

# Infectivity Studies of Avocado Sunblotch Disease Causal Agent, Possibly a Viroid Rather than a Virus

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## ABSTRACT

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The causal agent of avocado sunblotch disease was mechanically transmitted from avocado to avocado by the razor-slash technique. Infectious material in various extracts from diseased fruit tissue also was transmitted to avocado by this method. The lack of typical virions in diseased tissues and in tissue extracts, the lack of association of microorganisms, and the pronounced thermal stability of the infectious agent suggested a nature other than viral. In extraction procedures not involving phenol, the infectious agent was in higher concentration in high-speed supernatants than in high-speed pellets, suggesting a low molecular weight substance. Also, the agent in such extracts appeared to have some sensitivity to RNase. Apparently, the causal agent has many properties characteristic of viroids that are not characteristic of viruses.

The disease of avocado called sunblotch has been known in California for at least 50 yr. Initially it was considered to be a physiological disease (1) or a genetic disorder (10). In 1931, Horne and Parker (11) reported that the causal agent could be transmitted from diseased scions to healthy rootstocks and suggested that the disease "belongs to the group of plant diseases called infectious chloroses." By 1941, graft transmissibility had been well established and the disease was generally considered to be caused by a virus (12). Frequently, avocado trees recover from the disease but continue to be symptomless carriers of the causal agent. Wallace and Drake (21) reported an unusually high rate of transmission of the virus in seed from recovered trees (90–100%) compared with a low rate (0–5.5%) in seed from trees with symptoms of the disease. Transmission of the virus from tree to tree by natural root grafting in the field has been considered in certain instances (12,22). Pollen transmission of the causal agent also has been shown recently (3).

Since the causal agent of the disease had long been considered to be a virus, we have made concerted efforts for a number of years to isolate and characterize the virus. Concurrent with these studies, many attempts were made to mechanically transmit the virus by the usual techniques. Our inability to transmit the virus mechanically precluded assaying for virus activity in isolation and characterization studies. By 1974, three lines of evidence suggested that the sunblotch disease agent might be a viroid rather than a virus. The first was our inability to find typical virions in electron microscope studies of tissue extracts or thin sections

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of diseased tissue. The absence of virions is a criterion for viroids (6–8) and was one of the first findings by Diener and Raymer (9,15) in their early studies of potato spindle tuber viroid (PSTV) and by Semancik and Weathers (17) in studies of citrus exocortis viroid (CEV).

Electron and light microscope studies also failed to associate any microorganisms with the disease. This evidence fulfills a second criterion for the viroid nature of the causal agent (6–8).

In the past, we attempted to inactivate the sunblotch causal agent in seed, budwood, and budlings by heat therapy (P. R. Desjardins, R. J. Drake, and J. M. Wallace, *unpublished*). We failed, and the results indicated that the agent has a pronounced thermal stability; in fact, it could withstand any heat treatment regimen that the avocado tissue could. PSTV (6) and CEV (16) are both resistant to thermal inactivation. Also, higher temperatures shorten the incubation time of cucumber pale fruit viroid symptom expression (20). Therefore, thermal stability of the sunblotch agent should be considered as additional evidence that it may be a viroid.

Two recent papers (2,19) suggest a viroid nature for the sunblotch causal agent based on detection of low molecular weight RNA in diseased tissue extracts by gel electrophoresis, but infectivity of the RNA was not shown.

Because the abrasive-rubbing technique of mechanical inoculation with tissue extracts had given negative results, the razor-slash technique was used for inoculation of test seedlings directly from infected plants. Semancik and Weathers (17) had successfully transmitted CEV by this technique. In a preliminary report (4), we described successful transmission of the sunblotch agent from avocado to avocado by this method. We now

describe additional infectivity studies, some of which, we feel, provide additional evidence of the viroid nature of the causal agent of sunblotch disease.

## MATERIALS AND METHODS

**Razor-slash technique.** An alcohol-sterilized razor blade was passed through a diseased avocado shoot and immediately passed through shoot tissue of a healthy test seedling. This was repeated 10 times on each seedling.

**Freeze-dried technique.** This was essentially the technique described by Ragetli et al (13) for transmission of the potato spindle tuber viroid. Pieces of tissue showing symptoms were excised from infected leaves, ground to a fine powder in liquid nitrogen, and freeze-dried. When not used immediately, the preparations were stored at  $-20^{\circ}\text{C}$ . The fine dried powder was rubbed onto leaves of test seedlings with and without the use of an abrasive (aluminum oxide, 600 grit). When dry, frozen powder was used as inoculum; the leaves were not rinsed. In some tests, the freeze-dried powder was resuspended in small volumes of phosphate buffer (pH 7.0) and the suspension was rubbed on the leaves, which were then rinsed with water.

