs from the point of inoculation, and the period of time required until systemic infection is detectable.

MATERIALS AND METHODS

Establishment of test plots. Young, commercially-grown CTV-free Valencia sweet orange trees on *Citrus macrophylla* rootstock were established at the USDA-ARS field site in Plymouth, FL, an area where commercial plantings are rare because of previous devastating freezes between 1983 and 1989. These trees were 1.5 to 2 m high and were tested by DAS-I ELISA to ensure their virus-free status, before the experiment. Four branches were labeled on each tree; one to be bud-inoculated and three to remain uninoculated (Fig. 1).

Inoculation and pruning. Inoculum consisted of buds cut from angular green wood from greenhouse-grown sweet orange plants infected with the Florida mild isolate T30, and inoculated to 0.7-0.9 cm diameter branches of recipient trees by T-bud graft. Each recipient tree received two infected buds on the same branch. There were five single-tree replications per treatment. Four treatments were repeated during fall 1997 and spring 1998. In treatment 1, trees were inoculated and left unpruned. In treatments 2 and 3, trees were inoculated and the inoculated limb pruned 15 cm below the point of inoculation 3 and 8 weeks post-inoculation, respectively. Trees in treatment 4 were mock-inoculated with healthy buds to serve as controls for potential natural infections.

Sampling. Tissue samples were collected from all treatments at 3 and 8 weeks post-inoculation. Tissues consisted of leaf petioles from the inoculated and pruned branches, bark patches were also taken from similar locations on unpruned branches, and

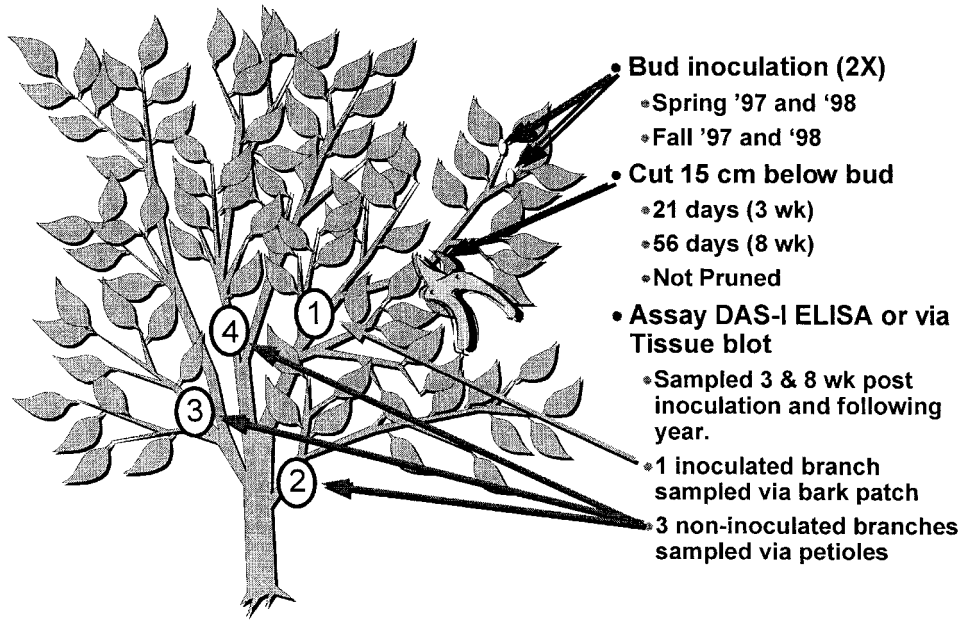

Fig. 1. Experimental design and sampling scheme for *Citrus tristeza virus*.

leaf petioles collected from uninoculated branches. Periodic sampling was timed to occur following new flushes (Fig. 1). Samples were taken on 15 Oct 97, 18 Nov 97, 10 Apr 98, 26 Aug 98, and 2 Mar 99. Tissues were stored dry over silica gel or at -80°C until processing.

