Sensitive Detection and Characterization of Viroid, Virus, and Virus-like Agents of Avocado

Continuing Project: Year 5 of 5

Project Leader: J. Allan Dodds (909) 787-4491
e-mail: dodds@ucr.edu
Department of Plant Pathology
University of California, Riverside
Riverside, CA 92521

Cooperating Personnel: D. M. Mathews, dmathews@ucrac1.ucr.edu; (909) 787-3864

Benefit to the Industry

Avocado sunblotch symptoms have been known since the early 1900's and cause discoloration and physical distortion of leaves and fruit (Coit, 1928; Desjardins, 1987). Fruit may be unmarketable due to these symptoms. This disease is caused by the avocado sunblotch viroid (ASBVd), a small (247 nt) RNA pathogen. For many years the only way to survey for this disease was to biologically index plants, which consumed not only time (1-3 yrs), but greenhouse space. During the course of our research we have developed and modified the use of RT-PCR for the routine detection of ASBVd. Testing can be done in as little as 24 hrs and uses plant material directly from the trees of interest. Our method has been approved by the CDFA for the registration of foundation stock trees and growers can now apply for a permit for this purpose. Since this test has been made available it has resulted in preventing the dissemination of sunblotch infected material into the industry on several occasions. Many of these trees were those at the UC South Coast Research and Extension Center and the identification and removal of sunblotch infected trees is an important aspect of producing new avocado varieties for the industry that are meanwhile free of this pathogen. The search for other viral diseases of avocado is ongoing and characterization of double-stranded RNAs readily found in most trees is leading toward the possible identification and determination of their origin and how they may impact the crop.

Objectives

1. Cloning and sequencing of dsRNAs found in avocado.
2. Testing of nurse seeds/seedlings for the presence of avocado sunblotch viroid (ASBVd).
3. Time course/progression of infection of ASBVd in greenhouse grown avocados.
4. Identification of symptomless carriers and strain characterization of ASBVd.

Summary

This year in addition to the 4 research objectives above, we have also continued to test trees for ASBVd through our testing service. We tested a total of 220 trees received from 1 researcher and 4 growers and found 41 positive trees (18.6 %). If you are interested in having trees tested for sunblotch, please contact Deb Mathews using the information above for pricing and scheduling

1. Cloning and sequencing of double-stranded (ds) RNAs found in avocado.

The presence of dsRNAs in plants typically (but not always) indicates the presence of viruses. Several unique dsRNAs have been known in avocado for 20 years (Jordan et al., 1983). There are 3 pattern types (Figure 1) which may occur singly or in various mixtures of each, including all 3 in the same tree. Over 85% of the trees we have tested contain one or more of these dsRNAs. Although these dsRNAs have not been shown to be viral-like in nature (no associated particles, no obvious disease syndrome), one (pattern type 1) has shown to be partially related to the avocado genome (Cook et al., 1994). Our research has shown that one region of the type 1 dsRNA does show significant sequence similarity to that of the chloroplast genome of many plant species (Mathews and Dodds, unpublished results). This relationship to known sequences was the only one found in the initial cloning and sequencing of each of these dsRNA types.

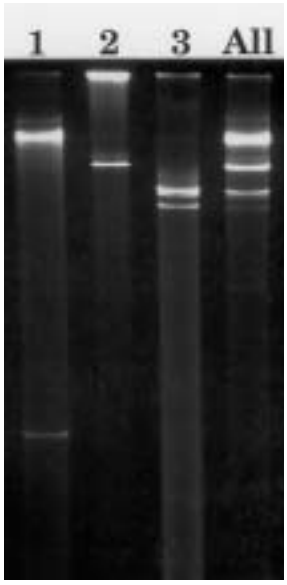

Figure 1. Double-stranded RNAs from 4 different avocado trees. Each tree contains dsRNA patterns 1, 2 or 3 individually or contains all 3 patterns in the same tree (All).