**Extraction and fractionation methods.** Frozen, infected fruit tissue (100–150 g) from the cultivar Hass was used in all extractions of the causal agent. Only portions of the fruit with typical sunblotch symptoms were used. The methods for initial extraction were essentially modifications of the methods described by Diener (5) and by Semancik and Weathers (18); the fractionation procedures, while similar, differed somewhat. In the work reported here, the rationale for our interest in the infectivity of various specific fractions was to provide information for assaying more highly purified fractions in later characterization studies. Although the four extraction methods are only briefly described, several details should be kept in mind: 1) When phenol or chloroform-butanol was used in the initial extraction, only the aqueous phase was used for further fractionation. 2) Low-speed centrifugation steps were used after initial extraction, after all dialysis steps, and after all alcohol precipitation steps. 3) Concentration by rotary evaporation was always followed by a dialysis step. 4) All pellets from low- and high-speed centrifugations and all alcohol precipitates

were resuspended in buffer if they were to be tested for infectivity. 5) Two or three volumes of alcohol per fraction volume were used to obtain alcohol precipitates.

For the initial extraction, either 0.1 M tris-HCl buffer, pH 8.9, or TKM buffer (0.1 M tris, 0.1 M KCl, and 0.0001 M MgCl<sub>2</sub>), pH 7.4, was used. For resuspension of sediments and precipitates, 0.01 M solutions of these buffers were used. In the initial extraction medium, various proportions of 5% sodium lauryl sulfate (SLS) and 0.1 M EDTA were also included. All initial extractions were done in a high-speed blender.

**Method 1.** Tissue was extracted in 1 vol of tris-HCl buffer (including SLS and EDTA), pH 8.9, and 3 vol of water-saturated phenol. The aqueous phase was collected and given a low-speed centrifugation; the sediment from this was phenol-extracted, and low-speed centrifugation was repeated. The aqueous phase from the second phenol extraction was centrifuged, and the two low-speed supernatants were combined. A reddish

brown precipitate formed in the standing suspension, which was then centrifuged at 12 K rpm for 15 min in a Sorvall SS-34 rotor. The resulting sediment was resuspended in buffer and recentrifuged, and the supernatant and sediment from this centrifugation were designated fractions 1 and 2, respectively, for infectivity assay. The supernatant from the centrifugation of the original suspension was concentrated to two-thirds its volume by rotary evaporation. An aliquot was saved for assay and designated fraction 3. The remainder was subjected to two alcohol precipitations, and the final resuspended alcohol precipitate was designated fraction 4 for infectivity assay.

**Method 2.** The extraction medium was composed of 105 ml of 5% SLS, 105 ml of 0.1 M EDTA, 390 ml of tris-HCl buffer (pH 8.9) containing 1.2 g of Macaloid, and 0.9 g of dithioerythritol. The extract was blended, then centrifuged at 10 K rpm for 10 min in a Sorvall GSA rotor. The low-speed sediment was saved for

assay. The supernatant was subjected to 35 K rpm for 1 hr in a Beckman No. 35 rotor. The high-speed pellet was resuspended and used for assay. An aliquot of the high-speed supernatant was saved for assay, and the remainder was concentrated 10-fold by rotary evaporation. The high-speed supernatant thus concentrated was dialyzed against TKM buffer (pH 7.4) and used for infectivity assay.

**Method 3.** The extraction medium was composed of 150 ml of TKM buffer (pH 7.4), 50 ml of 5% SLS, 50 ml of 0.1 M EDTA, and 250 ml of chloroform-butanol (1:1 mixture). The mixture was blended, then the aqueous phase was separated by low-speed centrifugation, collected, and subjected to high-speed centrifugation for 1 hr at 30 K rpm in a Beckman No. 30 rotor. High-speed pellets were resuspended in TKM buffer, combined, and assayed for infectivity. The high-speed supernatant was concentrated 10-fold by rotary evaporation. During dialysis against TKM buffer after the concentration step, a precipitate formed in the dialysis tubing. This was removed by centrifugation, resuspended, and assayed for infectivity. The supernatant of the centrifuged dialysate was subjected to alcohol precipitation. After resuspension in TKM buffer, the alcohol precipitate was assayed for infectivity.

