A STUDY ON

AVOCADO SUNBLOTCH DISEASE

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by

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Thesis (PhD., Microbiology and Plant Pathology) - University of Natal, Pietermaritzburg, 1980.

Submitted in partial fulfilment of
the requirements for the degree of

Doctor of Philosophy

in the

Department of Microbiology and Plant Pathology

Faculty of Agriculture

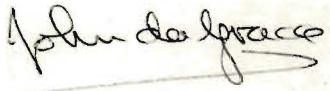
University of Natal,

PIETERMARITZBURG

- December 1980 -

- DECLARATION -

I hereby certify that this research is the result of my own investigation.



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- ACKNOWLEDGEMENTS -

I wish to express my sincere thanks to the following:

- Professor M. M. Martin, my supervisor, for his encouragement, guidance and criticisms, especially during the preparation of this thesis;
- Dr J. H. Grobler, Director of the Citrus and Subtropical Fruit Research Institute (CSFRI), Nelspruit, for permission to use results of research conducted at the Institute;
- Dr L. J. van Lelyveld, CSFRI, my co-supervisor, for his invaluable guidance during the biochemical studies;
- Mr S. P. van Vuuren, CSFRI, for his expert technical assistance, and for his encouragement and criticisms;
- H. L. Hall and Sons, Mataffin, for permission to use their trees as sources of infected material;
- Mr B. Q. Manicom and Miss G. Monti for their help in preparing samples for electron microscopy;
- Mr V. H. Bandu and Mr M. Kundalran for their assistance with the electron microscopy and the preparation of photographs;
- Mrs S. E. Hancock for typing this thesis; and
- my wife, Sheila, for her support and encouragement throughout this study.

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- ABBREVIATIONS USED IN TEXT -

ASV	avocado sunblotch viroid
CEV	citrus exocortis viroid
DEDTC	(sodium) diethyldithiocarbamate
DMACA	4-dimethylaminocinnamaldehyde
DNA	deoxyribonucleic acid
EM	electron microscope
IAA	indoleacetic acid
MBA	N,N'-methylenebisacrylamide
PAGE	polyacrylamide gel electrophoresis
PAL	phenylalanine ammonia-lyase
PE	pectinesterase
PO	peroxidase
PPO	polyphenoloxidase
PSTV	potato spindle tuber viroid
PVP	polyvinyl pyrollidone
RNA	ribonucleic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TMV	tobacco mosaic virus
Tris	tris(hydroxymethyl)-aminomethane
TYMV	turnip yellow mosaic virus
YTD	young tree decline

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INTRODUCTION

Although sunblotch disease of the avocado (Persea americana Mill.) was first observed in 1914 by Barrett in southern California (cited by Whitsell, 1952), the first description of the disorder was published by Coit in 1928. He noted that the symptoms bore a resemblance to sunburn, but to distinguish the condition he named it 'sunblotch', attributing the disease to the effects of the sun producing genetic changes. Nevertheless, infectivity tests by grafting were soon initiated (Horne, 1929), and in 1932 Parker and Horne reported the graft transmissibility of sunblotch, disproving Coit's theory and implicating a virus-like agent. Despite this demonstration, the belief that there was a non-pathogenic cause persisted for some years. Calkins (1936) considered that 'abuse', apparently the harsh treatment of plants after grafting, was responsible.

Until recently sunblotch was the only recorded virus or virus-like disease of avocados. Alper, Bar-Joseph, Salomon and Loebenstein (1978) isolated a symptomless strain of tobacco mosaic virus from avocados, while another avocado disease which may yet prove to have a viral etiology is black streak disease (Ohr, Platt and Zentmyer, 1976 a ; 1976 b).

In addition to California, sunblotch has been recorded in Florida (Stevens, 1939 ; 1941) and Texas (Schroeder and Frolich, 1958), Israel (Coit, 1928 ; Comelli, 1960; Alper, Loebenstein, Bar-Joseph and Cohn, 1975), Mexico (Trask, 1948), Peru (Zentmyer, 1957 ; 1959), South Africa (Loest and Stofberg, 1954 ; Brodrick, Pretorius and Frean, 1974), Australia (Trochoulias and Allen, 1970), Venezuela (Rondón and Figueroa, 1976) and Sri Lanka (Abeyesekera, M., personal communication).

Although sunblotch has not been found in New Zealand (Sale, 1977) research performed there on quarantined material indicated that the cause may be a viroid (Thomas and Mohamed, 1979). Dale and Allen (1979) in Australia reported a similar finding. Both groups detected, by polyacrylamide gel electrophoresis (PAGE) a small RNA species in extracts of infected material which was absent from healthy tissue.

SYMPTOMS

1. Symptoms on the green stem: A few yellow, depressed, longitudinal streaks appear on the young growth, those arising in the early part of the flush tending to broaden to surround the stem (Horne, Parker and Rounds, 1941). The streaks have also been noted sometimes to have a

reddish (Whitsell, 1952) or whitish appearance (Wallace, 1952) and to be frequently below and in line with the leaf petiole. While on some trees these symptoms are pronounced and widespread, others have only a few (Horne and Parker, 1931).

2. Symptoms on the fruit: Streaks somewhat similar to those on the green stem appear on the fruit, being more pronounced from the stem end to the middle of the fruit (Horne et al., 1941; Whitsell, 1952). When fruit of the darker varieties colour up the streaks remain lighter than the surrounding skin, appearing as red or purple depressions. Haas (1938) reported that when Fuerte avocado fruit with external symptoms are cut open the pulp darkens quicker than unaffected fruit.

Not all the fruit on a diseased tree display symptoms, but affected fruit is not marketable (Horne et al., 1941).

3. Symptoms on the leaves: Leaf symptoms in the field are rare, but infected trees sometimes produce clusters of leaves showing white variegation (Wallace, 1958). The white areas expand less than the green so that the leaves become deformed (Horne et al., 1941). Wallace and Drake (1956) reported that seedlings which develop from immature seed frequently have white, poorly developed leaves, closely resembling sunblotch. However, tests conducted on these plants proved that this condition, called 'albinism', is unconnected with sunblotch. Earlier, albinism had been confused with the disease (Haas, 1952).

4. Symptoms on older portions of the tree: In contrast to the smooth bark of healthy trees, the bark of affected trees shows a rectangular cracking, giving it a very rough appearance (Whitsell, 1952). These cracks begin to appear about three years after inoculation (van Vuuren, S.P., personal communication). Affected branches have a deficient development of their woody tissue (Horne et al., 1941). They are weak, bend easily, and the tree develops a sprawling, bushy and decumbent style of growth.

Olson, Maxwell and Cooper (1958) reported a bud-perpetuated rootstock disorder in Texas, which they called 'Azteca disease', characterised by deep grooves and cracks in the bark of West Indian rootstocks. Later this was shown by indexing to be caused by the sunblotch agent (Olson, 1963). In South Africa, Pretorius (1972 a ; 1972 b ; 1973) described a condition very similar to Azteca affecting Guatemalan rootstocks. He also found that in trees with rough scion bark there was an accumulation of starch in the rootstock. Later he reported a correlation between cracking of the scion bark and sunblotch fruit symptoms (Pretorius, 1972 c), confirming an earlier observation by Whitsell (1952).

5. 'Recovery' growth and symptomless carriers: Frequently a tree with sunblotch symptoms produces one or more apparently normal and vigorous shoots (Horne et al., 1941; Wallace, 1967). Despite the absence of symptoms, indexing tests have shown the agent to be present in these branches. Parker and Horne (1932) reported that symptomless shoots growing out of stems with symptoms all developed mild symptoms after two to three years. This may not have been the same as the 'recovery' growth described above which, according to Wallace (1967), never develops any symptoms.

The first mention of a symptomless carrier tree was by Stevens (1939). He reported that when apparently healthy Taft avocado grafted to sunblotch-free West Indian avocado seedlings, were later topworked with Nabal and Taylor from 'reliable' disease-free sources, the trees all developed sunblotch symptoms. The Taft was suspected of being a symptomless carrier. Coit (1949) described a similar phenomenon also involving Taft, and since then symptomless carriers of other avocado cultivars have been recorded (Wallace and Drake, 1953; Wallace, 1958; Schroeder and Frolich, 1958; Wallace and Drake, 1962; Wallace, 1967). Normal indexing procedures proved the presence of the pathogen in these trees.

TRANSMISSION

1. Transmission by grafting and budding: Parker and Horne (1932) were the first to demonstrate that sunblotch could be transmitted by grafting or budding. Symptoms can develop from three months to two years after inoculation (Wallace, 1958). Drake and Wallace (1973) have also successfully transmitted sunblotch by embryo grafting. Infected sunblotch stem tissue was cut to fit wedges made in the cotyledons of half-seeds with thinned points reaching into the centre of the seeds. Twelve of the 21 resulting seedlings developed symptoms.

2. Seed transmission: Parker and Horne (1932), following their successful transmission of sunblotch by grafting, planted seed from trees showing symptoms but could observe no symptoms in the resultant seedlings. Horne et al. in 1941, however, reported sunblotch symptoms in unbudded seedling trees. In the absence of any indication of an insect vector, Zentmyer (1946) and Wallace (1950) concluded that seed transmission does occur.

The first experimental proof of seed transmission involved seed taken from symptomless carrier trees (Wallace and Drake, 1953). The seedlings remained symptomless initially even after cutting back. However, when

sunblotch-free scions were grafted onto other seedlings from the same source, a large percentage of the scions developed symptoms within 18 months. It was concluded that the agent had passed through the seed.

A clearer understanding of seed transmission has since emerged (Wallace, 1958; Wallace and Drake, 1962). There appear to be two types of seed transmission. One, of rare occurrence, is the type that earlier studies had attempted to find involving seed from trees showing symptoms (Parker and Horne, 1932; Horne et al., 1941). Of the seedlings from such trees, from zero to about five per cent develop symptoms of the disease, the rest being truly sunblotch-free. The other type involves the seed of symptomless carriers; up to 100% of such seedlings are in turn symptomless carriers, but when used as rootstocks, typical sunblotch symptoms develop in the scion.

The reason for the two types of seed transmission is not known, but Wallace and Drake (1962) suggest that it may be related to the concentration of the causal agent in the parent tree. The incubation time in transmission tests is longer for symptomless carriers than for material with symptoms, indicating that there may be a lower concentration of the agent in the former. In the case of trees showing symptoms, the authors suggested that a higher concentration of the causal agent may damage host tissue thus preventing entry of the agent into the seed. Conversely a low concentration could lead to the absence of symptoms in symptomless carriers. However, Thomas and Mohamed (1979) suggest that the sunblotch agent may be more concentrated in symptomless carriers since they could more easily detect the small molecular weight RNA in this tissue than in material with symptoms. However, recent evidence (Semancik and Desjardins 1980) suggests that this RNA detected by Thomas and Mohamed (1979) is not the viroid RNA, but rather an associated species.

Wallace (1967) has suggested a possible origin of the symptomless carrier, namely the 'recovery' growth, the seedlings from such branches being all infected but symptomless, i.e. symptomless carriers.

3. Pollen transmission: Since seed transmission is now well documented, attention has turned recently to the possibility that the agent could be introduced into the seed through pollen. Desjardins, Drake, Atkins and Bergh (1979) enclosed a healthy avocado tree in an insect-proof cage with two symptomless carriers and two symptom bearing plants, and then introduced a colony of honey bees. A low percentage (1,8 - 3,125%) of

seedlings from the pollen recipient tree exhibited symptoms of sunblotch, the disease being verified by indexing.

4. Mechanical transmission: Early attempts to transmit sunblotch mechanically on pruning tools (Parker and Horne, 1932; Horne et al., 1941) or by sap inoculation (Horne et al., 1941; Boyce, 1956; Wallace and Drake, 1962) were unsuccessful, although Parker and Horne (1932) reported one instance where transmission was achieved by placing macerated infected tissue under the bark of a healthy plant. Recently Desjardins, Drake and Swiecki (1980) have transmitted sunblotch from avocado to avocado by means of knife cuts.

5. Insect and nematode transmission: There is good circumstantial evidence (Zentmyer, 1946; Wallace and Drake, 1953) that insect, and presumably nematode, transmission of sunblotch does not occur, whilst Alper et al. (1975) were unable to demonstrate transmission by the nematode Xiphenema brevicolle.

INDEXING METHODS

Testing for the presence of sunblotch is very important to nurserymen, especially with respect to symptomless carriers.

There are two ways in which indexing is presently performed:

(a) Buds or strips of young bark from the tree under test are grafted onto at least ten sunblotch-free seedlings (three to five months old) of a variety that develops clear symptoms (Wallace, 1958; Burns, Drake, Wallace and Zentmyer, 1968; 1969). The seedlings are cut back, allowed to grow for six months and then cut back again. If after 12 - 18 months none of the seedlings shows sunblotch symptoms the probability is strong that the tree is not a carrier of the disease.

(b) The second method involves indexing seedlings of the tree under test (Wallace, 1958). Known sunblotch-free material is grafted onto at least 25 such seedlings and the scions are observed for 12 months or more for symptom development.

As both the above techniques can take more than a year to yield results, Schwarz and van Vuuren (1970) conducted a study to determine whether indexing for sunblotch could be based on the presence of a specific phenolic marker, as is the case with citrus greening disease (Schwarz, 1968). Some indication of a specific marker was found, but the results were not sufficiently conclusive for the technique to be recommended for indexing.

TISSUE CULTURING

Meristem-tip culturing has proved a useful method of freeing certain plants of viruses. However, all attempts to produce avocado plants from stems, leaf petioles and cotyledons have been unsuccessful (Desjardins, 1958; Schroeder, 1972). Some citrus viruses and viroids can now be eliminated by shoot-tip grafting (Roistacher, Navarro and Murashige, 1976), and an attempt has been made to use this technique to free a potentially important avocado rootstock cultivar, 'Huntalas', of sunblotch (Desjardins and Drake, 1979). A few plants were produced but they did not survive transplanting (Desjardins and Drake, 1980).