Further cloning and sequencing along with analysis using BLAST and Smith-Waterman Similarity searches has shown that there are several regions of viral-like sequences that show these dsRNAs do now or once did, function much like viruses. The type 1 dsRNA has regions that show the structure for a typical polyprotein and sequences typical of viral 3'ends. One clone hybridized not only to the replicative form dsRNA, but also to a small subgenomic dsRNA found in the type 1 pattern, which confirms the proposed 3' end like sequences. Only a few clones have been recovered to date from the type 2 dsRNA pattern due to the presence of a large amount of host nucleic acids in the extract. The amount of these interfering nucleic acids seem to be host dependent, and the particular variety that contained the highest titre of the type 2 dsRNA also unfortunately contained the greatest amount of unwanted material as well. However, the clones analyzed so far have shown the presence of an RNA polymerase gene and an ssRNA binding protein in the type 2 dsRNA, functions commonly found in viruses, since they supply their own replication enzymes. An RNA-dependent RNA polymerase domain has also been found in the sequences for the type 3 dsRNA. This is the first genetic evidence that these avocado dsRNAs are viral-like in nature. Additional dsRNAs are being purified and new specific primers are being built in order to continue this research.

2. Testing nurse seeds/seedlings for ASBVd.

Nurse seeds/seedlings are not screened for the presence of ASBVd and therefore pose a potential threat to the industry. If seeds were collected from a symptomless carrier tree, resulting nurse seedlings would most likely not be readily identifiable as infected with ASBVd and could result in the subsequent grafting and dissemination of infected trees. We proposed a random subsampling of available nurse seedlings from several nurseries to try and determine if infected seeds were being used and at what frequency. With the help of Ben Faber and Gary Bender a total of 491 nurse seedlings were collected and tested from 6 different nurseries. Samples were pooled in groups of 15 trees (2 lvs per tree) to optimize the number of trees that could be tested in a single sample. Our research has shown that we can readily detect 1 infected leaf in a pool of 40 leaves, so we decided to use a potential 1/30 detection limit (1 infected leaf from one of the 30 lvs collected from 15 trees) for these young trees where the titre of the viroid may be low. All of the samples tested negative for the presence of ASBVd, while positive controls using 1 infected leaf in 30 total leaves were readily scored as positive for the pathogen. While all of the samples from this year were negative for ASBVd, we believe that this survey should be performed on an annual basis in order to continually screen each years nurse seedlings for sunblotch.

3. Time course/progression of infection of ASBVd in greenhouse grown avocados.

In a continuing effort to improve our detection system for ASBVd, it is important to know what type and age of tissue is optimum and how long after grafting samples should be collected in order to allow time for the pathogen to move into the scion. Much of our previous research has been on mature field trees and in smaller greenhouse trees that had long established infections of sunblotch. We purchased both one year old "hot house" seedlings (12-18 in. tall) (3 each of varieties 'Hass' on Duke 7 and Toro Canyon and 'Lamb Hass' on Duke 7) and 2 year old (3-4 ft tall) sleeved trees (5 each of varieties 'Hass', 'Lamb Hass' and 'Zutano' on both Duke 7 and Toro Canyon) to use for inoculation studies. Three different methods of inoculation have been used: 1) grafting of healthy scions onto ASBVd infected symptomless carrier trees (2 yrs old, Figure 2); 2) patch grafting of infected bark from both symptomless carrier and symptomatic ASBVd positive trees onto healthy greenhouse trees (both 1 and 2 yr old trees, Figure 3); and 3) drill inoculation using a drill bit to drill into an infected symptomless carrier tree and then immediately into a healthy 2 yr old tree (Figure 4). Prior to the inoculations all "healthy" trees were tested by RT-PCR to ensure that they were free of ASBVd, as well as all sunblotch "source" trees, to ensure that they would provide sufficient inoculum for the experiments.

