Project Concluded: Final Report

Development of a Rapid System for Detection of Stubborn Disease in the Field

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Stubborn disease of citrus, caused by Spiroplasma citri, is an important disease of citrus in the hot, arid inland areas of California and Arizona. The two methods most commonly used for detection of stubborn have been biological indexing and culture in a cell-free medium. Indexing for stubborn disease of citrus is difficult since it requires the somewhat tricky side-graft or leaf vein method, and it takes several months to obtain results. Culturing is also somewhat time consuming, sometimes requires several attempts, and can produce false positives from contamination. Culturing is the test of record for CDFA and most regulatory agencies.

As reported previously, we have developed a PCR-based test that is reliable and faster than the traditional culture method. Our method utilizes commercial kits for some steps in the process, thus simplifying the procedure. Cost of the kits and other supplies is about $5 per test. Our procedure is as reliable as the standard culture method and is more sensitive.

Orchard with stubborn incidence that was tracked throughout the growing season. Replants of severely infected trees is evident.

During the initial phases of this project, we utilized a pre-PCR culture in order to amplify the bacterium sufficiently for a successful PCR procedure. This initially involved an extraction of DNA from the culture medium. We were later able to improve the methodology so that PCR was possible directly from the medium. In the final year of the project, we were able to further refine the extraction and amplification protocols so that the PCR could be conducted directly from tissue (fruit, budwood) taken from the trees. This procedure has proven to be as sensitive as the culture method. We have been able to detect the spiroplasmas in some instances where culture did not or the titer was so low that the reaction did not occur in the normal timeframe.

However, we are still working out a sampling methodology. We have confirmed previous local reports that there are seasonal fluctuations in the titer of the *Spiroplasma* within citrus trees, with peaks during the warmer months. Based upon our last year's work, it takes the *Spiroplasma* about 6 months to become established in an inoculated plant at near optimal temperatures. Therefore, it will take several months for the *Spiroplasma* to 're-colonize' the tree, which would mean that the best time for sampling would be towards the late summer or early fall. We believe that the best time period for testing for stubborn in the San Joaquin Valley is between July and October.

Previous reports on stubborn indexing indicated that the pathogens were not evenly distributed throughout the tree. During FY 2004, we also observed that association of the pathogen and symptomatic areas is not 100%. This also complicates development of an appropriate sampling strategy. Because of these factors, the number of samples taken from the tree becomes more important than the location or the size of the individual sample. In known positive trees, only about 15-20% of the samples of individual budsticks produce a positive reaction, either by culture or PCR. Therefore, a pooled sample of about 10 busticks taken from around the tree is recommended. Samples should be taken as much as possible from visually symptomatic areas. The highest percentage of positives is obtained if it is possible to sample from behind symptomatic fruit.

We have also developed an anti-body for stubborn. However, the first iteration from the lab was not a high enough titer, so a second antibody was developed. We have not developed an ELISA at this point, but have utilized the antibody for immuno-capture PCR, which is more sensitive but also a more involved assay. A serological test would useful for a large scale testing and we may work more on the development of this assay in the future. However, the sampling issues would be the same for an ELISA test as for the PCR test, and due to the lower sensitivity of ELISA, might be even more of an issue.

Our work to this point has shown that there is some genetic diversity to be found from isolates from various areas. We do not yet know whether or not this diversity has any practical significance. We may continue to work with growers and farm advisors to identify new (to us) blocks of infected trees and to evaluate the genetic diversity using the AFLP markers that we have found useful for this.

It has become apparent over the last several years that there is probably more stubborn within the state than previously realized. With the incidence of CTV quite low in the San Joaquin Valley, stubborn may be the disease producing the largest yield losses. We previously detected stubborn-positive trees in the block being utilized for the "Economic Impact of Tristeza" project. This last year of the project, we sampled the entire block. These samples have not yet been processed completely. Although the samples were taken somewhat late in the season, it is hoped that we will be able to overlay the stubborn incidence over CTV and separate the economic impacts of these two diseases.

This constitutes the final report for this project. Resources permitting, we hope to continue to work in this area at a reduced scale. Any growers or extension personnel that are interested in testing for stubborn should contact us. Furthermore, any personnel from state or university entities that are interested in learning the PCR technique should contact us for tech transfer.