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Authors
Gottwald, T. R.
Taylor, E. L.

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Using Survival Analysis to Predict the Risk of Infection in a *Citrus tristeza virus* Epidemic

T. R. Gottwald and E. L. Taylor

USDA, ARS, Horticultural Research Laboratory, 2001 S. Rock Road, Fort Pierce, FL 34945, USA

ABSTRACT. The spatial and temporal characteristics of *Citrus tristeza virus* (CTV) epidemics have been described previously for both the CTV/Toxoptera citricida and CTV/Aphis gossypii pathosystems. For the CTV/T. citricida pathosystem, aggregation of infected trees develops due to transmission of CTV by viruliferous aphids within local areas of influence. Long distance spread of CTV has been documented for both pathosystems. However, the threat of infection from CTV-infected trees to neighboring trees is unknown. Survival analysis was used to examine the probability of survival (remaining in a non-infected state) through time of CTV-free trees when located at various distances of proximity to CTV-infected trees. A risk index was calculated via a modified Cox proportional hazards model to estimate the probability of survival through time of CTV-free trees when located at various distances from trees that became CTV-infected in prior years. The risk of becoming infected was related to previous reported 'local areas of influence'. Within a planting, infection by CTV of a substantial proportion of newly infected trees could be attributed to trees determined to be infected 6 mo previously within a 'local area of influence' of approximately 24 m radius, and that 'survival' decreased significantly through time.

Index words. Virus transmission, CTV spread, CTV increase, comparative epidemiology, Toxoptera citricida, Survival Analysis, Cox Model, hazard.

*Citrus tristeza virus* (CTV) epidemiology has been studied with increasing rigor over the past two decades. Spatial and temporal analyses of CTV epidemics have been conducted previously and concluded that for virus increase and spread two broad pathosystems exist based primarily on the predominant vector species that are present (1, 5, 13, 20, 22, 23, 24, 25). Using data from intensely-mapped multi-year studies of CTV incidence in eastern Spain and Florida, we found that for CTV epidemics with *Aphis gossypii* as the predominant CTV vector, CTV incidence progressed from low levels (~0.05) to high levels (~0.95) in 8 to 15 years (11, 13, 14, and Gottwald, Garnsey, and Irey unpublished).

Data from Costa Rica and the Dominican Republic showed that, although CTV-positive trees did not often influence immediately adjacent trees, virus transmission was common within an area that extended two to eight trees in all directions, i.e. a ‘local area of influence’ (11, 14). Where asymmetry was indicated, this area of influence was somewhat elliptical. The spatial and temporal analyses gave some insight into the underlying processes of CTV spread in the presence of *Toxoptera citricida* and suggested that CTV spread was predominantly to trees within a local area. Patterns of longer distance spread were not detected within the confines of the plot sizes tested. However, longer distance spread has been documented up to 4.2 km from known sources in studies in Costa Rica (15).

Studies in Spain indicated there was little evidence for aggregation of CTV-infected trees, and the spatial patterns of CTV-infected trees could not be distinguished from random (11, 13). Virus spread did not occur preferentially to trees adjacent to those already infected. Rather, new infections probably arose from both inter- and intra-plot transmissions (11). Gibson (8) recently reevaluated the same data from Eastern Spain and Gottwald et al. (12, 13) examined CTV data from...
Costa Rica and the Dominican Republic. Using all of the Spanish CTV data collectively, Hughes et al. (17) found that the data could be fitted by binomial distributions with a separate mean incidence for each assessment, but with a common aggregation parameter, equal to 0.03. The low value of the aggregation parameter and the proximity of the data points to the theoretical binomial line in a plot of observed variance against binomial variance, are indicative of a random pattern at the quadrat scale (17). Gibson (8, 9, 10, 14) and Gottwald et al. (14), examined the data using a spatio-temporal stochastic model based on Markov chain, Monte Carlo integration methods. These stochastic model analyses reinforced the inter- and intra-plot transmission theory and provided some evidence that one component of spread was likely due to short-distance transmissions from nearby trees, which was not apparent from the analytical methods used by Gottwald et al. (11, 12).