EVIDENCE FOR THE VIROID NATURE OF SUNBLOTCH

Alper et al. (1975) observed virus-like particles in two parenchyma cells of the bark during an electron microscope study, but because of their low frequency concluded that these were not the causal agent. They were also unable to isolate any virus particles.

Recently, evidence indicating that the causal agent may be a viroid was reported simultaneously by Thomas and Mohamed (1979) and Dale and Allen (1979). Both groups, using PAGE, detected a small species of RNA in infected tissue that was absent in healthy. The former authors detected it in symptomless carrier leaf material and in bark tissue with symptoms, but not in leaves from plants with symptoms. Dale and Allen (1979) detected it in both types of leaf tissue.

Palukaitis, Hatta, Alexander and Symons (1979) have since reported that this RNA species is a covalently closed circular molecule, smaller than citrus exocortis and chrysanthemum stunt viroids. They also found that hybridisation with ^{32}P-labelled complementary DNA was the most sensitive method for detection Almost simultaneously further details were reported by Mohamed and Thomas (1980) who estimated the molecular weight to be 65 000 daltons and, in fractionation studies, found that the RNA is associated with the chloroplasts and endoplasmic reticulum.

The observation by Desjardins et al. (1980) that mechanical transmission is associated with low molecular weight fractions showing some RNase sensitivity has provided strong support to the viroid hypothesis.

Semancik and Desjardins (1980) have since detected five low molecular weight RNA species in extracts of infected tissue, ranging from 61 000 to 185 000 daltons, using ethidium bromide to stain the gels instead of toluidine

blue as used by the other groups. They suggest that the smallest species (ASV_5), which coincides with the single species reported by previous workers, may be a non-infectious viroid-associated RNA, similar to those associated with cadang-cadang of coconuts (Randles, 1975) and citrus exocortis (Semancik and Desjardins, 1980), and that the larger three (ASV_{1-3}) are aggregates of it. The fourth species, ASV_4 , they regarded as most likely to be the presumed viroid.

It is thus highly likely that the avocado agent is a viroid, but final proof awaits the successful transmission of the isolated agent to healthy avocado. Such tests have been initiated (Mohamed and Thomas, 1980; Semancik and Desjardins, 1980).

AIMS OF THE PRESENT STUDY

This study on avocado sunblotch disease was initiated in 1975, and had two basic aims, the one practical and the other more academic.

The first was the development of more rapid methods of indexing for sunblotch to serve in support of an urgently needed avocado improvement scheme for South Africa, similar to the citrus programme then already in operation (von Broembsen and da Graça, 1976; von Broembsen, da Graça, Lee and Waller, 1978). Under such a programme avocado nurserymen will require sources of sunblotch-free scion and rootstock material in order to produce certified trees. A rapid indexing method would be of great value in this regard.

When the study was initiated, little was known about the biochemical and ultrastructural effects of sunblotch on its host, whilst the nature of the causal agent was uncertain. The second aim, therefore, was to attempt to close some of the gaps in these aspects of our knowledge of sunblotch.

Contact with research workers in California (U.S.A.), Israel and New Zealand was maintained during the study, and material was sent to New Zealand to aid their research (Thomas and Mohamed, 1979 ; Mohamed and Thomas, 1980).

CHAPTER 1

GENERAL STUDIES ON AVOCADO SUNBLOTCH DISEASE

1.1 INTRODUCTION

Despite avocado sunblotch having been known for some decades the only aspects to have received much attention have been symptomology, graft and seed transmissibility, and more recently, viroid research. Several other aspects of the disease have not yet been studied, and certain of these were therefore investigated.

Apart from the external symptoms, the only other reported effect of sunblotch on the avocado fruit is an increased rate of darkening of cut infected fruit compared with healthy (Haas, 1938). It was also noted by the present author during a seed collection exercise that fruit from symptomless 'recovery' growth branches appeared to be larger than those from branches with symptoms. Studies were therefore initiated into the effect of sunblotch on fruit size and fruit maturation.

Due to the difficulty in transmitting sunblotch mechanically such properties as thermal inactivation, dilution end point and longevity *in vitro* have not been reported. Therefore to obtain an indication of the thermal sensitivity of the causal agent, thermal inactivation *in vivo* was tested.

It was frequently also noted by the present author that transmission of sunblotch occurred even when the grafted tissue died. An experiment was therefore initiated to establish the graft contact time for the pathogen to move into the test plant. Recently Desjardins *et al.* (1980) have established that sunblotch can be mechanically transmitted.

No host other than avocado has been reported for sunblotch, although unsuccessful efforts to transmit the disease to other plant species, especially herbaceous, have been made (Alper *et al.*, 1978). It seemed probable that the most likely additional hosts would be found amongst plants closely related to the avocado, i.e. the family Lauraceae. One of these relatives is the false dodder, *Cassytha* (Schroeder, 1967), which occurs naturally on the avocado (Schroeder, 1978). Attempts were therefore also made to use this plant as a vector for sunblotch.

The standard method of indexing for avocado sunblotch is to graft buds from suspect plants onto healthy avocado seedlings of the cultivar Hass (Burns *et al.*, 1968; 1969). This is usually done in screenhouses where

temperatures are uncontrolled, and although symptoms can appear within a few months they often take one to two years to develop (Wallace, 1958). Two further cultivars were therefore tested as possible indicators. The effects of controlled glasshouse temperatures and timing of cutting back on the rate of symptom development were also studied.

1.2 MATERIALS AND METHODS

1.2.1 Fruit Studies

1.2.1.1 Size and oil determinations

Fifty Edranol avocado fruit of each of the following categories were picked at the start of the harvesting season:

(i) Fruit from apparently healthy trees;
(ii) fruit with sunblotch symptoms;
(iii) blemish-free fruit from branches with symptoms; and
(iv) fruit from 'recovery' growth branches.

Fruit mass was determined, and the length and diameter across the widest part of each fruit, measured.

Oil content was determined using the method described by Swarts (1976). Five fruit from each category were used and each was bisected along a longitudinal axis to yield ten half-fruit replicates per fruit category. Lateral cuts (5 mm deep and 4 mm apart) were made on one side of the exposed surface after removal of the skin. A thin slice was then taken and the resultant blocks of flesh collected in a petri dish. This was repeated until 100 g of each replicate had been collected. These were then oven dried (70° C) to constant weight. Moisture content was then calculated. Swarts (1976) reported a very close correlation between oil and moisture content of avocado fruit, the sum of these being remarkably constant for a given cultivar. The oil content was therefore determined by subtracting the moisture content from 90,9 , the constant for Edranol.

1.2.1.2 Pectinesterase determinations

Because of the differences detected in oil content it was decided to investigate pectinesterase activity as a possible measure of fruit maturity. The method used to determine milliequivalents of ester

hydrolysed per min per g [(PE.u) g] was based on those described for citrus fruit (Rouse and Atkins, 1955; Robertson, 1976).

Ten grams of pulped fruit flesh of each of the four fruit categories described in 1.2.1.1 above were added to 20-mℓ aliquots of 1% citrus pectin in 0,1 M NaCl previously adjusted to pH 7,5 with 0,1 N NaOH . The pH was then re-adjusted to 7,5 . For 30 min temperature was maintained at 30° C and pH at 7,5 , the latter by titrating against 0,02 N NaOH. Enzyme activity was then calculated as follows:

$$(PE.u)g = \frac{m\ell \text{ NaOH} \times \text{normality}}{\text{mass} \times \text{time}}$$

$$= \frac{m\ell \text{ NaOH} \times 0,2}{10 \times 30} \quad ,$$

and expressed as $(PE.u)g \times 10^4$.

1.2.2 *In vivo* Thermal Inactivation and Rate of Movement into Indicator Plants

Pieces of young twigs 10 cm long and 1 cm in diameter cut from branches of a sunblotch-infected Edranol avocado tree showing symptoms, were placed in a steam bath set at various temperatures in the range 46° C to 56° C for various times. In a follow-up experiment temperatures ranging from 50° C to 90° C were used. Residual infectivity after treatment was tested by grafting bark strips from the treated twigs onto healthy Edranol seedlings. Three bark strips were grafted onto each of three seedlings, thus testing nine heated strips per treatment. The plants were immediately cut back and the new growth observed for symptoms over a two-year period. Cutting back was repeated every six months. In this experiment, and all others reported in this chapter, cutting tools were sterilized between samples with 10% commercial bleach solution containing sodium hypochlorite (Roistacher, Blue and Calavan, 1969).

In the experiment to determine the rate of movement of the sunblotch agent from a bark strip into the indicator host, seedlings were each inoculated with three infected bark strips. After intervals ranging from one to 21 days the strips were removed, and the plants watched for symptom development over two years.

1.2.3 Host Range

The following members of the family Lauraceae were tested as possible hosts of sunblotch:

Persea Schiedeana Nees ..... - Coyo
P. indica Spreng. .....
Cinnamomum zeylanicum Nees .. - Cinnamon
C. camphora Nees & Eberm. ... - Camphor
Ocotea bullata E. Mey. ..... - White stinkwood
Cryptocarya liebertiana Engl. - Wild quince

Between five and eight seedlings, or in the case of P. Schiedeana grafted plants, of each of the above species, were graft inoculated with three bark strips from a sunblotch-infected Edranol avocado tree. The plants were then observed for the development of any disease symptoms. After two years all plants were tested for sunblotch by grafting bark strips onto healthy Collinson seedlings. Subsequently, in a repeat attempt to transmit sunblotch to camphor three further seedlings were graft inoculated from avocado.

After confirmation of the transmission of sunblotch to cinnamon (see Results) attempts were made to transmit the disease from this host to C. camphora, O. bullata and C. liebertiana.

Specimens of false dodder (Cassytha filiformis L.) were established on four sunblotch-infected avocado seedlings, and were then trained across onto four healthy avocado seedlings so that each infected plant was linked to a diseased plant via the phanerogam.

False dodder growing on infected avocado seedlings were also trained onto plants of the following species:

Citrus jambhiri Lush. ..... - Rough lemon
C. aurantium L. ..... - Sour orange
Vinca rosae L. (Catharanthus roseus) - Periwinkle
Chenopodium quinoa Wild.
Datura stramonium L.

Attempts were made to establish true dodder plants (Cuscuta sp.) onto infected avocado seedlings, but their growth on the latter was very poor and it was not possible to conduct any transmission tests.

1.2.4 Improvement of Current Indexing Techniques

1.2.4.1 Comparison of three cultivars

Hass has been recommended as the most suitable indicator for sunblotch (Burns et al., 1968; 1969). For some experiments in this study, however, insufficient Hass seedlings were available and it was therefore decided to test by comparison with Hass two other cultivars, Fuerte and Collinson, as possible indicators.

Three sources of sunblotch inoculum were used: Edranol branches with symptoms, Edranol 'recovery' growth branches and shoots of the Mexican rootstocks of these trees. The last two are symptomless carriers. Each inoculum source was inoculated to eight seedlings of each cultivar, each seedling receiving three bark pieces. The plants were kept in a warm glasshouse (29 ± 2° C) and observed at approximately weekly intervals for symptom development during one year.

1.2.4.2 Response of Collinson to temperature

Because of enhanced rate of symptom development obtained in the above experiment compared with indexing conducted under uncontrolled temperature conditions, a further experiment was initiated to determine the influence of glasshouse temperature on symptom development. In addition the effect of different cutting-back times and changes in temperature were investigated.

Ninety three-month-old Collinson seedlings were used as indicators; 45 were placed in a glasshouse set at 30/28° C (day/night) and the remaining 45 in a cooler glasshouse at 20/18° C (day/night). The same three sunblotch sources described above in the previous experiment were used. Three bark strips of each of the source materials were grafted to each of 15 indicator plants in each temperature regime. After grafting, all seedlings of the six groups (three inoculum sources, two temperature regimes) were immediately cut back. After three months the new growth of 10 plants in each group was cut back, and half of these plants transferred from 30° C to 20° C and vice versa. Of the remaining 30 plants not cut back at three months, those that had not developed symptoms by six months were then cut back, whilst remaining at the same temperature regime. With only minor deviations, inspections were conducted on a weekly basis for symptom development over a period of one year.

1.2.4.3 Response of Hass to temperature

To test the response of Hass seedlings as indicators to the different temperatures and cutting-back times, a similar experiment to the one described in 1.2.4.2 above was initiated. The sources of inoculum and

number of replicates were the same as noted in 1.2.4.2, and the only change made in the treatments was the omission of the temperature transfers.

An attempt to test Fuerte seedlings in a similar experiment was unsuccessful because of the poor growth of the plants at the higher temperature.

1.3 RESULTS

1.3.1 Fruit Studies

1.3.1.1 Size and oil determinations

Figure 1.1 shows representative examples of the four fruit categories.