ELISA. Each sample consisting of 0.25 to 0.5 g of tissue was placed in 5 ml of PBS-Tween buffer and pulverized for 30 s in a Kleco Tissue Pulverizer (Kinetic Laboratory Equipment, Visalia, CA). Extracts were assayed for presence of CTV by double antibody sandwich indirect (DAS-I) ELISA (3, 4), or via tissue blot immunoassay (7) of pruned branches. A mixture of monoclonal antibodies 11B1 and 3E10 was used as a universal probe (3, 5), and detects isolate T30 plus also all other known isolates in Florida. A second probe was the monoclonal MCA13, which reacts to most decline-inducing and stem-pitting isolates of CTV, but does not react to T30 (11); it was included as a check on possible natural infections.

RESULTS

The ELISA results are summarized in Table 1. In trees that were inoculated in the fall of 1997 and left unpruned, four of five trees were systemically infected by the following spring and all five were infected by the fall of 1998. Trees inoculated in the fall of 1997 that subsequently had the inoculated branch removed 15 cm below the point of inoculation 3 weeks post inoculation, remained CTV-free through the last assay made in the spring of 1999. However, when similar trees were pruned 8 weeks post-inoculation, two of five trees tested CTV-positive the following spring and all were positive by spring of 1999. Trees inoculated in the spring of 1998 had no detected systemic infections during the remainder of 1998, but three of five unpruned trees**,** three of five trees pruned 3 weeks post-inoculation, and three of five trees pruned 8 weeks post-inoculation tested positively in the spring of 1999.

Tissue-blot assays of stem sections of inoculated branches above and below the point of inoculation did not detect the virus.

None of the control trees, grafted with CTV-free buds became ELISA-positive during the experiment. No MC13-positive isolates were detected in any trees in the plot. Thus the infections detected apparently all

TABLE 1
CITRUS TRISTEZA VIRUS (CTV) DETECTION BY DAS-I ELISA IN FIELD GROWN SWEET ORANGE TREES GRAFT-EXPOSED FOR DIFFERENT INTERVALS, I.E., 3-WEEK, 8-WEEK AND PERMANENT EXPOSURE TO GRAFT INOCULATION

Date bud-grafted	Pruned exposure duration	15-Oct-97	17-Nov-97	10-Apr-98	26-Aug-98	2-Mar-99
Fall 97	unpruned	0	0	4/20 (4)	9/20 (4)	20/20 (5)
Fall 97	3 wk	0	0	0	0	0
Fall 97	8 wk	0	0	2/20 (2)	11/20 (5)	19/20 (5)
Fall 97	Control	0	0	0	0	0
Spring 98	unpruned	0	0	0	0	7/20 (3)
Spring 98	3 wk	0	0	0	0	9/20 (3)
Spring 98	8 wk	0	0	0	0	4/20 (3)
Spring 98	control	0	0	0	0	0

The experimental design consisted of five-tree replications with four branches assayed by DAS-I ELISA per tree. Trees were exposed to CTV infected buds for 3 weeks, 8 weeks or permanently (remained unpruned) following grafting or remained ungrafted (control). Data represent the number of CTV-positive assays over the total number of assays, i.e., 20. Data in parenthesis are the number of trees that became CTV-positive out of the five trees inoculated.

resulted from experimental inoculation, although the possible movement of T30 within the plot cannot be completely excluded.

DISCUSSION

The results indicate that in 2-3 yr-old trees, CTV infections initiated either in the spring or in the fall, apparently remain serologically undetectable until the following spring or later. Even during the following spring only some of the trees may have detectable infections. The proportion of trees infected and the proportion of the canopy infected within individual trees continues to increase over an extensive period of time. Lack of any infections in uninoculated controls suggests that all infections detected resulted from our inoculations and that the variability is associated with differences in rates of movement and/or replication. While initial movement from the inoculation site was apparently slower in the fall than the spring, based on our failure to detect passage of CTV beyond 15 cm below the point of inoculation in the fall, the total incubation period until trees were largely systemically infected was shorter. Fall infections were first detected the following spring in inoculated branches, and systemically throughout tree in the summer of the second season. Spring infections were first detected by ELISA the following spring but were often detected simultaneously in inoculated and uninoculated branches.