Survival analysis is a class of statistical methods for studying the occurrence and timing of events and is often applied to the study of deaths. Survival analysis has been used for many years in medical studies and by the insurance industry for generation of actuary tables. However, this analytical method has served many disciplines and has been referred to by other names in other disciplines, for example event history analysis (sociology), duration analysis or transition (economics), reliability analysis or failure time analysis (engineering), etc. (2, 18). Survival analysis has been used only recently in botanical epidemiology to examine plant disease epidemics and the factors affecting these epidemics through time, such as the effect of roguing diseased plants (4, 21).

This paper explores the contribution of short distance transmissions of CTV and the influence they have on the overall spatial pattern of disease that develops through time. A secondary objective of the study was to examine co-migration of different CTV isolates and the effect they have on one another. Although these isolates can be differentiated serologically, they were not considered to be competitive in the way cross-protecting isolates are.

MATERIALS AND METHODS

The three data sets analyzed in this study are a subset of those used previously for spatial and spatio-temporal analyses of CTV (11, 12). The data came from three separate plots from within established commercial plantations in northwest Costa Rica where T. citricida was the predominant vector species. All three plots consisted of 400 trees (20 rows x 20 trees per row) in a rectangular planting pattern within larger commercial plantings and the trees ranged from 1 to 5 years old at the beginning of the study. Plot CR1 was a Pineapple sweet orange scion on Cleopatra mandarin rootstock, plot CR2 was Valencia sweet orange on Cleopatra mandarin rootstock, and plot CR3 was a Valencia sweet orange on grapefruit rootstock. No aphid control procedures were applied in any of the plots and T. citricida was present in all locations throughout the data collection period. Within these plots, CTV incidence varied from 0.02 to 0.92 (2 to 92%).

Sample collection and ELISA. Sampling and serological analysis methods were as previously reported (7, 13). Plots were sampled in the spring and fall of each year. Samples consisted of four leaf petioles from young, nearly fully expanded leaves taken from the periphery of each tree and each tree was tested independently. Extracts were assayed for the presence of CTV via double antibody sandwich indirect (DAS-I) ELISA as previously reported (7). Briefly, leaf samples were placed in 5 ml of PBS-Tween buffer and pulverized for 30 sec in a Kleco tissue pul-
Verizer (Garcia Manufacturing, Visalia, CA). General CTV detection utilized a mixture of the monoclonal antibodies 3E10 and 11B1 (3, 6, 7). CTV isolates were differentiated as mild or T30-like if they were non-reactive, or severe (causing decline or stem-pitting) or T36-like if they were reactive with the selective monoclonal antibody MCA13 (19). Maps were prepared for each plot by assessment date, indicating the location of T30 and T36 type infected trees. T36-induced decline is rare in Costa Rica because much of the citrus is grown on rootstocks other than sour orange, which is particularly susceptible to decline. Isolates from the Costa Rica plots were accessioned into the USDA, ARS Beltsville Exotic Pathogen collection, and both host range and genetic marker analyses (16) were conducted. Marker analysis indicated that MCA13 non-reactive isolates were genetically similar to the mild Florida isolate were typical of with T30 (Genbank Accession AF260651) and MCA13 reactive isolates were genetically similar to the severe Florida isolate, T36 (Genbank Accession U16304) (16), which is consistent with information from Costa Rica farm managers who indicated that much of their original propagation material came from Florida, where isolates with these genotypes are common (16).

**Survival analysis.** Methods were applied first to determine the general survival characteristics of both the T30 and T36 isolates in each plot using the Kaplan-Meier Survival model described by,

\[
S(t) = \prod_{t_i \leq t} \left[ \frac{r_i - d_i}{r_i} \right]
\]

where \( t \) = time in months, \( \Pi \) denotes the geometric mean, \( r \) = the hazard ratio, and \( d \) = disease status (0 or 1) for the \( i^{th} \) individual tree.

Next the data were tested to determine if a prior infection by the T30 type isolate affected subsequent co-infection by the opposing T36 type isolate. Finally the data were examined to gauge if trees with existing CTV infection from previous assessment periods could be used to explain the occurrence of new infections that occurred within different distances from the potential infection source (defining the area of influence). Radii of 8, 16, 24 and 32 m surrounding infected trees were queried. The number of CTV-positive trees for each isolate found within those areas that existed during the previous assessment period was enumerated. For individual trees occurring near the edge of the plot, the radii often extended beyond the plot boundaries. To adjust for edge effects in the data, an edge correction calculation was performed to adjust the number of CTV-positive trees within each area defined by the radii. Using these parameters, the covariate that was tested via survival analysis was the number of prior infections within areas described by these radii.