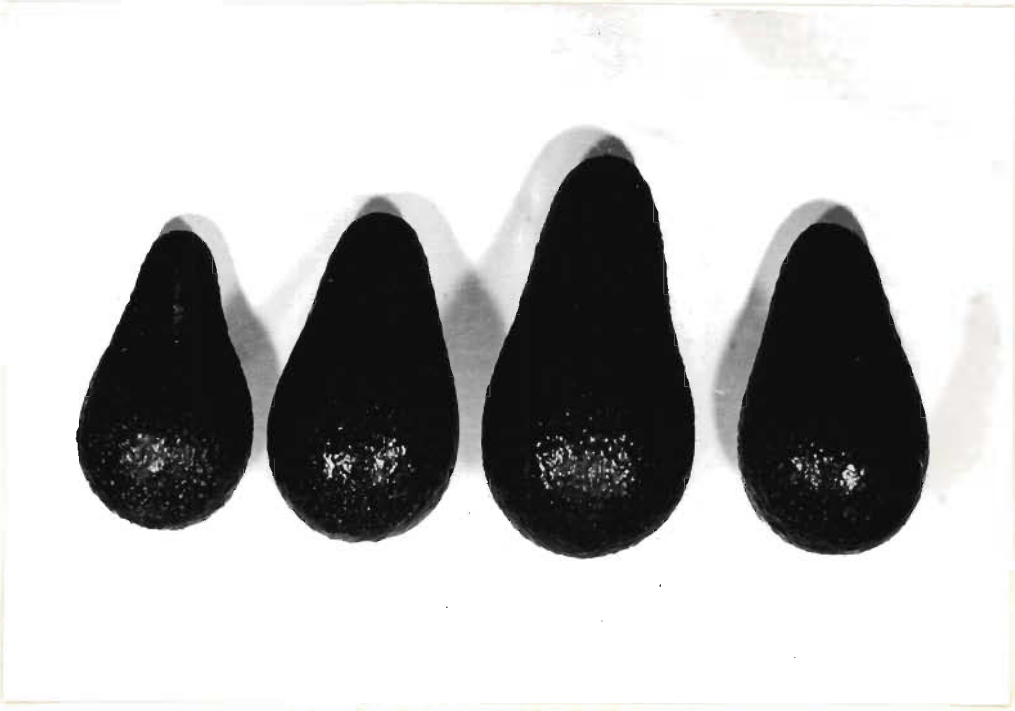


FIGURE 1.1 Edranol avocado fruit. Left to right: fruit with sunblotch symptoms, blemish-free fruit from a branch with symptoms, 'recovery' growth fruit and fruit from an apparently healthy tree

The mean mass and size of fruit produced on 'recovery' growth branches significantly exceeded those of the other fruit categories, whilst fruit showing symptoms were significantly smaller than either symptomless fruit from branches with symptoms or apparently healthy trees (Table 1.1). No differences

between healthy and blemish-free fruit were found.

TABLE 1.1 Effect of sunblotch on the mass and size of Edranol avocado fruit

Fruit Category	Mean mass (g)	Mean length (mm)	Mean diameter (mm)
Sunblotch symptoms	269,9a	114,7a	72,2a
Apparently healthy	315,9b	125,6b	74,5b
Blemish-free, diseased	315,5b	124,4b	74,9b
'Recovery' growth	418,3c	147,3c	80,9c

Different letters indicate significance at the 1% level.

The oil content determination similarly showed that fruit from 'recovery' growth branches had a significantly higher oil content than did the other fruit types tested (Table 1.2).

TABLE 1.2 Effect of avocado sunblotch on the oil content of fruit

Replicate	PERCENTAGE OIL CONTENT			
	Sunblotch symptoms	Apparently healthy	Blemish-free (diseased)	'Recovery' growth
1	15,7	16,9	17,1	22,7
2	14,0	18,3	14,3	20,9
3	16,9	16,5	15,5	22,7
4	11,9	17,1	17,3	20,9
5	16,7	18,1	15,7	21,3
6	14,7	15,7	15,9	19,3
7	19,5	16,1	14,7	21,1
8	16,7	19,3	16,3	20,7
9	16,1	14,9	15,5	20,3
10	15,9	15,3	14,1	21,7
Mean	15,81a	16,82a	15,64a	21,16b

Different letters indicate significance at 1% level.

1.3.1.2 Pectinesterase determinations

The highest pectinesterase activity was also found in fruit from 'recovery' growth, whilst the apparently healthy fruit had significantly lower activity (Table 1.3).

TABLE 1.3 Pectinesterase activity in apparently healthy and sunblotch-infected fruit

Replicate	PECTINESTERASE ACTIVITY [(PE.u) g x 10^4]			
	Sunblotch symptoms	Apparently healthy	Blemish-free (diseased)	'Recovery' growth
1	6,46	3,66	7,53	7,73
2	7,06	4,19	6,13	10,19
3	6.66	5,26	7,06	8,92
4	5,53	3,46	4,99	7,13
5	5,99	4,46	9,06	10,92
Mean	6,34a	4,21b	6,95a	8,98c

Different letters indicate significance at 5% level.

1.3.2 Thermal Inactivation and Rate of Movement into the Indicator

The sunblotch agent was found to be inactivated *in vivo* by briefly treating tissue for 15 minutes at 56° C. Lengthier treatment for 120 minutes at 48° C also inactivated it (Table 1.4). In only one case, viz. 50° C for 15 minutes, did the treated inoculum tissue survive to graft successfully.

The results of the test to determine the time taken for the agent to move into the indicator plant from the inoculum bark pieces showing symptoms are given in Table 1.5. Where bark strips were left in position for two or more weeks, symptoms developed in the indicators.

TABLE 1.4 Thermal inactivation of avocado sunblotch in detached twig tissue

EXPERIMENT I			EXPERIMENT II		
Temp. (°C)/Time (min)	No. +/4	Graft survival	Temp. (°C)/Time (min)	No. +/4	Graft survival
Control	4	0	Control	4	0
46/150	4	0	50/120	0	0
46/120	4	0	50/15	3	1
48/120	0	0	55/60	0	0
48/90	4	0	55/30	0	0
50/90	2	0	55/15	1	0
50/60	3	0	60/60	0	0
52/60	0	0	60/30	0	0
52/30	4	0	60/15	0	0
54/30	0	0	65/30	0	0
54/15	3	0	65/15	0	0
56/15	0	0	70-90/15	0	0

TABLE 1.5 Effect of varying contact time between sunblotch-infected inoculum tissue and indicator seedlings

Removal time of inoculum tissue (days)	No. +/3
1	0
2	0
3	0
8	0
10	0
14	2
21	2

1.3.3 Host Range

Two of the species graft inoculated with sunblotch from avocado, Cinnamomum zeylanicum and Persea Schiedeana, developed symptoms similar to those on sunblotch-infected avocado.

Seven months after inoculation one of the cinnamon seedlings developed yellow depressed streaks on the stem of a side shoot. Two months later a second cinnamon plant developed streaks. The stems of both plants later developed yellow blotches and a lumpy appearance (Fig. 1.2). The avocado seedlings inoculated with bark tissue from these two plants developed typical sunblotch symptoms within 12 months.

FIGURE 1.2 Cinnamon seedling with avocado sunblotch disease. Shoot on the right has depressed streaks on the stem (arrows) and a lumpy appearance

Two P. Schiedeana plants developed yellow depressed streaks on the main stem 10 months after inoculation (Fig. 1.3), and back inoculation to avocado indicator seedlings confirmed the presence of sunblotch.

FIGURE 1.3 Persea Schiedeana plant with stem symptoms of avocado sunblotch disease. Yellow streak indicated by the arrow

Of the avocado seedlings inoculated from the remaining four related species under test, one inoculated from an Ocotea bullata and another from a Cinnamomum camphora developed typical sunblotch symptoms. The O. bullata showed leaf curling and twig die-back (Figs. 1.4 and 1.5), but the camphor seedling remained symptomless. One of the three camphor seedlings inoculated in the subsequent experiment developed a light pink, depressed stem streak five months after inoculation (October 1980). This plant has not yet been indexed. None of the plants inoculated with infected cinnamon tissue developed symptoms.

FIGURE 1.4 Healthy Ocotea bullata leaf (left) and four curly leaves from a seedling infected with sunblotch

FIGURE 1.5 Twig die-back and curly leaves on Ocotea bullata seedling with sunblotch

In the false dodder experiment, one of the indicator avocado seedlings developed characteristic symptoms of sunblotch four months after being linked to an infected avocado via the phanerogam. The remaining four plants were still symptomless 16 months after the experiment had begun. No obvious disease symptoms developed on the false dodder. None of the other plant species tested developed any disease symptoms.

1.3.4 Improvement of Current Indexing Techniques

Comparing the three cultivars Hass, Fuerte and Collinson, as indicators of sunblotch, no differences in incubation period were found where symptomless carrier sources, i.e. 'recovery' growth and rootstock shoots, were used as inoculum (Table 1.6). However, where the indicators were inoculated from branches with symptoms, the Hass and Collinson seedlings developed symptoms far more rapidly than did the Fuerte. The mean symptom development time for Fuerte was approximately the same for each sunblotch inoculum source. To determine whether the misses (see Table 1.6) could influence the interpretation of the results, they were given the value of 366 days, and the means were recalculated. The overall picture remained the same.

The effects of glasshouse temperature and time of cutting back on symptom appearance in Collinson seedlings is shown in Table 1.7.

All 30 of the plants kept continuously at 30/28° C developed symptoms, 28 of them within eight months. Cutting back the plants three months after inoculation caused those inoculated with the symptomless carrier sources to develop symptoms more rapidly than those cut back at six months, but it slowed down development in the indicator plants inoculated from branches with symptoms.

Only two of the plants kept continuously in the cool glasshouse developed symptoms, and these only towards the end of the experiment. Of the plants transferred to or from the higher temperature after cutting back began to develop symptoms, mostly from the samples with symptoms.

The results of a similar experiment using Hass indicator seedlings, but omitting transfer to the higher or lower temperature, are shown in Table 1.8 .

The above results are similar in most respects to those obtained with the Collinson seedlings. In both cases there was good symptom development in the hot glasshouse, while symptoms only began to appear in plants in the cool glasshouse towards the end of the experiment. Cutting back at three months was also found to speed up symptom appearance in indicators inoculated from

TABLE 1.6 Comparison of the incubation periods in Fuerte, Hass and Collinson seedlings inoculated from three sunblotch sources

Cultivar	Replicate	INCUBATION TIME (DAYS)[a]		
		With symptoms	'Recovery' growth	Rootstock shoot
Fuerte	1	133	247	253
	2	276	247	276
	3	276	253	282
	4	276	260	282
	5	288	276	309
	6	323	276	330
	7	323	330	337
	8	330	-	-
	Mean	278,1	269,9	295,6
Hass	1	85	247	247
	2	100	260	247
	3	119	260	253
	4	253	267	260
	5	253	267	260
	6	282	323	295
	7	288	330	323
	8	-	-	-
	Mean	197,2	279,2	269,3
Collinson	1	63	156	243
	2	84	175	261
	3	133	267	267
	4	133	267	282
	5	169	282	288
	6	-	345	288
	7	-	-	330
	8	(died)	-	338
	Mean	116,6	267,2	291,0

[a] - = no symptom development within one year, and were taken as misses.

TABLE 1.7 Effect of temperature and cutting back on the incubation period in Collinson avocado indicator seedlings inoculated from three sunblotch sources

Treatment	Replicate	INCUBATION TIME (DAYS)[a]		
		With symptoms	'Recovery' growth	Rootstock shoot
30/28° C only; 6 mth cut back	1	87	175	136
	2	87	237	156
	3	87	237	175
	4	87	333	175
	5	111	365	230
	Mean	90,0	269,4	174,4
30/28° C only; 3 mth cut back	1	143	141	143
	2	149	149	149
	3	149	149	153
	4	175	153	163
	5	175	206	181
	Mean	158,2	159,8	157,8
30/28° C 3 mth cut back; transfer to 20/18° C	1	329	329	-
	2	329	-	-
	3	365	-	-
	4	365	-	-
	5	-	-	-
	Mean	347,0	329	-
20/18° C only; 6 mth cut back	1	335	-	-
	2	365	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
	Mean	350,0	-	-
20/18° C only; 3 mth cut back	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
20/18° C 3 mth cut back; transfer to 30/28° C	1	156	-	329
	2	192	-	-
	3	192	-	-
	4	259	-	-
	5	-	-	-
	Mean	199,9	-	329

[a] - = no symptom development within one year.

TABLE 1.8 Effect of temperature on the incubation period in Hass avocado indicator seedlings inoculated from three sunblotch sources

Treatment	Replicate	INCUBATION TIME (DAYS)[a]		
		With symptoms	'Recovery' growth	Rootstock shoot
30/28° C only; 6 mth cut back	1	97	234	181
	2	174	253	203
	3	234	277	234
	4	234	277	234
	5	234	365	253
	Mean	194,6	281,2	221,0
30/28° C only; 3 mth cut back	1	160	345	160
	2	203	358	160
	3	203	365	174
	4	253	-	234
	5	253	-	234
	Mean	214,4	356,0	192,4
20/18° C only; 6 mth cut back	1	-	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-
20/18° C only; 3 mth cut back	1	356	-	-
	2	-	-	-
	3	-	-	-
	4	-	-	-
	5	-	-	-

[a] - = no symptom development within one year.

rootstock shoots, but no significant differences were found in the plants infected from the source showing symptoms. The 'recovery' growth indicators were very slow to develop symptoms in the three month cut back treatment, in contrast to the Collinson study.

1.4 DISCUSSION

Avocado fruit with sunblotch symptoms are unsightly and unmarketable and, in addition, the yellow streaks usually turn black and split open as the fruit ripens (Whitsell, 1952). This study reports that the fruit produced on 'recovery' growth branches, which are symptomless, are significantly larger than healthy fruit. However, the increased size is no advantage commercially since fruit with a diameter exceeding 80 mm are unsuitable for export (Hughes, J. P., personal communication). Another disadvantage is the higher oil content in these fruit which renders them unsuitable for long cold storage (Boyes, 1953). According to Swarts (1976), the longer that healthy fruit hang on the tree the higher is their oil content, which can rise to 35 - 40 %. In the present study the oil content of fruit picked from 'recovery' growth at the beginning of the harvest season was already over 20 %, compared to 16 % in the other fruit categories. Two further relevant observations were made in the orchard from which the samples for this study were collected:

(i) Yield from 'recovery' growth branches in terms of fruit numbers was much lower than from either branches with symptoms or apparently healthy trees; and

(ii) 'recovery' growth branches appeared to flower earlier. The latter may account for the higher oil content and apparently more mature state of the 'recovery' growth fruit.