Inoculation type 1 will imitate the grafting of healthy material onto symptomless carrier rootstocks. Four scions of different varieties ('Hass', 'Lamb Hass', 'Zutano' and either Duke 7 or Toro Canyon) were grafted onto each of 2 infected Topa-Topa symptomless carrier trees and a healthy Topa-Topa tree (12 grafts total). Initial samples for testing were small (approx. 3mm X 4mm) pieces of bark removed from the scion material. After the new buds on the scions began to produce leaves (as early as the third assay-5 weeks post inoculation), small pieces of leaves were used. At each harvest date, a sample was also taken from the infected "rootstock" portion

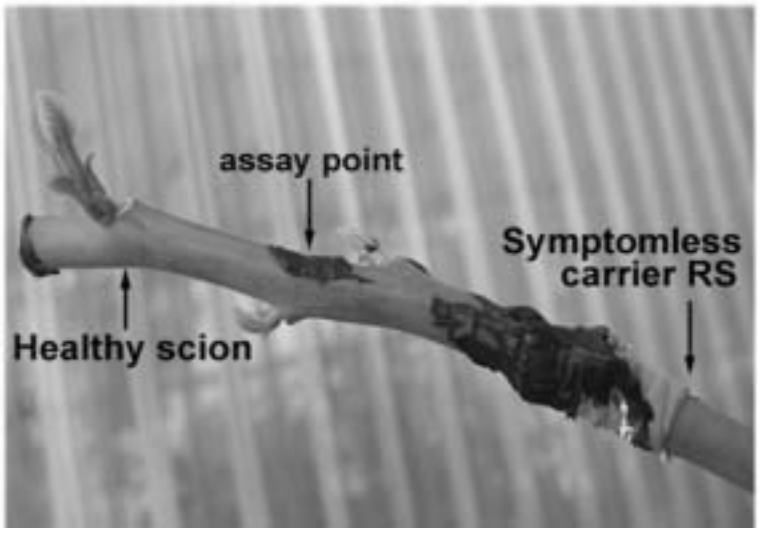

Figure 2. Inoculation type 1: Grafting of healthy scions onto ASBVd infected symptomless carrier rootstock. Assay point shows where first patch of bark was removed for testing, then covered with tree seal.

of the tree using a similar size and age of material to ensure that ASBVd could be detected in that type of tissue. Bark samples from all scions were still negative for ASBVd at 2 and 4 weeks after inoculation, while the rootstock positive control samples were all positive for sunblotch (Table 1). All healthy rootstock and scion samples tested negative for ASBVd as expected. By week 5 however, a small (10-15 mg) leaf sample from one of the 'Zutano' scions grafted onto one of the symptomless carrier trees was scored strongly positive for ASBVd (Table 1). After these initial results are obtained that indicate how long it takes to reliably detect the movement of the pathogen into the healthy scion, additional replications will be done. The development of symptoms on the scions will also be observed, since ASBVd in symptomless carrier trees may not remain symptomless upon the grafting of a new variety.

Table 1. Movement and detection of ASBVd from infected symptomless carrier trees into healthy grafted scions of different varieties.

Rootstock variety and time of assay (wks) post inoculation											
		SC1[1]				SC2[1]				Healthy	
Scion	2	4	5		2	4	5		2	4	5
'Hass'	-	-	-		-	-	-		-	-	-
'Lamb Hass'	-	-	-		-	-	-		-	-	-
'Zutano'	-	-	-		-	-	+[2]		-	-	-
Duke 7	-	-	-		NT	NT	NT		-	-	-
Toro Canyon	NT[3]	NT	NT		-	-	-[2]		NT	NT	NT
Rootstock source control	+	+	+		+	+	+		-	-	-

[1] Symptomless carrier tree infected with ASBVd, variety 'Topa Topa'.

[2] Leaf tissue assayed for week 5 on SC2 scions of Toro Canyon and 'Zutano'. All other assay times used bark patches as assay material.

[3] Not tested, this variety not grafted onto this source material.