The semi-parametric Cox proportional hazards model was fitted to the data, which specifies the hazard for an individual tree \( i \) at time \( t \) as,

\[
h_i(t) = h_0(t) \exp(\beta'X_i)
\]

where \( h(t) \) is an unspecified baseline hazard function, \( X \), a vector of time-constant covariates values and \( \beta^\prime \) the vector of covariate coefficients that are estimated by partial likelihood (2, 18). The potential effect of a covariate is quantified using the hazard ratio (HR), expressed in terms of an exponential of the corresponding estimated \( \beta \) coefficient for one unit change in the value of the given variable. An HR value of 1 (\( \beta^\prime = 0 \)) indicates no significant effect of the covariate tested. The explanatory covariates tested were 1) the number of infected trees within an area of influence in a prior assessment (time period), and 2) if a tree with a prior infection of the isolate T30 had a significant effect (positive...
or negative) on the subsequent infection by a T36 type isolate. The hazard function modified for this purpose was,

\[ h_i(t) = h_0(t) \exp(\beta'X_i(t)) \]

with \(X_i(t)\), the vector of values at time \(t\) of the time-dependent covariates as well as the values of the time-independent covariates; and \(\beta'\) is the vector of associated coefficients (4). Analysis was performed using the Survival library of S-PLUS (Data Analysis Products Division, Mathsoft, Inc.).

RESULTS AND DISCUSSION

The spatiotemporal progress of the two strains of CTV in the three Costa Rica plots has been analyzed and presented previously (12) (Fig. 1). CR1 began with a low incidence of the T30-like (MC13-) isolate and the T36-like (MC13+) isolate did not appear until the third assessment, after approx. 11 mo. For plot CR2, the incidence of both T30-like and T36-like isolates was present at moderate incidence at the beginning of the study, and by the end of the study infection was asymptotic. The incidence of both isolates at the beginning of the study in plot CR2 was similar to that at the final assessment for CR1. Therefore, CR1 can be viewed as typical of the first half of a CTV epidemic and CR2 as typical of the latter half. Plot CR3 presents an interesting situation in which six rows on one side of the plot (about 1/3 of the total plot) were heavily infected with the T30-like isolate at the beginning of the monitoring period and the remaining 2/3 of the plot had very few prior infected trees at the onset (8 and 8 trees infected with T30-like and T36-like CTV, respectively). Thus, within CR3 there was an opportunity to watch the evolution of infection by the T36-like CTV isolate within an area (2/3 of the plot) where it was on nearly equal footing with the T30-like isolate and an opportunity to follow invasion by the T36-like isolate into an area nearly completely pre-populated with the T30 isolate.

Survival analyses of these plots for both isolates individually agree with the characteristics of disease

![Fig. 1. Spatial point pattern maps of three plots in which disease incidence of two CTV isolates was followed over multiple assessments. White squares indicate CTV-free trees, grey squares indicated presence of CTV T30 isolate, black squares indicate presence of CTV T36 isolate that may or may not be co-infected with T30, and square with 'X' indicate missing tree. See text for details.](image)
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Progress presented above. Survival of trees in the virus-free condition as estimated by the Kaplan-Meier model, decreased with time as expected relative to both isolates (Fig. 2). In all cases the Kaplan-Meier model fit the data well (Table 1). For CR1, the T30-like isolate appeared prior to the T36-like isolate by about 11 months as seen by the first occurrence of the T36-like isolate in the corresponding graph (Fig. 1). As a consequence, in CR1 survival fell below 50% approximately 33 and 38 mo into the study for T30-like and T36-like isolates, respectively (Fig. 2A). The temporal difference in survival corresponds well to the difference in initial onset of virus infection for each isolate. For CR2, incidence of both isolates was greater at the onset of the monitoring period, i.e., 0.194 and 0.202 incidence for T30-like and T36-like CTV respectively. Because the incidence was higher at the initial