The finding in the present study of higher PE activity in 'recovery' growth fruit picked at the start of the harvest season could similarly indicate that the fruit was in a more advanced state of maturity compared with the other fruit categories, since increased PE activity has been shown to be associated with fruit ripening in tomatoes (Hobson, 1963 and 1974; Buescher and Tighelaar, 1975) and bananas (de Swardt and Maxie, 1967). However, Zaubermann and Schiffmann-Nadel (1972) and Awad and Young (1979) have reported that PE activity in the avocado declines continuously as the fruit matures, but they offered no explanation for the observed difference in this respect between the avocado and other fruits. On the basis of this information maturation in the 'recovery' growth fruit showing the higher PE activity, could be regarded as being comparatively delayed. However, a closer examination of Zaubermann and Schiffmann-Nadel's (1972) results shows that the decrease in activity after the start of normal harvesting is moderate and in one year insignificant over three months, and that therefore PE is probably an unreliable indicator of relative states of maturity.

The *in vivo* thermal inactivation study established that avocado sunblotch was inactivated at 56° C for 15 minutes. No comparable information is available regarding the *in vivo* thermal stability of known viroids. However, *in vitro* studies have shown viroids to be very heat stable. Potato spindle tuber viroid has a thermal inactivation point in NaCl extracts of between 75 and 80° C, or in phenol-treated preparations between 90 and 100° C (Diener and Raymer, 1971). Chrysanthemum stunt viroid is similarly inactivated in sap at between 90 and 100° C (Brierley, 1952), while flaming blades contaminated with citrus exocortis viroid failed to cause inactivation (Roistacher *et al.*, 1969). However, high thermal stability may not be a characteristic of all viroids, and also the heat stability of the avocado sunblotch agent may well be greater in the extracted form.

The inoculum tissue survived to effect a graft union in only the mildest treatment of 50° C (Table 1.4), while the sunblotch agent was inactivated at 56° C. Thus there appears to be little chance of eliminating the pathogen in plant tissue by heat therapy. Desjardins *et al.* (1980) similarly commented recently that the sunblotch agent could withstand any heat treatment that the avocado tissue could.

The successful infection of the indicator plants despite death or removal of inoculum tissue (Tables 1.4 and 1.5) affords strong evidence that sunblotch should be mechanically transmissible. However, several attempts by the present author at mechanical transmission by knife cuts have failed. Recently Desjardins *et al.* (1980) reported a low percentage of transmission using the razor-slash technique.

The successful graft transmission to cinnamon appears to be the first instance of sunblotch infection of a plant species other than the avocado. The other three species found to be susceptible, *Persea Schiedeana*, *Cinnamomum camphora* and *Ocotea bullata*, are all members of the same family as the avocado, namely Lauraceae. Unfortunately none developed symptoms more rapidly than the avocado and all are therefore not to be preferred as indicator species. The non-infection of *P. indica* and *Cryptocarya liebertiana* does not necessarily reflect that they are non-hosts. Transmission to the other species occurred at a fairly low rate, and hence transmission to these two species might well have occurred had more plants been available for experimentation.

The transmission of sunblotch from avocado to avocado by the false dodder raises the possibility of transmitting the pathogen by this means to other plants, especially herbaceous plants. Attempts by this author to do this were, however, not successful. Possibly the false dodder is an inefficient vector

of sunblotch, as only one of the four avocado plants tested became infected.

The experiments conducted to improve current indexing procedures showed the following:

(i) Collinson seedlings are as suitable as Hass which is the variety recommended in California (Burns et al., 1968; 1969). Collinson has the advantage of earlier maturing fruit (Rounds, 1950). In the present study many of the Hass seedlings grew poorly, and this could possibly account for the slightly different results obtained between the Hass and the Collinson.

(ii) Indexing at 30/28° C (day/night) yields results far more quickly than at lower temperatures, enabling the test to be completed in eight months. Fuerte, which is a frost resistant cultivar (Chandler, 1964), is not suited to these high temperatures.

(iii) Cutting back indicators of symptomless carriers three months after inoculation, instead of at six months, generally results in quicker symptom development.

Until such time as a quick laboratory technique for sunblotch indexing becomes acceptable, e.g. PAGE detection of a viroid causal agent, incorporation of the above improvements into current indexing procedures is recommended, and has been adopted by the CSFRI, Nelspruit (van Vuuren, S. P., personal communication). These would also prove useful in establishments not equipped for sophisticated analyses.

The observation that symptoms develop more rapidly in indicators inoculated from branches with symptoms than from symptomless carriers confirms the earlier finding by Wallace and Drake (1962). These authors suggested that there may be a lower concentration of the pathogen in symptomless carrier tissue. Another possible explanation for the symptomless condition could be that tissue is invaded before it is sufficiently mature to be susceptible to injury, as appears to be the case with curly top virus in tobacco (Benda and Bennett, 1964).

CHAPTER 2

BIOCHEMICAL EFFECTS OF AVOCADO SUNBLOTCH DISEASE

2.1 INTRODUCTION

Changes in the phenol metabolism of many infected plants have been studied and interpretations made as to their significance in resistance. This information has been brought together in reviews by several authors (Cruickshank, 1963; Kosuge, 1969; Kuć, 1972).

In the only study to date in this field on avocado sunblotch, Schwarz and van Vuuren (1970) reported the detection by thin layer chromatography of an increased level of an unidentified phenol in the young bark of sunblotch-infected trees.

As this work has never been followed up, a study was initiated on changes in phenol metabolism of sunblotch-infected avocados, primarily with the aim of developing a rapid laboratory method for detecting trees symptomlessly infected with the disease. The areas chosen for study, which are well documented for a wide range of other host/pathogen combinations, were total soluble phenols and individual phenol make-up, anthocyanin/leucoanthocyanin levels, activities and isoenzyme patterns of peroxidase (PO), polyphenoloxidase (PPO) and indoleacetic acid (IAA) oxidase, and phenylalanine ammonia-lyase (PAL) activity. Total soluble protein was also analysed since it is necessarily determined during enzyme studies. Water soluble phenol levels and cation status were also determined as they have been found useful in diagnostic studies on citrus blight (young tree decline) (Wutscher, Cohen and Young, 1977).

2.2 MATERIALS AND METHODS

2.2.1 Phenols

2.2.1.1 Bark total soluble phenols

The method followed was based on one developed by Pirie and Mullins (1976) for grape tissues. Half-gram samples of the following avocado bark samples were homogenised with an Ultra Turrax macerator in 15 mℓ methyl alcohol-HCl (50% methyl alcohol + 0,5 mℓ HCl; pH adjusted to 1,0 with HCl):

(i) Young bark of two-year-old Hass seedlings:

(a) healthy tissue;
(b) yellow streak tissue;
(c) green tissue adjacent to yellow streak; and
(d) green tissue distant from yellow streak.

(ii) Young bark of two-year-old Edranol seedlings:

(a) healthy tissue; and
(b) symptomless carrier tissue.

(iii) Mature bark of 15-year-old Edranol trees:

(a) healthy tissue;
(b) tissue with rough bark symptoms; and
(c) symptomless 'recovery' growth tissue.

The homogenate was centrifuged at 13 000 g for 20 minutes in a Sorvall SS-34 rotor. The supernatant was poured off and made up to 25 mℓ . This was diluted 1 : 10 , and the absorbance at 280 nm was read in a Beckman DK-2A spectrophotometer. The readings were converted to µM gallic acid from a standard curve.

2.2.1.2 Bark and wood total water soluble phenols

The method used was based on that described by Wutscher et al. (1977). Bark samples weighing approximately 5 g were collected from five Edranol trees showing both typical sunblotch symptoms as well as 'recovery' growth (Fig. 2.1), and also from five healthy trees. Wood samples were collected from the same trees by drilling two opposite, 2,5 cm deep holes into each tree. All samples were dried in open vials at 70° C for 24 hours, after which 100 mg of each dried sample was shaken in 10 mℓ deionised water for 2 hou diluted ten times, and the absorbance read at 280 nm in a Beckman DK-2A spectro photometer.

FIGURE 2.1 Branch of a sunblotch-infected Edranol avocado tree displaying rough bark symptoms and symptomless 'recovery' growth

2.2.1.3 Bark and leaf phenol identification

Ibrahim and Towers (1960) described a method for the recovery and identification of plant phenolic acids, and this method was used in this study on the effect of sunblotch on the cultivar Edranol.

Ten-gram samples of mature bark, young bark and leaf tissue from healthy trees, trees with rough bark symptoms, and 'recovery' growth branches on infected trees (Fig. 2.1), as well as young bark from healthy and infected seedlings with symptoms were each homogenised in 20 mℓ 80% ethyl alcohol with an Ultra Turrax macerator, and boiled and refluxed for 2 hours. The extracts were then filtered through a Büchner funnel and washed with 100% ethyl alcohol. The extract was dried under reduced pressure in a Büchi Rotavapor-R rotary evaporator at 40° C and then dissolved in about 45 mℓ distilled water. For alkaline hydrolysis, 30 mℓ 2N NaOH was added, shaken for 2 minutes and left overnight in a nitrogen atmosphere.

The phenols were then ether extracted. For this purpose the pH of the hydrolysed mixture was first adjusted to pH 2,0 with concentrated HCl. Seventy millilitres of diethyl ether was placed in the bottom flask of a continuous extractor and extraction was performed at a setting of 30° C for 2 hours. The ether extract obtained was shaken with 50 mℓ 5% $NaHCO_3$ for 2 minutes, allowed to separate and the aqueous fraction collected. After adjusting the pH to 2,0 the ether extraction was repeated. The final extract was taken to dryness and dissolved in 1 mℓ absolute alcohol and stored in a deep freeze.

The samples were all found to contain a resinous material which caused problems during chromatography. To reduce the amount of this material the alcohol was evaporated off, the samples dissolved in distilled water, centrifuged at 8000 g for ten minutes, shaken with 20 mℓ ethyl acetate, avaporated to dryness and dissolved again in absolute alcohol.

The phenols were then separated by two-dimensional descending chromatography using large sheets of Whatman No. 1 chromatography grade paper. Tests determined that 100 µℓ of sample was suitable for spotting. The solvent used for the first direction was the upper phase of benzene : acetic acid : water (6 : 7 : 3), whilst for the second direction 2% aqueous formic acid was used. After drying the sheets were examined under the ultraviolet light of a CAMAG Reprostar at 254 and 366 nm.

To aid in identifying the phenols, solutions of the following standards were each spotted on chromatography paper: sinapic acid, ferulic acid, caffeic acid, p-coumaric acid, aesculetin, p-hydroxybenzoic acid, gentisic acid, vanillic acid, α-resorcyclic acid and protocatechuic acid.

The most strongly fluorescing spots of the mature bark extracts were cut out and eluted in 5 mℓ distilled water and their absorbance read in a Beckman DK-2A spectrophotometer.

Duplicate sheets were sprayed with diazotised p-nitroaniline (Ibrahim and Towers, 1960).

2.2.1.4 Leaf total anthocyanins/leucoanthocyanins

Van Lelyveld, Brodrick and Bester (1979) detected changed levels of anthocyanin and leucoanthocyanin in the leaves of avocado trees displaying symptoms of Phytophthora root rot. It was therefore decided to determine, using the cultivar Edranol, whether sunblotch infection has a significant effect on endogenous levels of these compounds. The method of assay was based on that described by Mulder (1977).

Symptomless avocado leaves from healthy branches, branches with bark symptoms and 'recovery' growth branches on infected trees were picked, washed and powdered in liquid nitrogen. A 10 g amount of each leaf powder material was macerated in 100 mℓ methyl alcohol, filtered through a Millipore filter and washed with 50 mℓ 50% methyl alcohol. Each extract was then evaporated to about 5 mℓ, made up to 50 mℓ with distilled water, centrifuged at 50 000 g for 30 minutes and the supernatant decanted.

Two millilitres of 3% bovine serum albumin was added to 0,2 mℓ extract in centrifuge tubes, which were then shaken for 20 seconds and allowed to stand for 10 minutes before being spun at 50 000 g for 30 minutes. The supernatant, which contained the anthocyanins, was poured off into sample bottles, and 10 mℓ hydrolyzing agent (450 mℓ n-butanol + 25 mℓ concentrated HCl) was added to each. The same volume was added to the leucoanthocyanin-containing pellets, which were resuspended by shaking. All the sample bottles were placed in boiling water (97° C) for 40 minutes. After cooling, absorbances at 550 nm were read in a Beckman DK-2A spectrophotometer.

2.2.2 Proteins

2.2.2.1 Bark and leaf PO, and bark IAA oxidase, PPO and PAL activities

The study began with both leaf and bark tissue, but with the exception of PO, the leaf tissue results were extremely variable, possibly due to the difficulty in ensuring leaves of the same age (van Lelyveld and Bester, 1978), so that the study of IAA oxidase, PPO and PAL activities was restricted to mature bark.

(a) Extraction. Mature bark samples were collected from four Edranol avocado trees showing both typical sunblotch symptoms as well as 'recovery' growth branches. Fifteen gram samples were taken, one of rough bark on branches showing symptoms and one of smooth bark from 'recovery' growth branches. In addition samples were taken from four healthy trees. All samples were from branches of approximately the same age.

All dead and corky material was removed from the bark and discarded. Each 15 g sample was cut into small pieces (0,25 mm^2) and placed in a deep freeze. Leaf material was similarly collected, cut and stored. Those from branches with symptoms were themselves symptomless; leaf symptoms in the field are rare (Wallace, 1958).