The results suggest that systemic movement of CTV occurs within a few weeks after inoculation, but that the amount of virus involved may be quite low since none was found in immunoblot assays of stem sections. These results are similar to those reported by Price (12) in greenhouse tests. Immunoblot tests on stems of recently inoculated small container grown plants also failed to detect CTV in stems below the point of inoculation (S. M. Garnsey, unpublished data). Presumably, loci of infection are soon established in the roots or other lower parts of the tree (1, 14), following this systemic spread, although the detailed assays needed to confirm this were not conducted in this study. Infection in most of the upper scaffolds apparently occurs only after trees have passed through a quiescent winter period, and the virus then moves into and replicates extensively in new bark and shoots. This would be consistent with the results reported for a single field tree by Burnett (2), and the general scenario is consistent with observations on patterns of systemic infection of other viruses (9).

The observation that trees which were inoculated at the same time may show extensive differences in incubation period is consistent with other field observations. For example, large numbers of 8-10 yr-old CTV-free trees in a commercial citrus planting in south Florida, were deliberately bud-inoculated with T30 in hopes of inducing protection against anticipated natural infections of decline isolates. Assays of representative samples over time indicated that only a small proportion became systemically infected with T30 within the first year, with new infections appearing in each of the following three seasons (M. Irey, pers. comm.). This observation is corroborated by the findings of Bar-Joseph and Nitzan (1) who found highly variable detection of CTV throughout the canopies of individual field trees. However, it can be easily demonstrated for potted trees in the greenhouse, that systemic infections are detectable serologically in 1-4 mo (Garnsey, unpublished data).

Tree size, health, weather, and horticultural treatments such as pruning or irrigation all probably effect virus replication and movement. The considerable variation in latent period between initial and systemic infection has broad implications. Because of this latency,

when assessing and mapping virus incidence in field trees by ELISA, we are not looking at what exists at the time of assessment, but are actually obtaining a view of virus infection as it existed some time in the past. In addition, because of the variable time-lag for detection relative to individual trees, this historical view is temporally 'fuzzy'. Thus spatial maps based on ELISA testing and the overall CTV incidence at a given point in time are somewhat uncertain.

Extended and variable latent periods also pose tactical problems for eradication programs. Even if all detected infections are eliminated, one must assume that other sub-clinical infections may develop over the next few years, and if natural spread occurs from these before they are detected and eradicated, the cycle is repeated. More detailed sampling using more sensitive detection methods could improve detection of these latent infections, but is unlikely to be practical on a large scale.

Historically, rooting of stem sections from recently inoculated plants also failed to detect reproducing virus from sections through which long-distance movement apparently occurred suggesting that failure of detection is not simply a matter of subclinical virus titers that remain undetectable by modern ELISA methods (12).

Only graft inoculation was used in our study, in part to ensure consistent rates of infection. Initial patterns of movement may differ somewhat following inoculation by aphid transmission. We show that the virus can begin to migrate from bud-grafts in less than 21 days. With graft inoculation a relatively large number of donor cambial cells come in contact with the receptor cambium, but direct phloem linkage is probably not established for at least 8 days (12) and in some cases much longer (1). With aphid-transmission, infection may occur rapidly, and virus is introduced directly into the phloem although the amount of virus inoculated may be small. Multiple infections at several sites in the tree canopy may also occur when aphid populations are high. It is unknown if the differences in the mode of infection (budding versus aphid transmission) significantly impacts the time required for infections to become sufficiently systemic that they can be readily detected.

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