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DETECTION OF AVOCADO SUNBLOTCH VIROID AND ESTIMATION OF INFECTION AMONG ACCESSIONS IN THE NATIONAL GERMPLASM COLLECTION FOR AVOCADO

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ABSTRACT

Reverse transcription-polymerase chain reaction (RTPCR) was used to determine the incidence of infection by avocado sunblotch viroid (ASBVd) in the germplasm collection at the National Germplasm Repository at Miami (NGR-Mia). Of the 429 avocado plants growing at the repository, 81 (18.9%) are infected with the viroid. The 429 plants represent 237 accessions. There are multiple plants of some accessions and for 42 accessions (17%) every plant is infected with the viroid. There was no apparent relationship between host race and the incidence of infection.

Symptoms of sunblotch disease on avocado (*Persea Americana* Mill.) manifest as a general decline of tree vigor with sunken, yellow areas on the fruit that lessen its marketability. The disease was first described as infectious, rather than physiological, by Home and Parker (1931). The infectious agent has since been determined to be an RNA viroid known as Avocado Sunblotch Viroid (ASBVd) (Dale and Allen, 1979; Thomas and Mohammed, 1979). The viroid is transmitted by budding and grafting, including natural root grafting (Home etal, 1941; Whitsell, 1952), seed (Wallace and Drake, 1962), and pollen (Desjardins *et al*., 1979). Since desirable clones and selections are vegetatively propagated and distributed to growers, and because of the latent character of the disease, economic consequences to growers in south Florida and elsewhere are potentially devastating.

Several methods have been employed for ASBVd detection. Wedgebud grafting of suspect tissue onto viroid-free rootstocks is used, but symptom expression may take up to several months (Whitsell, 1952). Polyacrylamide-tube-gel electrophoresis of viroid extracts has been used to detect the viroid (Palukaitis *et al.,* 1979). This test is much faster than wedgebud grafting but not completely reliable. Molecular hybridization with 32P cDNA probes specific to ASBVd is much more sensitive and reliable than either of the above methods (Palukaitis *et al*., 1981; Bar-Joseph *et al*., 1985).

While hybridization to radiolabelled probes has been shown to be a rapid, reliable, and

sensitive indexing procedure, the cost and safety issues involved with using radioactivity can be prohibitive when screening large germplasm collections. Also, levels of the viroid in infected trees can be highly variable within and among infected trees, and in various tree organs (Bar-Joseph *et al*., 1985). Viroid titers also vary between seasons, between leaf age (Palukaitis *et al*.,1979), between branches within a tree, between leaves and flowers (Allen and Dale, 1981). This range in viroid concentration, found in both symptom-bearing and symptom-free infected trees, demonstrates the need for a test sensitive enough to detect extremely low levels of viroid.

An adaptation of the polymerase chain reaction to amplify mRNA sequences from cDNA has been developed (Veres *et al*., 1987) and used in the detection of pome fruit viroids (Hadidi and Yang, 1990). This method, reverse-transcriptase polymerase chain reaction, or RT-PCR, is extremely sensitive, allowing detection of very small amounts of RNA from total nucleic acid extracts without hybridization and probing. These assays are easy to perform, and are economical enough to allow large number of samples to be examined.

RT-PCR has been used to detect ASBVd in infected trees using 0.5-1 ng/ul total nucleic acids (Schnell *et al*., 1995). Here, we describe the use of RT-PCR to screen the avocado germplasm collection located at USDA-NGR, Miami, FL for presence of ASBVd.

MATERIALS AND METHODS

Plant material. Unopened flower buds were collected from all flowering trees in the germplasm collection at NGR, Miami, FL. Soft, flushing leaf material was harvested from those trees which were as yet too immature to flower. In addition, leaf tissue was collected from the related species *P. schiedeana* Nees, *P. nubigeana* Mill, and two related *genera Cinnamomun* and *Laurus.* One hundred mg of flower buds, or 200 mg of leaf tissue, were weighed and stored at -80C until extraction. All 429 trees in the *Persea* collection at Miami were sampled in this manner.

RNA extraction. RNA was extracted from flower buds and leaves using Amresco Total RNA kit (Amresco, Solon, OH). Pelleted RNA was dissolved in 50 µℓl nuclease-free distilled water, and stored at -20C.