The method used for enzyme extraction was based on that described by van Lelyveld and Pretorius (1973) as modified by Milne, van Lelyveld and de Villiers (1977), which has since been shown to be statistically reliable (van Lelyveld and Bozalek, 1979). Each test sample of cooled bark pieces was mixed with 1 g carbowax-4000 (van Lelyveld, Brodrick and Esterhuizen, 1975) and 100 mℓ acetone (-20° C) and then homogenised with an Ultra Turrax macerator for 1 minute. The homogenate was filtered through a Büchner funnel and the acetone powder thus recovered was washed with an additional 150 mℓ acetone (-20° C), and dried of all the acetone. It was then homogenised for 45 seconds with 30 mℓ cold 0,01M acetate buffer (pH 5,0), and filtered through fine gauze cloth. A 22-mℓ aliquot of the filtrate was taken and centrifuged at 50 000 g for 30 minutes (4° C). The resulting supernatant was shaken with 70 mℓ acetone (-20° C) and centrifuged at 25 000 g for 30 minutes (4° C). The pellet was resuspended in 22 mℓ cold 0,01M acetate buffer (pH 5,0) and centrifuged at 25 000 g for 30 minutes (4° C) to remove all insoluble matter. This final solution was stored in a deep freeze for use in the various enzyme determinations.

(b) PO activity (van Lelyveld and Pretorius, 1973). Into test tubes containing 20 mℓ acetate buffer (pH 5,0) , 1 mℓ 0,1N H_2O_2 and 1 mℓ 0,04M guaiacol was placed 0,02 mℓ enzyme extract. The rate of colour development was calculated from absorbances at 420 nm measured in a Beckman DK-2A spectrophotometer during 5 minutes activity. Calculations were based only on the initial steepest increase in absorbance.

(c) IAA oxidase activity (van Lelyveld and Pretorius, 1973). The incubation mixture comprised 3 mℓ distilled water, 4 mℓ 0,01M acetate buffer (pH 5,0) , 1 mℓ 0,009M p-coumaric acid (dissolved in 50% ethyl alcohol), 1 mℓ 0,004M H_2O_2 , 2 mℓ 0,0015M IAA (dissolved in 50% ethyl alcohol) and

2 mℓ enzyme extract. Each such mixture was incubated at room temperature for 1 hour during which period a 1 mℓ sample was removed every 20 minutes. The amount of IAA remaining was in each case ascertained by adding 4 mℓ of reagent comprising 2 mℓ 0,5M $FeCl_3$ + 100 mℓ 35% $HClO_3$. After 15 minutes the absorbance at 530 nm was read in a Beckman DK-2A spectrophotometer.

For the standard, 30 mg IAA was dissolved in 100 mℓ 50% ethyl alcohol

Readings after 20 minutes' incubation were found to be satisfactory for determining enzyme activity.

(d) PPO activity (van Lelyveld and Pretorius, 1973). The incubation mixture comprised 10 mℓ 0,01M acetate buffer (pH 5,0), 0,1 mℓ 0,13% $CuSO_4$ and 2 mℓ 1% catechol, to which was added and mixed 1 mℓ enzyme extract. The rate of colour development was followed by recording absorbance at 480 nm in a Beckman DK-2A spectrophotometer over 2 minutes. Activity calculations were again based only on the initial steepest increase in absorbance.

(e) PAL activity (Farkas and Szirmai, 1969). Into a set of test tubes standing in a water bath at 40° C was placed 1 mℓ clear enzyme extract, 1 mℓ 50 mM L-phenylalanine and 1 mℓ 0,01M acetate buffer (pH 5,0). Using a Beckman DK-2A spectrophotometer the increasing absorbances at 290 nm, due to the formation of cinnamic acid, were recorded every 20 minutes for 1 hour. Results were calculated by using the molar absorption coefficient of trans-cinnamic acid.

(f) Total soluble protein. The total soluble protein content was determined by the method of Lowry, Rosebrough, Farr and Randall (1951), employing the following two reagents:

(i) 2% Na_2CO_3 in 0,1N NaOH , and 0,5% $CuSO_4$, $5H_2O$ in 5% sodium citrate, mixed in a ratio of 30 : 1 ; and

(ii) one part Folin-Ciocalteau reagent added to two parts water.

As standards, a dilution series from a 20 mg/10 mℓ bovine serum albumin solution was prepared.

Five millilitres of reagent (i) was mixed with 0,5 mℓ enzyme extract, or serum for the standards, in test tubes and allowed to stand for 10 minutes, after which 0,5 mℓ of the Folin-Ciocalteau solution was added. The mixture was shaken vigorously and allowed to stand for 10 minutes to allow for maximum colour development. Absorbances at 600 nm were read on a Beckman DK-2A spectrophotometer. Standards were then plotted and

the protein concentrations of the enzyme extracts determined from the graph.

2.2.2.2 PAGE of PO, IAA oxidase and PPO isoenzymes and total soluble proteins

Anodic polyacrylamide gel electrophoresis was carried out following the method devised by Davis (1964). The following stock solutions were prepared:

A. 48 mℓ 1N HCl
36,6 g Tris
0,23 mℓ TEMED
Water to 100 mℓ
(pH 8,9)

B. 5,98 g Tris
0,46 mℓ TEMED
± 48 mℓ 1N HCl
(to pH 6,7)
Water to 100 mℓ

C. 28,0 g acrylamide
0,735 g MBA
Water to 100 mℓ

D. 10,0 g acrylamide
2,5 g MBA
Water to 100 mℓ

E. 4 mg riboflavin
Water to 100 mℓ

F. 40% sucrose solution

The small pore gel was prepared by mixing 1 part A, 2 parts C, 1 part water and 4 parts 0,14% ammonium persulphate. The large pore gel consisted of 1 part B, 2 parts D, 1 part E and 4 parts F. The running buffer used was tris-glycine (pH 8,9), and bromophenol blue was used as a tracer dye.

The gels were run at an initial current of 2 mA per tube for the large pore gel, and 4 mA per tube in the small pore gel. A concentration of 160 µg total soluble protein was used for each sample.

PO isoenzymes were resolved by placing the gels in 0,04M guaiacol for 30 minutes, and then substituting 0,1N H_2O_2 . After colour development the gels were preserved in 7% acetic acid (van Lelyveld and Brodrick, 1975 b).

IAA oxidase isoenzymes were resolved by incubating the gels for 30 minutes in the following medium:

18,24 mℓ 2M acetic acid
1,6 mℓ 2M sodium acetate
8 mℓ distilled water
8 mℓ 5 mM p-coumaric acid
8 mℓ 4,5 mM H_2O_2
8 mℓ 5 mM IAA (in 50% ethyl alcohol) .

The gels were then stained with 4-dimethylaminocinnamaldehyde (DMACA) (0,5% in 1N HCl), and preserved in 7% acetic acid (Gove and Hoyle, 1975).

PPO isoenzymes were resolved by equilibrating the gels in 10 mM catechol and 0,1% m-phenylenediamine (Kahn, 1976).

Staining for total soluble protein was done with 1% amido Schwartz in 7% trichloroacetic acid (van Loon, 1973) for 1 hour. The gels were destained in 7% acetic acid and then scanned in an ISCO model 1310 gel scanner fitted with a UA5 absorbance monitor.

2.2.3 Bark and Wood Cations

The same samples used for total water soluble phenol determination (see Section 2.2.1.2) were used for cation analysis. Samples of 2 g dry mass were ashed overnight in a furnace at 450° C (Wutscher et al., 1977). The ash was then dissolved in 50 mℓ 5% HCl and cation determinations were made on a Techtron atomic absorption spectrophotometer. The following were analysed: Na , K , Ca , Mg , Fe , Zn , Mn and Cu .

2.3 RESULTS

2.3.1 Phenols

2.3.1.1 Bark total soluble phenols

Tables 2.1 , 2.2 and 2.3 , respectively, show the results of the total soluble phenol tests conducted on Hass seedlings, Edranol seedling and Edranol trees. Higher total soluble phenol levels were detected in young bark only in association with symptoms (Tables 2.1 and 2.2). In mature bark, however, levels were not significantly different in healthy and infected tissues (Table 2.3).

TABLE 2.1 Total soluble phenols in the young bark of healthy and sunblotch-infected Hass avocado seedlings

Replicates	PHENOLS (µM EQUIVALENTS OF GALLIC ACID)			
	Healthy	Yellow streak	Green (adjacent to yellow) streak	Green (distant from yellow) streak
1	56	86	91	78
2	45	106	110	84
3	70	85	71	76
4	62	72	99	108
5	56	76	62	52
Mean	57,8a	85,0b	86,6b	79,6b

Different letters indicate significance differences at 1% level.

TABLE 2.2 Total soluble phenols in the young bark of healthy and symptomless carrier Edranol avocado seedlings

Replicates	PHENOLS (µM EQUIVALENTS OF GALLIC ACID)	
	Healthy	Symptomless carrier
1	53	60
2	68	62
3	36	56
4	53	74
5	59	51
Mean	53,8 [1]	60,6 [1]

[1] Not significant at 5% level.

TABLE 2.3 Total soluble phenols in the mature bark of healthy and sunblotch-infected Edranol avocado trees

Replicates	PHENOLS (µM EQUIVALENTS OF GALLIC ACID)		
	Healthy	With symptoms	'Recovery' growth
1	140	142	87
2	84	75	85
3	96	76	70
4	90	158	71
5	76	76	103
Mean	97,2 [1]	105,4 [1]	83,2 [1]

[1] Not significant at 5% level.

2.3.1.2 Bark and wood total water soluble phenols

The levels of water soluble phenols found in healthy and infected wood and bark are shown in Table 2.4. No significant differences were found in the wood samples, but infected bark was found to have higher levels than the healthy.

TABLE 2.4 Water soluble phenols in healthy and sunblotch-infected Edranol avocado wood and bark

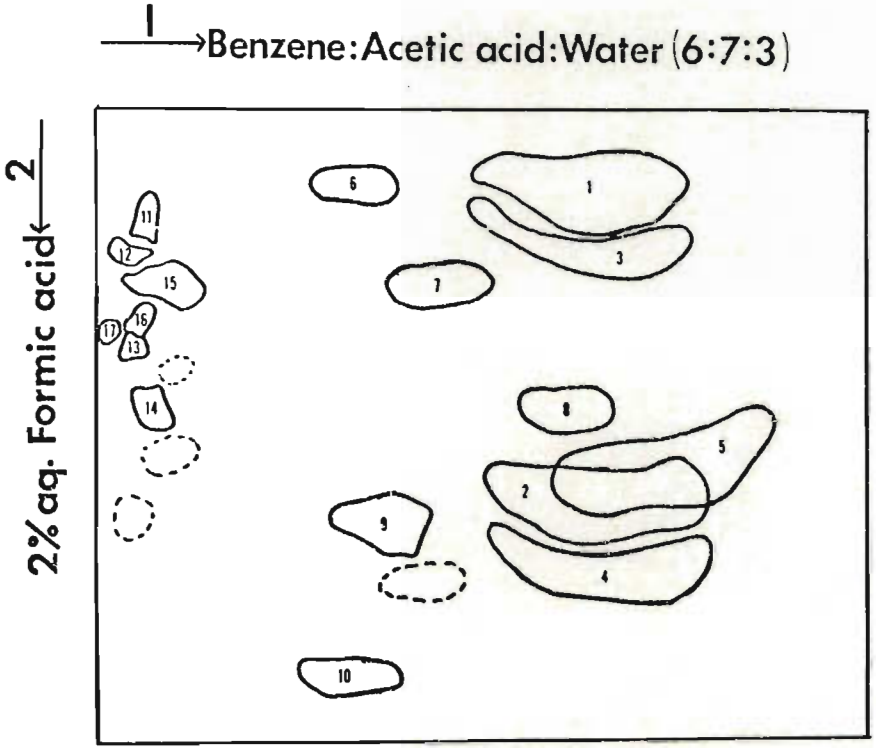
Tissue	Replicates	WATER SOLUBLE PHENOLS (µM GALLIC ACID EQUIVALENTS)		
		Healthy	With symptoms	'Recovery' growth
Wood	1	20	30	28
	2	24	29	31
	3	30	34	32
	4	31	39	38
	5	29	28	32
	Mean	26,8	32,0	32,2
Bark	1	36	38	51
	2	47	53	60
	3	22	63	48
	4	22	41	38
	5	25	64	39
	Mean	30,7a	51,8b	47,2b

Different letters indicate significance at 5% level.

2.3.1.3 Bark and leaf phenol identification

Chromatograms showing the individual phenols resolved from extractions of mature bark, young bark and leaves of healthy and diseased plants are summarized in Figures 2.2 , 2.3 and 2.4 respectively. Certain spots were identified by comparison with standards, whilst other identifications were based on results obtained by Ibrahim and Towers (1960) and van Lelyveld (1974).