Figure 3. Inoculation type 2: Patch grafting of ASBVd infected bark onto a healthy seedling.

The patch grafting of inoculation type 2 was done to give us a feel for the time course of infection using a relatively large amount of inoculum. When grafting citrus tristeza virus (CTV) using patch grafting, it usually takes a minimum of 6 weeks until they are scored as positive by ELISA. Although the RT-PCR assay used for ASBVd detection is much more sensitive than ELISA, the sunblotch viroid is a much different pathogen than CTV, so we cannot assume a similar progression of infection. The first samples collected (1 week post inoculation) were negative for ASBVd. Additional samples are being collected every week from 2 locations on the trees: one near (within 6 inches) and one far (at the terminal portion of the tree) from the point of inoculation.

Figure 4. Inoculation type 3: Drill inoculation of ASBVd into healthy seedlings.

Inoculation strategy 3 is meant to simulate the use of a drill for phosphorous acid application. A number 41 Cleveland drill bit (approx. 2mm in diameter) was used to drill approximately half way through a young ASBVd infected symptomless carrier tree (through the bark and into the pith). The drill bit was removed from the infected tree and immediately used to drill into a healthy 2 year old tree without cleaning. One such inoculation was used for each of 6 trees. The resulting holes were covered by wrapping with a piece of Parafilm. The small bit was used to avoid destruction of the small trees being used. The trees will be assayed every 2 weeks; the first 2 assays will be ready by the annual meeting in November 2003. As a positive control, the tissue material that accumulated on the drill bit from a test drill into the infected tree (3 replications) was collected (approximately 10-20 mg each) and analyzed by RT-PCR, then the drill bit was rinsed with extraction buffer and the resulting liquid was also tested for ASBVd to test for residual ASBVd after the visible debris was removed. All 3 of the debris samples from the test drillings were positive for ASBVd as well as the extract from the rinsed drill bit. This result shows that enough infected material does collect on the drill bit to be potentially infectious and that even after the physical matter is removed, ASBVd is still present on the bit.

4. Identification of symptomless carrier trees and strain characterization of ASBVd.

In the last year approximately 30 trees suspected of possibly being symptomless carriers (SC) of ASBVd were tested. These trees typically produce a large number of flowers, but set little or no fruit. None of the trees tested this year were positive for ASBVd. It has previously been shown that the dominant viroid present in SC trees has a slightly different RNA sequence than those that cause the typical bleaching or variegation symptoms (Semancik and Szychowski, 1994). Since we have found no new SC trees, we have not been able to analyze any new sequences from such trees. We do plan on analyzing any resulting infections from our inoculation experiments (see objective 3) to look at sequence changes as the SC strain of the viroid moves into new varieties and either stays SC or becomes symptomatic.

Conclusions

- DsRNAs found in avocado have identifiably viral-like genetic domains, but some also show regions of homology with the host genome. Long term co-evolution may have occurred between viruses and the host resulting in such chimeras.

- ASBVd is able to move relatively quickly into grafted materials and can be detected by RT-PCR in as little as 5 weeks post grafting ("inoculation" onto an infected source plant).

- Nurse seeds were not acting as a significant source of ASBVd this year, but the potential threat is still there and should be periodically surveyed for.

Literature Cited

Coit, J.E. 1928. Sun-blotch of the avocado. Calif. Avocado Soc Yearbook 1928: 27.

Cook, G., Kotza, J.M. and Nel, L.H. 1994. Double-stranded RNA comprising the putative avocado virus 1 has a high degree of sequence homology to the avocado genome. Plant Pathology 43:913-916.

Desjardins, P.R. 1987. Avocado Sunblotch. In: *The Viroids*, pp. 299-313, edited by T.O. Diener, Plenum Pub. Corp., New York, NY.

Semancik, J.S. and Szychowski, J.A. 1994. Avocado sunblotch disease: a persistent viroid infection in which variants are associated with differential symptoms. J. Gen. Virology 75:1543-1549.