**Method 4.** This was identical to Method 3 except that the resuspended, final alcohol precipitate was given various treatments and only these fractions were used for infectivity assay. One aliquot of the resuspended precipitate was left untreated. A second aliquot was treated for 1 hr at 37 C with RNase (2 µg/ml), then frozen until inoculations were made. A third aliquot was held in a boiling water bath for 15 min, brought back to its original volume (since evaporation had occurred), and frozen until inoculations were made.

Although inoculations were made using both the rubbing and razor-slash techniques, infections were obtained only with the latter method. Our infectivity data are from only the tests of seedlings inoculated by the razor-slash technique.

Attempts were made to inoculate a number of herbaceous plants directly from avocado by the slash technique and with various fractions obtained by the four methods. The species inoculated were *Nicotiana tabacum*, *N. sylvestris*, *N. glutinosa*, *Phaseolus vulgaris*, *Gomphrena globosa*, *Lycopersicon esculentum*, *Chenopodium quinoa*, *C. amaranticolor*, *Gynura aurantiaca*, *Vigna sinensis*, and *Petunia hybrida*.

## RESULTS

**Razor-slash technique.** The causal agent of sunblotch disease was successfully transmitted directly from avocado to avocado by this technique (Table 1) but not from avocado to any herbaceous

**Table 1.** Infectivity results with mechanical transmission directly from avocado to avocado by the razor-slash technique

| Experiment number | Avocado cultivar inoculated | Infected seedlings <sup>a</sup> | Incubation time (mo) <sup>b</sup> |
|-------------------|-----------------------------|---------------------------------|-----------------------------------|
| 1                 | MacArthur                   | 4/12                            | 16-22                             |
| 2                 | Hass                        | 1/14                            | 18                                |
| 3                 | Hass                        | 2/25                            | 10-16                             |

<sup>a</sup>Numerator = seedlings infected; denominator = seedlings inoculated.

<sup>b</sup>Time when first and last positive readings were obtained.

**Table 2.** Infectivity results with extraction and fractionation Method 2 and Method 3

| Method and inoculation fraction                           | Infected seedlings <sup>a</sup> | Incubation time (mo) |
|---|---------------------------------|----------------------|
| Method 2  |                                 |                      |
| Initial low-speed sediment                                | 2/12 <sup>b</sup>               | 12                   |
| High-speed pellet   | 2/12                            | 16                   |
| High-speed supernatant                                    | 4/12                            | 4.5-12               |
| Concentrated high-speed supernatant <sup>c</sup>          | 8/12                            | 12-16                |
| Method 3  |                                 |                      |
| High-speed pellet   | 0/5                             | ...                  |
| Dialysate precipitate <sup>d</sup>                        | 3/5                             | 4.5-6                |
| Alcohol precipitate of dialysate supernatant <sup>d</sup> | 12/15 <sup>e</sup>              | 4.5-6                |

<sup>a</sup>Numerator = seedlings infected; denominator = seedlings inoculated

<sup>b</sup>Fuerte seedlings used for Method 2.

<sup>c</sup>Concentrated by rotary evaporation.

<sup>d</sup>Fractions from dialysate after concentration of high-speed supernatant by rotary evaporation.

<sup>e</sup>MacArthur seedlings used for Method 3 except in this group, where 3 of 4 Hass seedlings and 9 of 11 MacArthur seedlings were positive.

**Table 3.** Infectivity results with extraction and fractionation Method 4

| Inoculum fraction                                  | Avocado cultivar | Infected seedlings <sup>a</sup> |      |
|--|------------------|---------------------------------|------|
|  |                  | 4 mo                            | 8 mo |
| Final alcohol precipitate, untreated               | Hass             | 1/7 <sup>b</sup>                | 3/7  |
|  | MacArthur        | 0/1                             | 0/1  |
| Final alcohol precipitate, plus RNase <sup>c</sup> | Hass             | 0/6                             | 3/6  |
|  | MacArthur        | 0/2                             | 0/2  |
| Final alcohol precipitate, 100 C for 15 min        | Hass             | 3/5                             | 5/5  |
|  | MacArthur        | 0/2                             | 0/2  |

<sup>a</sup>Numbers of infected seedlings at 4 and 8 mo after inoculation.

<sup>b</sup>Numerator = seedlings infected; denominator = seedlings inoculated.