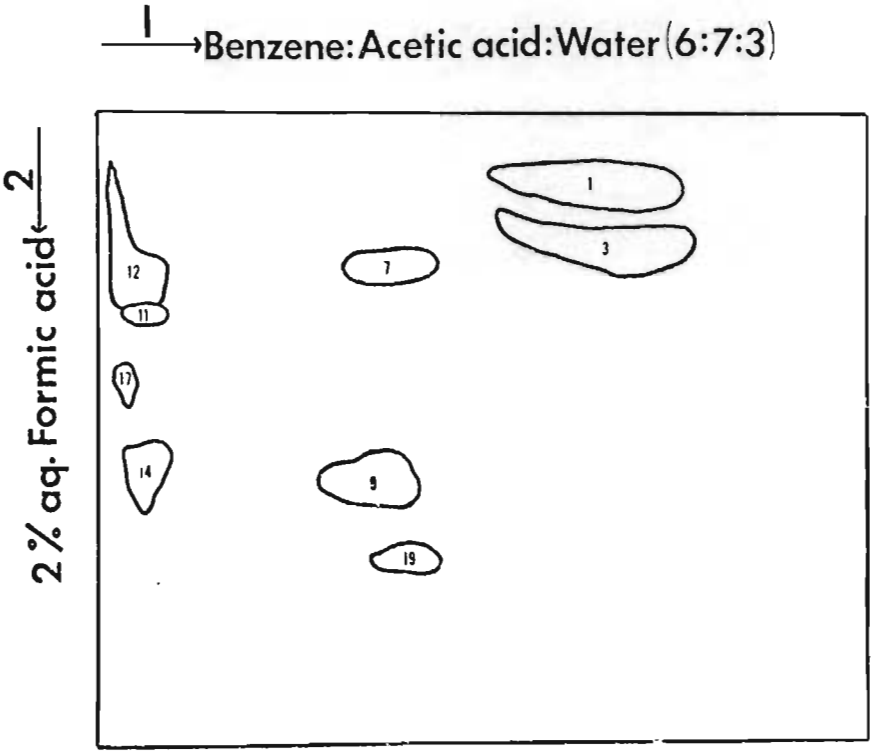
Two unidentified, non-fluorescing spots detected with p-nitroaniline, Nos. 15 and 16, were present in all diseased mature bark samples, but not in the healthy mature bark nor in any of the young bark and leaf samples.



1 sinapic acid
2 isomer, sinapic acid
3 ferulic acid
4 isomer, ferulic acid
5 vanillic acid
6 ?
7 ?
8 ?
9 p-hydroxybenzoic acid
10 ?
11 caffeic acid
12 aesculetin
13 ?
14 isomer, caffeic acid
15 ?
16 ?
17 α- resorcyclic acid ?

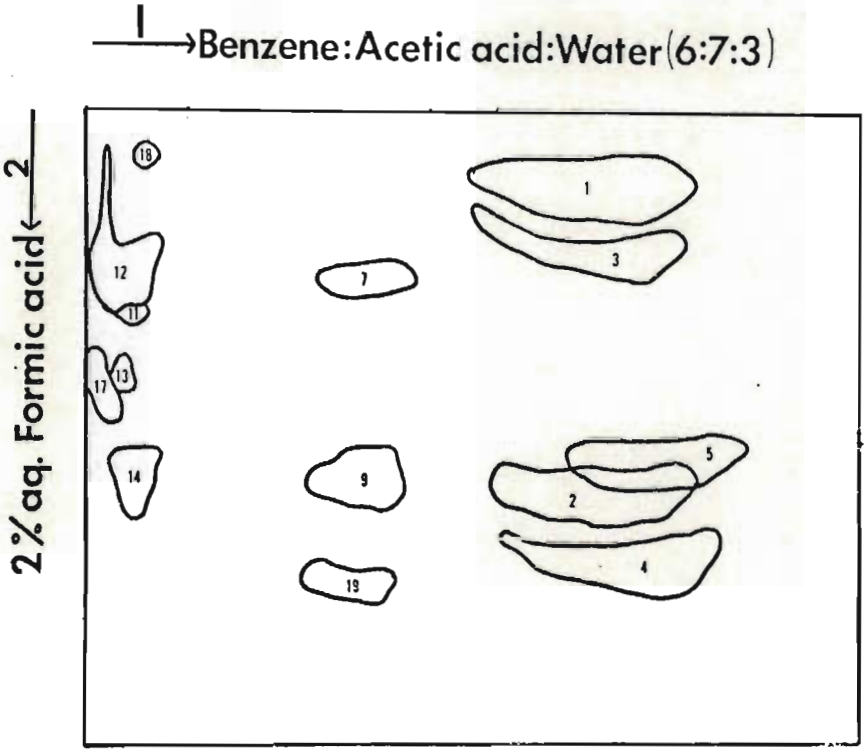
FIGURE 2.2 Summarized chromatogram of phenols in mature avocado bark. Nos. 1 - 14 and 17 were present in all samples; Nos. 15 and 16 were only present in infected bark both with symptoms and 'recovery' growth bark.

◯ - very faint, fluorescent spots



1 sinapic acid
3 ferulic acid
7 ?
9 p-hydroxybenzoic ac
11 caffeic acid
12 aesculetin
14 isomer, caffeic aci
17 α- resorcyclic acid
19 ?

FIGURE 2.3 Summarized chromatogram of phenols in young avocado bark. All spots were present in healthy and sunblotch-infected samples



1	sinapic acid
2	isomer, sinapic acid
3	ferulic acid
4	isomer, ferulic acid
5	vanillic acid
7	?
9	p-hydroxybenzoic acid
11	caffeic acid
12	aesculetin
13	?
14	isomer, caffeic acid
17	α- resorcyclic acid ?
19	?

FIGURE 2.4 Summarized chromatogram of phenols in young leaves of healthy and sunblotch-infected avocado trees. All spots were present in both healthy and diseased samples

Table 2.5 shows the results of quantitative tests conducted on individual phenols in the mature bark extracts that eluted in amounts detectable in the spectrophotometer. Only sinapic and ferulic acids showed any significant differences. Sinapic acid was lower in the 'recovery' growth tissue, while ferulic acid was lower in all diseased samples.

TABLE 2.5 Ultraviolet absorbance of avocado mature bark phenols separated by paper chromatography

Phenol	Absorbance λ (nm)	ABSORBANCE READINGS [a]		
		Healthy	With symptoms	'Recovery' growth
Sinapic acid	280	29,5a	33,4a	19,3b
Ferulic acid	310	24,0a	16,2b	12,3b
Caffeic acid	280	14,5	11,7	15,6
p-hydroxy-benzoic acid)	250	23,6	21,6	16,8
No. 10	280	14,6	18,8	19,0
No. 13	250	13,5	19,4	16,8

[a] Different letters for sinapic acid and ferulic acid indicate significance at 1% level.

2.3.1.4 Leaf total anthocyanins/leucoanthocyanins

The individual readings for anthocyanins and leucoanthocyanins in leaves were variable, therefore the readings were combined to give values for total pigment content (Table 2.6). No significant differences were found.

TABLE 2.6 Total red pigment (anthocyanin + leucoanthocyanin) in healthy and sunblotch-infected avocado leaves

Replicates	ABSORBANCE AT 550 nm		
	Healthy	With symptoms	'Recovery' growth
1	78,0	109,0	83,0
2	59,6	100,3	84,6
3	80,6	80,3	95,3
4	104,0	87,0	90,0
5	95,0	97,6	88,0
Mean	83,7	94,2	88,2

2.3.2 Proteins

2.3.2.1 Bark and leaf PO, and bark IAA oxidase, PPO and PAL activities

The detected activities of PO, IAA oxidase and PPO in mature bark of healthy and sunblotch-infected Edranol avocado trees are presented in Table 2.7.

TABLE 2.7 Activities of peroxidase (PO), indole acetic acid (IAA) oxidase and polyphenoloxidase (PPO) in mature bark extracts of healthy and sunblotch-infected Edranol avocado trees

Bark tissue	Replicate	PO activity [1]	IAA oxidase activity [2]	PPO activity [3]
With symptoms (rough bark)	1	12,50	1,84	1,40
	2	11,35	0,52	1,90
	3	18,35	0,52	2,95
	4	15,39	1,40	1,90
		14,39a	1,07a	2,04a
'Recovery' growth	1	6,60	3,16	1,80
	2	6,25	4,48	1,85
	3	7,60	5,80	1,45
	4	7,50	3,60	1,80
		6,98b	4,26b	1,72a
Healthy	1	5,25	5,36	1,15
	2	5,50	4,92	0,85
	3	5,25	4,04	1,05
	4	5,40	7,56	1,10
		5,35b	5,47b	1,04b

[1] Changes in absorbance at 420 nm per min.

[2] mg IAA oxidised by 2 mℓ extract

[3] Changes in absorbance at 480 nm per min.

Different letters indicate significance at 1% level

PO and IAA oxidase activities were significantly higher and lower, respectively, in material with symptoms than in symptomless and healthy. PPO activity was higher in both types of infected material regardless of whether symptoms were present or not.

Avocado leaf extracts, analysed for PO activity only, revealed no significant differences (Table 2.8), although the 'recovery' growth shows a tendency to higher activity.

TABLE 2.8 Peroxidase (PO) activity in extracts of symptomless leaves of healthy and sunblotch-infected Edranol avocado trees

Replicates	PO ACTIVITY (CHANGE IN ABSORBANCE AT 420 nm PER MIN)		
	Healthy	With symptoms	'Recovery' growth
1	15,0	11,0	25,0
2	15,0	19,0	20,0
3	21,5	33,0	22,0
4	14,0	10,0	21,0
5	13,0	15,0	17,0
Mean	15,7	17,6	21,0

The study on changes in PAL activity showed that, as with PO, the rough bark (diseased) samples displayed higher activity than the symptomless and healthy samples (Table 2.9). However, subjecting the very high activity values of replicates 3 of both diseased and symptomless bark to the t-test shows them both to be significantly higher than the other values recorded for the same type of tissue. On leaving them out of account when testing for statistical significance, PAL activity in the 'recovery' growth tissue is found to be significantly lower (5%) than the healthy, while that of the rough bark is still significantly higher (5%).

TABLE 2.9 Phenylalanine ammonia-lyase (PAL) activity in the mature bark of healthy and sunblotch-infected Edranol avocado trees

Replicates	PAL ACTIVITY (nmoles CINNAMIC ACID PRODUCED)[a]		
	Healthy	With symptoms	'Recovery' growth
1	1 390,5	927,0	772,5
2	1 545,0	1 545,0	154,5
3	1 081,5	2 935,5	1 545,0
4	463,5	1 390,5	618,0
5	309,0	2 008,5	927,0
6	618,0	618,0	772,0
7	618,0	927,0	154,5
8	463,5	927,0	154,5
9	618,0	772,5	154,5
10	1 390,5	772,5	618,0
Mean	849,75a	1 282,35b	587,1a

[a] Different letters indicate significance at 5% level.

2.3.2.2 Total soluble protein

Total soluble protein levels were significantly higher in both diseased and symptomless bark under both mid-summer and mid-winter conditions (Table 2.10).

TABLE 2.10 Total soluble protein levels in mature bark of healthy and sunblotch-infected Edranol avocado trees

Expt. date	Replicate	TOTAL SOLUBLE PROTEIN (mg per mℓ extract)		
		Healthy	With symptoms	'Recovery' growth
Jan. 1977 (Summer)	1	0,23	0,53	0,40
	2	0,20	0,48	0,40
	3	0,36	0,42	0,44
	4	0,34	0,52	0,43
	Mean	0,28a	0,49b	0,42b
July 1977 (Winter)	1	0,58	0,65	0,98
	2	0,44	0,69	0,56
	3	0,66	0,80	0,84
	4	0,58	0,51	0,61
	5	0,37	0,51	0,63
	6	0,41	0,97	0,69
	Mean	0,51a	0,71b	0,72b

Different letters indicate significance at 1% level.

2.3.2.3 PAGE of PO , IAA oxidase and PPO isoenzymes and total soluble protein

The anodic gel patterns of PO , IAA oxidase and PPO isoenzymes are shown in Figures 2.5 , 2.6 and 2.7 , respectively. The band designations are based on those of van Lelyveld (personal communication). In the PO gels bands a and b were found to be much more intense in the rough bark samples than in those of either the smooth 'recovery' growth or healthy bark. Band a_1 was slightly darker in all infected samples.

Band a_1 of IAA oxidase was much darker in all infected samples, both with symptoms and symptomless, indicating reduced activities. Bands a and b were darker only in the samples from trees with symptoms.

All infected samples, both with symptoms and symptomless, were found to contain more PPO isoenzyme bands than the healthy. In addition bands c, d and e were darker in all the infected samples.

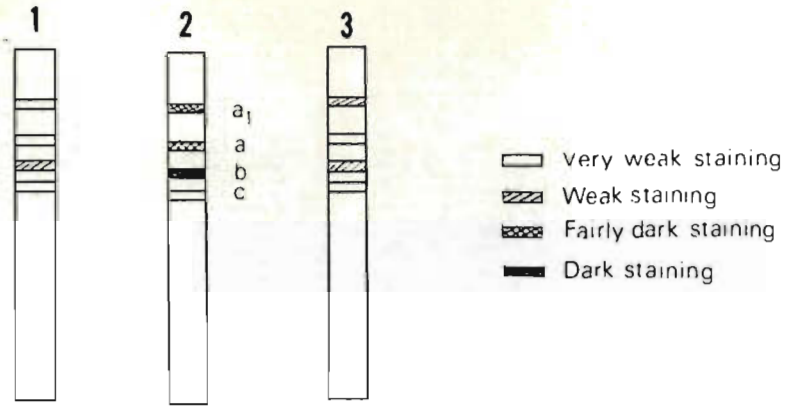


FIGURE 2.5 Gel-electrophoresis separation of peroxidase (PO) isoenzymes of the bark of sunblotch-infected and healthy avocado trees.