Primer design. The forward primer, complementary to nucleotides 68-87 in the central conserved region, has the sequence 5'-AAGTCGAAACTCAGAGTCGG-3'. The reverse primer, homologous to nucleotides 88-104, has sequence 5'GTGAGAGAAGGAGGAGT-3'. These primers have been previously described (Bar-Joseph *et al*., 1985; Schnell *et al*., 1995).

cDNA synthesis. For each sample, 3 µℓ of 5X 1st strand buffer (Gibco BRL, Gaithersburg, MD), 0.01 M DTT, 500 ng of forward primer, and 1.0 ng of RNA were mixed in a total volume of 15 µℓ. Reactions were incubated at 100C for 5 min, placed on ice for 2 min, then allowed to stand at room temperature for 1 hr to allow primer annealing. At the end of this incubation period, 10 µℓ of reaction mix (5X 1st strand buffer, 5 mM dNTPs, 75 µM ß-mercaptoethanol, 2 U of RNasin (Promega, Madison, WI), and 20 U of M-MLV reverse transcriptase (Gibco BRL)) were added to each

sample. The reactions were then incubated at 42C for 2.5 hrs.

Amplification of cDNA. Each reaction consisted of 2.5 |ll 10X PCR buffer (Perkin Elmer, Norwalk CT), 800 µM dNTPs, 0.05 U AmpliTaq DNA polymerase (Perkin Elmer), 2 µM forward primer, 1 µM reverse primer, and a 2.5 µℓ aliquot from the first strand cDNA synthesis, in a total volume of 25 µℓ. Cycling parameters consisted of 94C, 1 min; 40 cycles of 94C -1 min, 55C 2 min, 72C 3 min; followed by a final 5 min extension at 72C.

Electrophoresis. PCR products were electrophoresed through 2% agarose in 0.5X TBE buffer, and visualized with ethidium bromide staining. Samples that contained a band at approximately 250 bp (putative positives) were further separated in vertical 4% Metaphor (FMC, Rockland ME) agarose in IX TBE, and stained with EtBr, to confirm the size of the band.

DNA sequencing. To ensure that the amplification product was ASBVd, individual 247 bp bands were excised from agarose gels, and the DNA eluted and cloned into pBlueScript SK(+) (Stratagene, La Jolla, CA). Miniprep DNA was prepared using an alkaline PEG protocol. DNA was quantified spectrophotometrically and sequenced on an ABI 373A automated sequencer (ABI, Foster City, CA) using the Taq DyeDeoxy Terminator Cycle Sequencing Kit (Perkin Elmer). The partial sequence of the 247 bp fragment was compared to the GenBank database using the BLAST algorithm (Altschuletal. 1990).

RESULTS AND DISCUSSION

Two lines of evidence were used to demonstrate that the 247 bp RT-PCR product was ASBVd. First, acrylamide gel electrophoresis of the RT-PCR products from symptomatic and nonsymptomatic trees showed a 247 bp band only for symptomatic trees (Fig. 1). Likewise, the band was not evident for symptomless trees in the related genera *Cinnamomum* and *Laurus.* Second, when the sequence of the 247 bp fragment and the sequence of ASBVd J02020 were compared they differed at only two positions (Schnell *et al*. 1995). Slight variation in the sequence of ASBVd has been detected among different strains of this viroid (Semancik and Szychowski, 1994). Thus, the cloned fragment probably represents a new variant of the viroid.

The avocado germplasm collection at the NRG-Miami contains 237 accessions with a total of 429 plants. The largest group is a complex mix of hybrids between the races, followed by West Indian race, West Indian x Guatemalan hybrids, Guatemalan x Mexican hybrids, Mexican race, West Indian x Mexican hybrids, and Guatemalan race (Fig. 2).