<sup>c</sup>RNase concentration = 2 µg/ml.

species. The percentages of successful transmissions were somewhat low, however, and the incubation times rather long (10–22 mo). The long incubation times are comparable to those found by Randles et al (14) in studies of the infectious agent of cadang-cadang disease of coconut palms.

**Freeze-dried technique.** No infections of either avocado seedlings or herbaceous plants were obtained with this technique. Ragetli et al (13) obtained 100% infection of tomato plants with PSTV in freeze-dried tomato tissue. Why the technique did not work with the sunblotch agent is not known, but a low concentration of the agent in avocado tissue might be partly responsible.

**Extraction and fractionation techniques.** Four fractions resulting from Method 1 were inoculated to test seedlings, but infectivity was detected only in fraction 4. Three of the 10 seedlings inoculated died from salt accumulation, and only two of the seven that survived became infected. Fractions 3 and 4 were essentially the same material except that fraction 4 was more concentrated as a result of the alcohol precipitation step. This suggests that concentration steps are important to obtaining infectious fractions.

The results of inoculations with fractions from Method 2 and Method 3 are summarized in Table 2. The data for Method 2 indicate that some infectivity was found in the initial low-speed sediment, possibly resulting from association of the infectious agent with cellular constituents, as is the case with PSTV (6). With Method 2, some infectivity was detected in the high-speed pellet, but the infectious agent appeared to be in lower concentration in the pellet than in the supernatant, as fewer plants became infected and the incubation period was considerably longer than that for plants inoculated with the high-speed supernatant. With Method 3, no infectivity was found in the high-speed pellet 17 mo after inoculation of test seedlings, whereas plants inoculated with high-speed supernatant fractions became infected within 4.5–6 mo. CEV is also sometimes found in low concentrations in the high-speed pellet (17). Infectivity was found in high-speed supernatants obtained in both Method 2 and Method 3. The data for both methods also show that when the high-speed supernatants are further concentrated by rotary evaporation and alcohol precipitation, the percentage of infections is increased. Both concentration techniques were used in Method 3. These procedures not only increased the infection percentage but appeared to shorten the incubation period considerably.

The infectivity results for Method 4 are summarized in Table 3. This method was the same as Method 3 except that the final fractions were given additional treatments.

The data suggest that the sunblotch agent has a high thermal stability *in vitro* and also is somewhat sensitive to RNase, in that the incubation time was increased for the RNase-treated fraction.

## DISCUSSION

Our results show mechanical transmissibility of the sunblotch agent directly from avocado to avocado and when fractionated extracts are used as inoculum. The fact that the infectious agent appears to have a lower concentration in high-speed pellets than in high-speed supernatants, even when no phenol extraction is involved, suggests that the infectious agent is a low molecular weight substance. The results obtained by use of high-speed centrifugation fractions are more characteristic of viroids, such as PSTV (6) and CEV (17), than of typical viruses.

The infectivity data for the heat-treated fraction further confirm the pronounced thermal stability of the sunblotch agent. This is considered further evidence of the viroid nature of the agent.

The data suggesting some sensitivity to RNase, although not conclusive, are interesting when one considers that no phenol extraction was involved. Viroid sensitivity to inactivation by RNase depends on the ionic strength of the medium (6), sensitivity being the greatest in low-strength medium. Sensitivity to the somewhat low concentration of RNase used in our studies is more characteristic of a viroid than of a typical virus. Studies are under way to characterize the sunblotch agent by both physical and biochemical techniques and to investigate its possible RNase sensitivity more fully.

The reports by Dale and Allen (2) and Thomas and Mohamed (19) do not provide infectivity data on the low molecular weight RNA they describe, but the RNase sensitivity of the infectious agent as suggested by our studies supports their findings.

We feel the results of our infectivity studies provide additional evidence that the causal agent of sunblotch disease is probably a viroid rather than a typical virus. It is unlikely that even an unstable virus would be as heat-stable as the sunblotch agent.

The only method previously recommended for control of sunblotch has been the use of virusfree seed and budwood (12,23). Because the sunblotch agent can be transmitted by cutting tools, we recommend treatment of pruning tools and budding knives with 15% chlorine bleach and application of oil to prevent corrosion.

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## LITERATURE CITED

1. COIT, J. E. 1928. Sun blotch of the avocado, a

serious physiological disease. Pages 27-32 in: Calif. Avocado Soc. Yearb. 133 pp.