1 - healthy

2 - trees with rough bark symptoms

3 - symptomless ('recovery' growth)

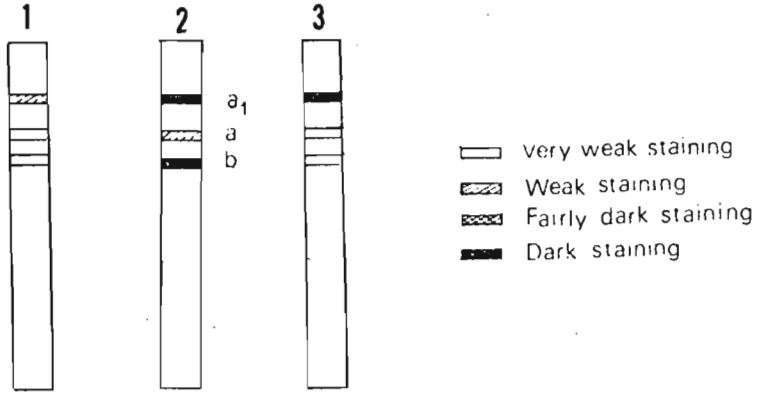


FIGURE 2.6 Gel-electrophoresis separation of indoleacetic acid (IAA) oxidase isoenzyme of sunblotch-infected and healthy avocado bark.

1 - healthy

2 - trees with rough bark symptoms

3 - symptomless ('recovery' growth)

Note: staining intensity is inversely related to activity.

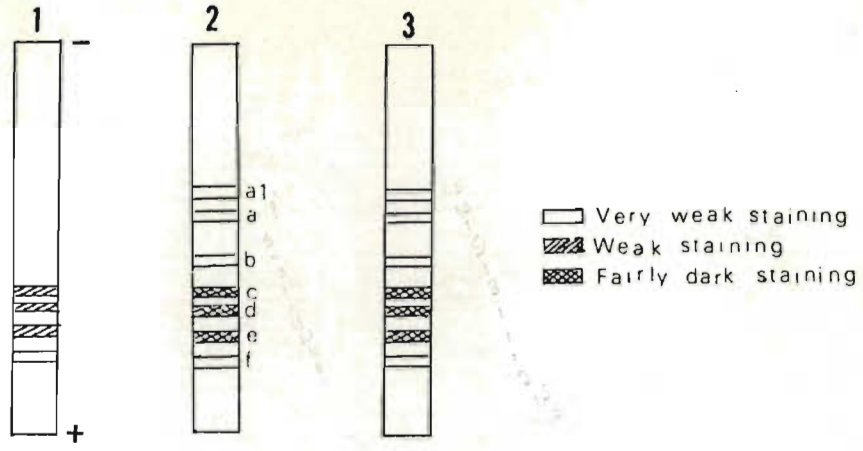


FIGURE 2.7 Gel-electrophoresis separation of polyphenoloxidase (PPO) isoenzymes of sunblotch-infected and healthy avocado bark.

1 - healthy

2 - trees with rough bark symptoms

3 - symptomless ('recovery' growth)

PAGE analysis of the total soluble proteins revealed only three protein bands (Fig. 2.8). The slowest moving band, a , represented a comparatively lower proportion of the proteins in the samples of infected tissue. However, correcting for higher levels of soluble protein found in infected bark, band a could be present in nearly equal amounts in healthy and diseased tissue, while bands b and c would be appreciably greater in the latter.

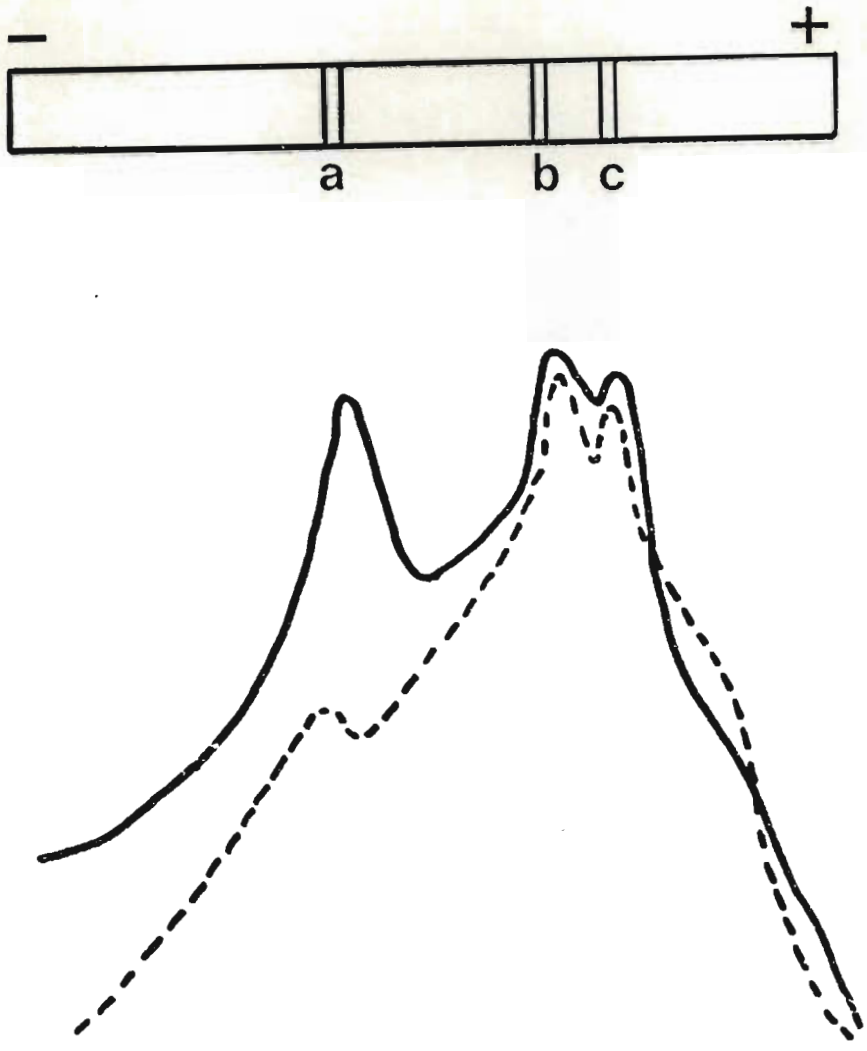


FIGURE 2.8 Gel electrophoresis pattern (top) and gel scan (bottom) of total soluble protein from mature avocado bark.

——— healthy

- - - sunblotch infected (identical traces were obtained for samples with and without symptoms)

2.3.3 Bark and Wood Cations

Table 2.11 shows the results of the element analyses conducted on the wood and bark. In the wood significant differences were found in the levels of Na , K , Mg , Fe and Cu , while in the bark only Fe and Cu were different.

TABLE 2.11 Analysis of elements in the wood and bark of healthy and sunblotch-infected Edranol avocado trees

Tissue	Replicate	Na %	K %	Ca %	Mg %	Fe ppm	Zn ppm	Mn ppm	Cu ppm
Healthy wood	1	0,005	0,163	0,075	0,028	8,8	4,0	4,5	3,9
	2	0,025	0,125	0,063	0,038	5,0	3,8	2,0	3,8
	3	0,075	0,138	0,050	0,035	1,3	14,0	2,5	3,9
	4	0,125	0,188	0,138	0,043	6,3	4,3	5,0	3,4
	5	0,025	0,188	0,100	0,035	6,3	6,8	5,0	3,9
	Mean	0,05a	0,16a	0,085	0,036a	5,5a	6,6	3,8	3,8a
Wood from trees with symptoms	1	0,125	0,163	0,100	0,035	13,8	4,3	3,5	8,6
	2	0,125	0,138	0,113	0,035	7,5	5,8	5,0	8,8
	3	0,125	0,188	0,075	0,040	13,8	6,5	5,5	13,3
	4	0,200	0,225	0,100	0,045	15,0	11,8	4,8	8,9
	5	0,075	0,213	0,113	0,048	7,5	31,5	3,0	6,4
	Mean	0,13b	0,19a	0,100	0,041a	11,5b	11,9	4,4	9,2b
'Recovery' growth wood	1	0,200	0,338	0,688	0,055	12,5	7,3	2,5	5,8
	2	0,200	0,475	0,138	0,078	3,8	9,8	2,0	4,5
	3	0,025	0,350	0,113	0,063	6,3	8,0	5,0	4,3
	4	0,075	0,463	0,088	0,085	5,0	5,8	2,5	4,5
	5	0,075	0,288	0,088	0,058	3,8	8,0	2,5	4,3
	Mean	0,12b	0,38b	0,103	0,068b	6,3a	7,8	2,9	4,7a
Significance levels		5%	5%	NS	1%	1%	NS	NS	1%

TABLE 2.11 (contd.)

Tissue	Replicate	Na %	K %	Ca %	Mg %	Fe ppm	Zn ppm	Mn ppm	Cu ppm
Healthy bark	1	0,52	0,82	3,93	0,08	105	10,8	17	6,2
	2	0,48	1,31	6,43	0,16	116	16,5	30	7,3
	3	0,37	0,55	2,41	1,10	90	11,8	15	4,6
	4	0,47	0,48	1,71	0,09	100	18,5	22	6,5
	5	0,48	0,88	2,86	0,07	103	14,5	19	5,4
	Mean	0,46	0,81	3,47	0,10	103a	14,3	20,6	6,0a
Bark with symptoms	1	0,32	0,68	4,14	0,15	108	12,8	12	6,8
	2	0,42	0,56	3,04	0,27	130	9,3	11	5,2
	3	0,45	0,66	2,32	0,14	144	17,0	18	7,4
	4	0,40	0,46	2,56	0,07	56	12,8	13	6,9
	5	0,45	0,60	3,21	0,15	42	14,5	22	6,6
	Mean	0,41	0,59	3,09	0,15	96a	13,3	15,2	6,6a
'Recovery' growth bark	1	0,53	0,58	2,32	0,17	56	22,3	17	9,6
	2	0,55	0,80	2,18	0,09	37	12,5	14	10,2
	3	0,44	0,71	1,78	0,10	48	15,8	21	8,6
	4	0,49	0,75	2,13	0,08	58	15,0	20	15,9
	5	0,40	0,90	2,06	0,10	52	28,3	29	12,3
	Mean	0,48	0,75	2,09	0,11	50b	18,8	20,2	11,3b
Significance levels		NS	NS	NS	NS	5%	NS	NS	1%

2.4 DISCUSSION

Systemic viral infections of several herbaceous plants have been reported to increase PO activity (Loebenstein and Linsey, 1961 and 1966; Melouk and Skotland, 1970; Wood and Barbara, 1971), the increases being smallest in symptomless infections and largest where necrosis occurs (Loebenstein and Linsey, 1966). However, van Loon (1976), working on Samsun NN tobacco, showed that ageing, trimming of plants, root inundation, drought and salt stress can also stimulate activity, and concluded that PO activity may be a reflection of physiological state. Furthermore, studies on PO activity in the bark of cachexia (xyloporosis)-infected mandarin (Citrus reticulata) (Jardeny, Monselise and Chorin, 1965), psorosis-infected citrus (Monselise and Chorin, 1965), exocortis-infected citron (C. medica) (Kapur, Gumpf and Weathers, 1974) and tristeza-infected citrus (Bar-Joseph and Loebenstein, 1973; Beltrán, Carbonell, Conejero and Primo, 1973), all of which are systemic virus infections causing bark damage, have each shown that increasing activity is correlated with increasing severity of bark symptoms. High PO activity in rough avocado bark resulting from graft-incompatibility has also been reported (van Lelyveld and Bester, 1979).

It appears, therefore, that in the case of avocado sunblotch the injury resulting in the bark cracking may have been the primary cause of the observed increased PO activity in this tissue (Table 2.7), the level of which was more than twice that in symptomless carrier and healthy bark tissues. Supporting this interpretation were the further observations that PO activity in symptomless bark tissue of 'recovery' growth, which also carries the causal agent (Wallace, 1967), was not significantly higher than that of healthy bark (Table 2.7) whilst activities in healthy and symptomless leaves also were not significantly different (Table 2.8).

Bates and Chant (1970) observed both increased PO activity and new isoenzymes in cowpeas systemically infected with TMV, but noted that the changes only occurred after the appearance of symptoms, and seemed to be associated with senescence. In contrast, quantitative but not qualitative, changes of PO enzymes were found in grapevines (Barna, 1973) and tobacco (Novacky and Hampton, 1968; Birecka, Catalfamo and Urban, 1975) systemically infected with mosaic-inducing viruses and in mechanically-injured tobacco (Birecka et al., 1975). Similarly, in the present investigation (Fig. 2.5) no qualitative PO changes were found but only quantitative enhancement, principally in the intensity of three of the four bands in the rough bark samples.

The absence of any significant PO increase in symptomless leaves from trees with mature bark symptoms (Table 2.8) is not unlike the situation of citrus infected with psorosis where smaller increases in PO activities were observed in leaves than in bark (Monselise and Goren, 1965).

Van Lelyveld and Brodrick (1975 a) detected higher IAA oxidase activity in the leaves of avocado trees affected by Phytophthora root rot, as did Smith, McCall and Harris (1968) in various plants infected with curly top virus. In the present study on avocado bark tissue, significantly reduced activity was noted in the rough bark from diseased trees compared to the smooth bark from 'recovery' growth (Table 2.7). This finding suggests either reduced enzyme presence or the intervention of an inhibitor of the IAA oxidase system, as occurs with scopoletin in sweet potato (Imbert and Wilson, 1970). Such reduction in IAA oxidase activity could be comparable in effect to increasing the level of endogenous IAA, which hormone may in turn play an important rôle in the typically abnormal growth, such as the decumbent habit, of diseased avocado trees having rough bark symptoms. Feldman and Hanks (1968) found a correlation between stunting of citrus on trifoliate rootstock by exocortis and decreases in growth promotor levels. In bark of xyloporosis-infected citrus IAA oxidase activity is also reported to be reduced (Monselise and Goren, 1964).