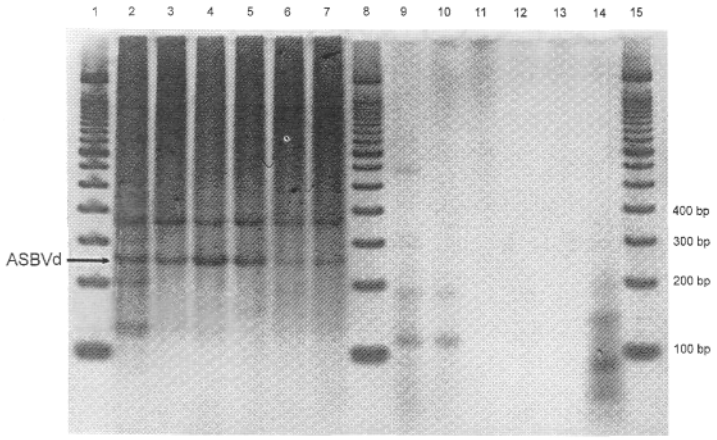

Figure 1. Polyacrylamide gel electrophoretic analysis of RT-PCR-amplified ASBVd cDNA from total nucleic acids from infected and non-infected avocado accessions, and two related genera. Lane 1, 8, & 15 100 bp ladder, lane 2 'Progresso Late', lane 3 'Wilson Popoenoe', lane 4 'Vero Beach#5', lane 6 'Vero Beach', lane 7 'Aycock Red#19', lane 9 'Morrocco#43', lane 10 'Romain#1', lane 11 'Tito Perla', lane 12 'Vero Beach SE2', lane 13 *Cinnamommum sp.*, lane 14 *Laurus nobiis.*

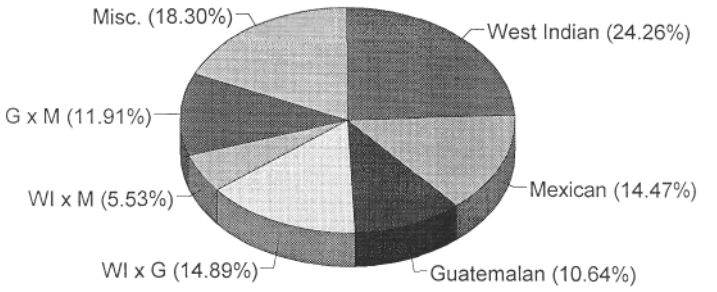

Figure 2. Distribution of the Avocado Germplasm Collection at the NGR-Miami by race.

Of the 429 trees the total infection rate is 18.9%. In 42 of the 237 accessions (17%) every plant is infected; however, in 16 accessions (7%) at least one of the plants is viroid free. The highest infection rates occurred in field W4-1 and WA2 with lower rates in W3-1 and WB3 (Table 1). Field W4-1 is the field where the original avocados collected by Wilson Popenoe in the 1920s were planted. These trees are about 70 years old and have had many opportunities for viroid transmission via root grafting, mechanical inoculation, and pollen transmission. The high percentage in WA2 is due to cold tolerant seedlings selected by R. J. Knight and sent to Texas for further evaluation then reintroduced. These plants were probably infected in Texas during cold tolerance testing.

When analyzed by race the Mexican accessions had the highest infection rate followed by the Guatemalan and West Indian. This difference probably resulted from a larger percentage of Guatemalan and Mexican accessions being planted in the field with the highest infection rate. The interracial hybrids varied from 17% to 25% (Table 2).

A sample of 19 seedlings from several maternal half-sib families with the seed collected from ASBVd positive mother trees were tested. Of these, 35% were found to be infected. This demonstrates that ASBV is transmitted at a high frequency to the offspring from the female parent. Further tests are planned to confirm these preliminary results and also to estimate transmission rates for infected male parents. Tissue culture methods have proven successful for the elimination of the Citrus Exocordis Viroid from *Citrus* germplasm. These methods are currently being modified for avocado and efforts to eliminate ASBVd will be made. Infected germplasm is not being distributed from the NGR-Miami. If successful cleanup via tissue culture can be accomplished, it will be possible to save this valuable germplasm.

Table 1. Field, number of avocado plants per field, and incidence of infection with ASBVd for the NGR-Miami.

Field	Total # trees	Infected # (%)
W3-1	38	6 (15.8)
W4-1	50	24 (48.0)
WA-2	97	27 (27.8)
WB-3	99	14 (14.1)
WB-4	115	3 (2.6)
WA-2 seedlings	19	7 (36.8)
Miscellaneous	11	0
Total	429	81 (18.9)

Table 2. Race, number of accessions, number of infected accessions, and percent of avocado germplasm infected by race at the NGR-Miami.

Race	Total # of Accessions	# with all Plants Infected (%)
West Indian	57	8 (14)
Mexican	34	7 (21)
Guatemalan	25	5 (20)
WI × G	35	6 (17)
WI × M	13	3 (23)
G × M	28	7 (25)
Complex hybrids	43	6 (14)
Total	235	42 (17)

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