2. DALE, J. L., and R. N. ALLEN. 1979. Avocado affected by sunblotch disease contains low molecular weight ribonucleic acid. *Aust. Plant Pathol.* 8:3-4.
3. DESJARDINS, P. R., R. J. DRAKE, E. L. ATKINS, and B. O. BERGH. 1979. Pollen transmission of avocado sunblotch virus experimentally demonstrated. *Calif. Agric.* 33(10):14-15.
4. DESJARDINS, P. R., J. M. WALLACE, and R. J. DRAKE. 1978. Avocado sunblotch studies. Pages 12-13 in: Summary of Avocado Research. Avocado Research Advisory Committee Meeting, April 1978, University of California, Riverside. 22 pp.
5. DIENER, T. O. 1971. Potato spindle tuber "virus." IV. A replicating low molecular weight RNA. *Virology* 45:411-428.
6. DIENER, T. O. 1979. Viroids and Viroid Diseases. John Wiley & Sons, New York. 252 pp.
7. DIENER, T. O., and A. HADIDI. 1977. Viroids. Pages 285-337 in: H. Fraenkel-Conrat and R. R. Wagner, eds. Vol. 11. *Comprehensive Virology*. Plenum Press, New York. 348 pp.
8. DIENER, T. O., A. HADIDI, and R. A. OWENS. 1977. Methods for studying viroids. Pages 185-216 in: K. Maramorosch and H. Koprowski, eds. Vol. 6. *Methods in Virology*. Academic Press, Inc., New York. 542 pp.
9. DIENER, T. O., and W. B. RAYMER. 1967. Potato spindle tuber virus: A plant virus with properties of a free nucleic acid. *Science* 158:378-381.
10. HORNE, W. T. 1929. Progress in the study of certain diseases of avocado. (Abstr.) *Phytopathology* 19:1144.
11. HORNE, W. T., and E. R. PARKER. 1931. The avocado disease called sun blotch. *Phytopathology* 21:235-238.
12. HORNE, W. T., E. R. PARKER, and M. B. ROUNDS. 1941. The nature of sun blotch and its practical control. Pages 35-38 in: *Calif. Avocado Soc. Yearb.* 150 pp.
13. RAGETLI, H. W. J., M. WEINTRAUB, and M. ELDER. 1973. Effective mechanical inoculation of plant viruses in the absence of water. *Can. J. Bot.* 51:1977-1981.
14. RANDELES, J. W., G. BOCCARDO, M. L. RETUERRA, and E. P. RILLO. 1977. Transmission of the RNA species associated with cadang-cadang of coconut palm, and the insensitivity of the disease to antibiotics. *Phytopathology* 67:1211-1216.
15. RAYMER, W. B., and T. O. DIENER. 1969. Potato spindle tuber virus: A plant virus with properties of a free nucleic acid. *Virology* 37:343-350.
16. SEMANCIK, J. S., T. J. MORRIS, and L. G. WEATHERS. 1973. Structure and conformation of low molecular weight RNA from exocortis disease. *Virology* 53:448-456.
17. SEMANCIK, J. S., and L. G. WEATHERS. 1968. Exocortis virus of citrus: Association of infectivity with nucleic acid preparations. *Virology* 36:328-330.
18. SEMANCIK, J. S., and L. G. WEATHERS. 1972. Exocortis virus: An infectious free-nucleic acid plant virus with unusual properties. *Virology* 47:456-466.
19. THOMAS, W., and N. A. MOHAMED. 1979. Avocado sunblotch—a viroid disease? *Aust. Plant Pathol.* 8:1-3.
20. VAN DORST, H. J. M., and D. PETERS. 1974. Some biological observations on pale fruit, a viroid-incited disease of cucumber. *Neth. J. Plant Pathol.* 80:85-96.
21. WALLACE, J. M., and R. J. DRAKE. 1962. A high rate of seed transmission of avocado sunblotch virus from symptomless trees and the origin of such trees. *Phytopathology* 52:237-241.
22. WHITSELL, R. 1952. Sunblotch disease of avocados. Pages 217-240 in: *Calif. Avocado Soc. Yearb.* 255 pp.
23. ZENTMYER, G. A., A. O. PAULUS, C. D. GUSTAFSON, J. M. WALLACE, and R. M. BURNS. 1965. Avocado diseases. Pages 8-9 in: *Calif. Agric. Exp. Stn. Circ.* 534. 11 pp.