Fig. 2. Kaplan-Meier survival graphs for Costa Rican plots (A, B, C), and hazard ratio analysis of survival curves for previous T30-like isolate infections and subsequent T36-like isolate survival (D, E, F).
assessment, the survival curves pass through the 50% survival point after 7 and 21 mo, respectively, considerably earlier than for plot CR1 (Fig. 2B). For plot CR3, the T30-like isolate was initially at much higher incidence (0.259) compared to the T36 isolate (0.015) at the beginning of the monitoring period, as depicted in (Figs. 1 and 2C). For CR3, the survival curves for the T30- and T36-like isolates passed through the 50% point after 12 and 38 mo, respectively. If the difference in virus incidence is taken into account, the temporal increase of the T30-like and T36-like isolates appear to be similar; however, it is not possible to accurately calculate the rate of virus increase for the T30-like isolate because as incidence of the T36 increases, the T30 isolate is obscured due to the inability of the ELISA assays to distinguish between a tree with a dual infection of both T30 and T36 and a tree infected with only T36-like CTV.

A second objective of this study was to examine the effect of a prior infection with T30-like CTV on subsequent coinfection by T36, indicating there is a synergistic interaction between the isolates. For this portion of the study, the Cox Proportional Hazards Model was used to estimate the effect of the covariate.

For CR1, prior infection by T30-like CTV had no effect on subsequent coinfection with T36-like CTV ($H_0: \beta = 0$ was not rejected $P = 0.9$, Table 2). This is most likely because the T36-like isolate was not detected in the plot until about mid way through the monitoring period. In addition, virus incidence of the T30 isolate was relatively low throughout the monitoring period and therefore the proportion of virus-free trees remained high. Thus, when the T36-like isolate spread within the plot, it was much more likely to encounter a virus-free tree than a T30-infected tree.

This is in contrast to plot CR2 which had much higher and nearly equivalent incidence of T30-like and T36-like infected trees at the onset of monitoring. For CR2 and CR3, $H_0: \beta = 0$ was rejected at $P = 0.0000$ and 0.0015 for CR2 and CR3, respectively. This implies that prior infection by the T30-like isolate had an effect on subsequent infection by the T36-like isolate (Table 2).

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TABLE 2
THE LIKELIHOOD OF INFECTION OF CITRUS TREES WITH T30-LIKE AND T36-LIKE CTV ISOLATES USING A COX PROPORTIONAL HAZARDS MODEL. PARAMETER ESTIMATES WITH EDGE EFFECTS CORRECTION.

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</tbody>
</table>

<sup>a</sup>Cox model Beta and hazard ratio estimates for (T30 to T36 Infection) the effect of prior infection by the CTV T30 isolate on subsequent infection by the CTV T36 isolate, (Radius) Beta and hazard ratio values for the effect of prior infection by the CTV T36 within the near vicinity on the subsequent disease status of non CTV-infected trees, and (Annuli) Beta and hazard ratio values for the effect of prior infection by the CTV isolate T36 within the near vicinity on the subsequent disease status of non CTV-infected trees described by concentric annuli 0-8, 8-16, 16-24, and 24-36 m radius.
covariate T30 increased, the survival of trees remaining in a T36-free state improved for both plots. Note that the hazard ratios for both CR2 and CR3 are less than 1. Consider the following analysis of the survival plot for CR3 (Fig. 2C). Probability of survival (remaining T36-free) of a non-infected tree at $t = 33$ months is 0.546 or 54.6%, or saying the reverse the probability of a non-infected tree becoming infected with T36 is 45.4%. This can be written as:

$$S_0(t) = 0.546, \ t = 33 \text{ mo}$$

Now let’s examine the effect of the pre-existing T30-like infection on subsequent co-infection by the T36-like isolate. The relationship is described by:

