Current research on *Spiroplasma citri* in California

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ABSTRACT. Stubborn disease, caused by *Spiroplasma citri*, has been a long-standing disease in most areas of California. Because of uneven distribution in the plant and seasonal fluctuations in titer, detection of *S. citri* by biological indexing or the traditional culture method is not always reliable. To optimize the detection of *S. citri*, we now culture the samples in liquid media to increase the number of spiroplasmas, extract the DNA, and assay for *S. citri* by PCR. This method has proven to be more rapid and sensitive than the traditional culturing method. Presently seasonal fluctuations in titer are being studied and comparisons are being made between the San Joaquin Valley and Southern California areas. Apparent genetic variability in the *Spiroplasma* from different areas of California has been identified using AFLP. This research also has lead to the observation that stubborn disease is more widespread in California than previously believed, and that the economic losses are probably greater also.

The disease referred to as 'Stubborn disease of citrus' was first observed in California about 1915 in Washington navel trees near Redlands. The first report of stubborn from outside of California was from Palestine in 1928. Stubborn is now known to be established in most warm, dry inland producing areas in California and Arizona, and is also a serious disease in most citrus-producing countries with suitable climates. However, in all cases there is great variability in the distribution and occurrence of stubborn in individual orchards (8).

Although symptoms and effects of stubborn were well known for many years, the causal agent was unknown and was thought to be a virus. However, in the late 1960s and early 1970s, the causal agent was identified as a mycoplasma-like organism (7, 12) and later further characterized and named *Spiroplasma citri* (13).

The symptoms of stubborn in field trees are variable and often so obscure that positive diagnosis requires confirmation by controlled testing. Biological indexing for stubborn was first reported by Calavan and Christiansen (2). Further information on this is found in Calavan (1) and Roistacher (11). Bio-indexing involves graft inoculation into sensitive varieties such as Madam Vinous sweet orange followed by incubation at warm temperatures. Symptoms develop in 2-3 mo. Inoculations for stubborn are most successful when done by side grafting, rather than by bud-grafting practiced with most graft-transmissible pathogens of citrus.

Culturing of *S. citri in vitro* was first described by Saglio *et al*. (12) and Fudl-Allah *et al*. (7). An alternative axenic medium was developed by Lee and Davis (9). Culturing requires a number of time-consuming and intricate steps and growth of *S. citri* usually takes 2-3 weeks. Contamination by non-target organisms can result in false positives.

Several serological techniques have been applied to the detection of *S. citri* (4, 5, 14). However, these techniques have not been established for use in California. A commercial kit is available but does not routinely detect the California isolates commonly used as positive controls by Citrus Clonal Protection Program (CCPP) and the National Clonal Germplasm Repository for

Citrus and Dates (NCGRCD) (Gumpf and Krueger, unpublished data). Therefore, it is unlikely that these techniques would prove effective for use in either an indexing program or a nursery testing program.

Polymerase chain reaction (PCR)-based techniques are promising for quick detection of *S. citri* since they are relatively rapid and are more sensitive than serological techniques. PCR and immuno-capture (IC)-PCR for detection of stubborn have been published by Saillard *et al*. (15, 16). However, some apparently positive stubborn trees gave negative results (16).

Osorio (10) attempted detection of stubborn in California using primers developed from the spiralin gene (6). Osorio, Creamer, Gumpf, and Krueger (unpublished data) compared several methods of DNA extraction for detection of stubborn and found detection under controlled greenhouse conditions was more reliable than from field trees. This was attributed to environmental factors and the irregular distribution of the pathogen in the tree as previously reported (3). Similar results were obtained by Rangel and Krueger (unpublished data) using other primers. Apparently the low levels of *S. citri* in the tissue, and seasonal variations therein, result in inconsistent PCR amplification.

Our current PCR method utilizes primers for the spiralin gene (6). Prior to PCR amplification, collected tissue is surface-sterilized and 'inoculated' into 'micro-culture' volumes of three to five ml of liquid media. DNA is extracted from a sub-sample of 0.5 ml and is used in the PCR assay. We can detect *S. citri* within a week after inoculation of the liquid media. This method is more reliable than PCR done without the pre-culture, and is faster and requires fewer resources than macro-scale culture or inoculation of indicator plants. Utilizing commercially-available kits for DNA purification and for the PCR reaction, and pre-cast mini-gels makes this method easy to implement.

Using our method, detection of *S. citri* is possible from bark (budwood) and fruits. Detection from symptomatic fruits is the most sensitive, as the symptomatic fruit are correlated with the presence of the pathogen in that portion of the tree and the pathogen is present in high concentrations in certain portions of the fruit. However, use of bark is preferable from the standpoint of sample handling and processing. Over the course of the year we have determined that the cooler months are not as efficient for the detection of *S. citri.* We have determined that the optimal time to detect *S. citri* is the summer months when the fruit has developed and matured. In the winter months (2002-2003), we observed that *S citri* does not do as well in culture. The cultures do not grow as fast, possibly because of the physiology of *S. citri* or a starting titer lower than that found in summer. Under greenhouse conditions, we were able to detect *S. citri* approximately 6 mo after an inoculation done in December from material collected in the field. This is the time period for the bacterium to replicate from the low levels present at the time of inoculation to concentrations high enough to be detectable. Under field conditions, the time period for this increase in titer is longer, since the temperatures are not as consistently optimal, and are variable from year to year.

The sampling for *S. citri* from budwood has over all has been consistent. There are some trees that consistently are positive when sampled over the course of several months. Inconsistent detection of *S. citri* could be attributed to either the low titer or the infection of *S. citri* being localized to a few branches of the tree (limited systemic infection). We are beginning work to further characterize within tree variations in distribution of *S. citri*. In the future, we will be try-

ing to refine the sampling system so that the level of training does not need to be as high and so that samples may be collected and sent to a lab for detection if appropriate.

When we began this project, we started accumulating positives and maintaining them in the greenhouse. At that time, we did not know the genetic diversity of the isolates and whether any additional isolates actually represented genetically diverse strains. Since the discovery of *S. citri* as the causal agent of stubborn disease in the 1970s, little has been determined about the genetic diversity of the organism. Previous work in California indicated that the spiralin gene, which is the basis for our assay, is useful for detection in a range of isolates. However, the range of genetic diversity of *S. citri* isolates studied was not known. It is possible that all isolates were similar and that the primers would not be as effective if more diverse strains were present. We are therefore attempting to assess the genetic diversity of the isolates that we have collected and been able to detect. Utilizing published sequences for various genes did not reveal any additional genetic diversity, due to a reduced genome in this simple organism. Utilizing AFLP markers, we were able to detect some apparent differences in the genomes of certain isolates. However, the significance of this apparent diversity has not been determined. The fact that the spiralin primer successfully detects isolates from these apparently different strains further reinforces the broadly applicable nature of the assay.

We are also working on development of an immunocapture method for detection of *S citri*. For various reasons, this work has not progressed as rapidly as has the work with the PCR technique. However, we have recently produced an antiserum that apparently is of sufficient titer to use for serological work. We have had some initial success in utilizing it for immunocapture PCR.

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