$$S_1(t) = 0.546^{0.687}, \ t = 33 \text{ months},$$

or $$S_1(t) = 0.660,$$

which is a measurement of survival, i.e., of the plant remaining in a non T36-like infected state. This means the probability of a T30-like infected tree becoming infected with a T36-like isolate is 34.0%, or about 11% less than in the previous example with no prior T30-like infection. This slightly higher survival value indicates that prior infection by the T30-like isolate has the affect of reducing the probability of new infections by the T36-like isolate. For CR2 this effect is a little more pronounced (Fig. 2B). In this case, the probability of survival of a non-infected tree at $t = 21$ months is 0.547 or 54.7%, and the probability of a non-infected tree becoming infected with the T36-like isolate is 45.3%. So:

$$S_0(t) = 0.547, \ t = 21 \text{ mo},$$

and

$$S_1(t) = 0.547^{-0.433}, \ t = 21 \text{ mo},$$

or $$S_1(t) = 0.770.$$

Thus, for CR2 at 21 mo, the probability of a non-infected tree becoming infected with a T36-like isolate is 45.3%, and the probability of a prior T30-like infected tree being infected with the T36-like isolate is 23.0%. Thus, it is 45.3-23.0 = 22.3% less probable for a tree to become infected with the T36-like isolate if it is pre-infected with the T30-like isolate. This clearly demonstrates the effect of prior infection by the T30-like isolate (Fig. 2E-G).

These effects are perhaps subtle, and, although there is an inhibitory affect of prior T30 infection on subsequent T36 infection, it is perhaps unnoticeable unless demonstrated statistically by survival analysis and hazard modelling methods. Certainly the affect of T30 on T36 infection is not at a level that would be conferred to cross protection. Yet the two isolates appear to be interacting in a slightly competitive manor. This appears to be the first time this isolate interaction/competition of CTV has been demonstrated at the field population level. There is a need to explore this affect at the cellular and molecular levels to determine how the mechanisms contributing to competition at those levels relate to CTV isolate interaction at the field level.

The final objective of the study was to examine the effect that prior infections of CTV had on the likelihood of infection within an area of influence (i.e., what is the source of infection. As described above, radii of 8, 16, 24, and 32 m were used to define successively larger areas of influence. The number of CTV-positive trees within each area during a prior assessment period was tested as a covariate to determine the effect on a tree’s survival (i.e., remaining in a virus-free state). All four radius categories were highly significant and were found to affect the disease status of CTV-free trees in the subsequent assessment period. The survival graphs show a decreasing survival (more rapid infection) associated with increasing radii (Fig. 3). The trend toward shorter survival time, as defined by the hazard ratio which increases through time, is shown in Table 2. What this tells us is perhaps intuitive, but difficult to actually demonstrate. For the CTV/T. citricida pathosystem, an increase in
Fig. 3. Survival curves describing plants remaining in a non-infected state within 8, 16, 24, and 32 m distance radii plant (A, B, C), and within 8, 16, 24, and 32 m radius distance annuli (D, E, F) from a CTV-infected plant.
prior-CTV infected trees within the immediate vicinity (a few tree spaces) has a significant effect on the subsequent probability of infection of non virus-infected trees within a local area of influence. When *T. citricida* is the predominant vector present, virus transmission to neighboring trees is expected because this vector is a colonizer of citrus (citrus is its preferred host), and transmission occurs preferentially to nearby trees (13, 15). Interestingly, although the hazard ratio increases with distance, the change in the hazard ratio:

\[
d(\text{HR})/d(r)
\]

where HR is the hazard ratio and \( r \) is radius from the non-infected tree, decreases with distance, indicating that the effect of previous infected trees diminishes with distance. This can also be tested by examining the hazard ratio associated with prior infected trees located within various range (distance) categories of 0-8, 8-16, 16-24, and 24-32 m (Fig. 3A-C), or as defined by concentric annuli from a non-infected tree (Fig. 3D-F). In this case the ‘change’ in the hazard ratio also decreases as the radius from the non-infected tree to the inner edge of the annuli increases and this change is more rapid than for the area calculations above. This further demonstrates a decrease in the effect of prior CTV-infected trees with distance from non-infected trees.

Within the scope of the present study, we did not assess strain interaction for the CTV/A. *gossypii* pathosystem. However, *A. gossypii* migrates through citrus plantations and does not generally colonize citrus trees. Thus we would not expect the same influence of prior virus-infected trees within the vicinity when *A. gossypii* is the predominant vector species. However, this is a subject for future studies.

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