The electrophoretic similarity between isoenzymes of IAA oxidase and PO was demonstrated by Gove and Hoyle (1975) and Shinshi and Naguchi (1975). In the present investigation (Fig. 2.5) it likewise appears on electrophoretic grounds, that IAA oxidase and PO may be the same enzyme but that its two active sites respond differently to the effects of avocado sunblotch infection. A Phytophthora-induced phenolic inhibitor of PO in avocados has been reported by van Lelyveld and Bester (1978), affording evidence that the system is under some inhibitory control as suggested above.

As with the PO isoenzymes the IAA oxidase isoenzymes (Fig. 2.6) were not qualitatively different in healthy and infected tissues but showed a quantitative increase in intensity (i.e. a decrease in activity) of all three bands in the rough bark material, and of only band a_1 in the symptomless recovery growth material. Such decrease in isoenzyme activities in diseased bark extracts correlates well with the observed reduction in overall IAA oxidase activity (Table 2.7). There is also limited evidence of similar correlation in the case of 'recovery' growth tissue.

With regard specifically to 'recovery' growth tissue it may be significant that the extracts derived from both the mature bark and leaf samples show a

consistent tendency, albeit non-significant, towards PO activities higher than those of the corresponding healthy tissues (Tables 2.7 and 2.8), which finding could correlate with the slight but consistent, increased intensity of the PO isoenzyme band a_1 shown in Figure 2.5. Similarly, in the case of IAA oxidase where there is evidence of a tendency for lower activity in 'recovery' growth bark (Table 2.7), the intensity of only isoenzyme a_1 is markedly altered, i.e. reduced, relative to the healthy tissue (Fig. 2.6). This near-complimentarity of the increase in PO activity with the reduction in IAA oxidase activity of the various isoenzyme bands represented in Figures 2.5 and 2.6 raises the question as to whether the reduction of IAA oxidase activity is accompanied by increased PO synthesis, or whether at the level of the individual molecule, IAA oxidase function is inhibited in favour of enhanced PO function.

Systemic viral infections have also been shown to be associated with increased PPO activity (Hampton and Fulton, 1961 ; Suseno and Hampton, 1966 ; Tomaru, Shiroya and Takanami, 1969 ; Barbara and Wood, 1972 ; Chernikov, 1977). However, Farkas, Király and Solymosy (1960) reported that increases in PPO activity in tobacco systemically infected with TMV were considerably lower than those associated with TMV-induced hypersensitivity, and Suseno and Hampton (1966), also studying tobacco systemically infected with TMV, found a correlation between increases in activity and symptom severity.

In the present study, which involved a systemic infection, the increased PPO activity associated with infection was apparently unrelated to presence or absence of symptoms since there was no significant difference between bark samples from branches with symptoms and those from latently infected branches (Table 2.7). The same conclusion may be drawn from the study on PPO isoenzymes (Fig. 2.7). Thus, the differences between the extract activities of sunblotch-infected bark and healthy bark therefore seem to be attributable directly to the infection without correlation to symptom severity. Note should be taken, however, of the consistently higher PPO activity of replicate 3 of the bark with symptoms (Table 2.7). Although the increase in intensity of the PPO isoenzymes in infected samples (Fig. 2.7) is paralleled by an increase in the level of faster moving soluble proteins (Fig. 2.8), an explanation for the latter increase cannot be given.

PAL is involved in increased aromatic biosynthesis in injured or infected plant tissue and several authors have reported increases in PAL activity in localized viral infections (Farkas and Szirmai, 1969 ; Fritig, Gosse, Legrand and Hirth, 1973 ; Vegetti et al., 1975). In the present study on sunblotch a significant PAL increase was detected only in bark with symptoms (Table 2.9).

Thus, as was found with PO, stimulation of this enzyme is probably primarily a response to tissue damage and not a cause of the symptoms. PAL is the first enzyme in the synthesis of the series of cinnamic acids (Towers, 1964), and the observed increased activity in rough bark (Table 2.9) may have a connection with the altered levels of ferulic and sinapic acids (Table 2.5). However, there is no evidence of correlation and therefore other factors must be involved. In 'recovery' growth bark sinapic and ferulic acid levels are significantly reduced (Table 2.5) and there is a strong possibility that PAL activity responsible for the synthesis of these phenols is also reduced in such tissues (Table 2.4).

With regard to phenols, higher total phenol levels were correlated with symptom severity in psorosis- and xyloporosis-infected citrus bark (Jardeny et al., 1965; Monselise and Goren, 1965), while the wood of citrus trees suffering from citrus blight contain very high levels of water soluble phenols (Wutscher et al., 1977; da Graça and van Vuuren, 1979). The analysis of water soluble phenols is attractive as a diagnostic technique because of its ease of execution. Higher total soluble phenol levels were detected in this study in young bark only in association with symptoms (Tables 2.1 and 2.2), whilst higher water soluble phenols were found in all infected mature bark samples (Table 2.4), although possibly not sufficiently large for diagnostic purposes. However, differences in total phenols in mature bark, whether diseased or symptomless, were not significant (Table 2.3).

No change in total authocyanins as a result of sunblotch infection was detected in this study. The reported increases associated with Phytophthora root rot in avocados (van Lelyveld et al., 1979) are not, therefore, indicative of a general response to pathogenic invasion.

The detection of two additional unidentified phenols by paper chromatography in infected mature bark (Fig. 2.2) raised the possibility that they might serve for rapid indexing for sunblotch. However, their absence in young infected tissues (Figs. 2.3 and 2.4) could indicate that they may have been produced other than in response to sunblotch infection. Neither of the two additional phenols fluoresced under ultraviolet light, having been detected by p-nitroaniline spray. Neither is therefore identical with the fluorescent phenol detected by Schwarz and van Vuuren (1970) in young infected avocado bark. No evidence for this latter phenol was found in any of the infected samples in this study.

Changes in levels of specific phenols as a result of infection, such as sinapic and ferulic acids (Table 2.5), have also been recorded for other

diseases. Schwarz (1965) reported the presence of a phenol, later identified as gentisoyl glucoside (Feldman and Hanks, 1969), in the bark of greening-infected citrus. In exocortis-infected citrus p-hydroxybenzoic and vanillic acids were lower in infected leaf and root tissue while sinapic and ferulic acids were increased (Feldman and Hanks, 1968). Differences in total phenols were not significant. Whilst sinapic acid levels were increased in both exocortis- and sunblotch-infected plants, the effect on ferulic acid levels was the opposite: whereas the former increased, the latter decreased. The increases in exocortis-infected plants occurred prior to symptom development (Feldman, Hanks and Garnsey, 1972), but symptom severity was correlated with higher coumarin levels (Feldman, Garnsey and Hanks, 1974).

Various individual phenols are known to have direct effects on oxidase enzymes. Scopoletin, a phenol not assayed in this study which typically increases in infected tissues (Farkas and Király, 1962; Sequeira, 1964), has been reported to have an inhibitory or a stimulatory effect on IAA oxidase in sweet potato, depending on its concentration (Imbert and Wilson, 1970), while ferulic acid is known to be an IAA oxidase inhibitor in pineapples (Gortner and Kent, 1958). However, Varga and Köves (1962) found that monophenols do not affect IAA oxidase activity whereas polyphenols are inhibitory. In the present study on avocado sunblotch, the significantly lower ferulic acid levels in all infected bark tissue, both with symptoms and symptomless (Table 2.5), might be expected to result in increased IAA oxidase activity in both types of infected bark tissue. Instead, however, IAA oxidase activity was decreased significantly in the diseased bark and possibly also slightly in 'recovery' growth tissue. Thus it would appear that some other factor(s), such as scopoletin, may be having a stronger influence, inhibiting IAA oxidase activity in tissue showing symptoms and/or activating it in symptomless tissue.

Very few studies have been conducted on the levels of elements in virus-infected plants. Bergman and Boyle (1962) found that tomato leaves infected with TMV had reduced levels of Mn , Zn and Cu . They suggested that changes in specific minerals may play a rôle in the induction of symptoms, possibly via enzymes. Rodriguez and Gallo (1968), studying the three citrus diseases, exocortis, xyloporosis and psorosis, observed that Zn levels were reduced in all three, K was lower in exocortis-infected leaves and B was reduced in both xyloporosis- and exocortis-infected tissue. In another citrus disease of unknown etiology, citrus blight (young tree decline), there is a marked accumulation of Zn , and a slight increase of Cu , in the wood (Smith, 1974). There is also a lower than normal level of Zn in the leaves, usually accompanied by Zn deficiency symptoms. In greening-infected leaves K was found to be higher and Ca and Mg lower (Koen and Langenegger, 1970).

In sunblotch-infected avocado trees there were changes in the levels of Na , K , Mg , Fe and Cu in the wood, and Fe and Cu in the bark (Table 2.11). In the wood of both diseased and symptomless trees Na was higher, while K and Mg were higher only in the symptomless carriers. Cu and Fe were higher in wood from trees with symptoms, whilst in the bark Cu was increased and Fe reduced in symptomless 'recovery' growth tissue. It is not possible to draw any conclusions relating cation levels to sunblotch symptomology. From an indexing point of view the changes were not as dramatic as that of Zn in YTD-infected citrus, and insufficient to serve as a reliable basis for indexing.

In conclusion, it can be said that most major alterations detected during this investigation, such as with PO and IAA oxidase, were associated with symptoms. The areas where differences between healthy and infected tissues, both with symptoms and symptomless, were found are probably unsuitable for diagnostic purposes. Increases in water soluble phenol levels, whilst significant, are not large enough as they are in blight-affected citrus (Wutscher et al., 1977); secondly, the detection of individual phenols is a tedious procedure and the absence of additional phenols in young infected tissues raises doubts as to its specificity, whilst the increased total soluble phenol levels and PPO changes may similarly be non-specific, the effects of symptomless TMV infection of avocados (Alper et al., 1978), for example, having not yet been investigated. In addition the subsequent evidence for the probable viroid nature of sunblotch (Thomas and Mohamed, 1979; Dale and Allen, 1979) offers the opportunity of a specific test for sunblotch.

Of particular interest, however, are the tendencies towards differences in some areas, such as the apparently higher PO activity and altered PO and IAA oxidase isoenzyme a_1 intensity in symptomless tissue, and the reduced PAL activity in symptomless 'recovery' growth bark. Further investigations of these may lead to an understanding of symptom development and the symptomless condition.

CHAPTER 3

ELECTRON MICROSCOPE STUDY ON SUNBLOTCH-INFECTED AVOCADO TISSUE

3.1 INTRODUCTION

Very little investigation of sunblotch-infected plant tissue by either light or electron microscopy appears to have been done. In the only published light microscope study Schroeder (1935) reported that there is a lack of development and differentiation of vascular tissue in the regions where external grooves are visible. No electron micrographs of infected tissues have been published, but Alper et al. (1975) reported that in only two parenchyma calls in bark tissue did they observe virus-like particles, while Desjardins et al. (1980) mention that their EM examinations revealed no micro-organisms. Neither paper comments on any ultrastructural changes induced by sunblotch in avocado tissue, but Desjardins (P. R., personal communication) observed gross disorganization making organelle identification difficult. Moll (J. N., personal communication) conducted a preliminary EM study on infected avocado bark with symptoms and observed extensive nacreous wall thickening in phloem sieve elements, but did not encounter any in healthy tissue.

The following is a report on an EM study of sunblotch-infected avocado leaf and stem tissue.

3.2 MATERIALS AND METHODS

Samples from the following tissues of P. americana cv. Hass seedlings were taken for electron microscopy:

(a) LEAF:
- (i) Yellow area of young expanding leaf with symptoms (Fig. 3.1)
- (ii) green area of young expanding leaf displaying symptoms (Fig. 3.1);
- (iii) young expanding healthy leaf;
- (iv) yellow area of fully expanded leaf with symptoms;
- (v) green area of fully expanded leaf displaying symptoms; and
- (vi) fully expanded healthy leaf.

(b) BARK: (i) Young bark with yellow streak; and

(ii) young healthy bark.

FIGURE 3.1 Two Hass avocado leaves with sunblotch symptoms.
Y = yellow area
G = green area

Five attempts were made at embedding each of the above tissue types, but only the last two attempts, involving approximately 10 pieces of tissue for each category, were suitable for sectioning and viewing in the EM. They were prepared as follows:

The samples were trimmed to about 1 mm^2 and fixed for 2 hours (leaf tissue) or 3 hours (bark tissue) in cold 6% glutaraldehyde in 0,1M sodium cacodylate buffer. They were then washed twice in 0,1M buffer, and post-fixed overnight in 2% osmium tetroxide. After post-fixation the pieces of tissue were washed in three 20-minute changes of deionised water, and then dehydrated

in two 15-minute changes of 2,2-dimethoxypropane (acidified with 0,5% HCl) (Thorpe and Harvey, 1979). They were then embedded in Spurr's resin (Spurr, 1969).

Approximately 10 tissue pieces of each of the sample categories were sectioned, using a diamond knife on an LKB UM III ultramicrotome. Sections were stained with 2% uranyl acetate and lead citrate, and then examined in an Hitachi HU-11E electron microscope or a JEOL 100 CX electron microscope (Figs. 3.25 and 3.26).

3.3 RESULTS

3.3.1 Leaf Tissue

Electron microscopy of avocado leaf tissue with symptoms revealed two very obvious ultrastructural changes. The one consisted of major disorganization of the chloroplasts, and the second was the appearance of paramural bodies. Both of these can be seen in the cell depicted in Figure 3.2

3.3.1.1 Chloroplasts

As shown in Figure 3.3 chloroplasts in uninfected leaf tissue have the normal arrangement of thylakoid grana and stroma lamellae. The chloroplast abnormalities described below were observed only in the yellow areas of infected leaves, and not in the symptomless green regions (Fig. 3.4). Ultrastructurally, there was no apparent difference between chloroplasts of expanding and expanded leaves.

Loss of lamellae seemed to occur in most yellow area chloroplasts, and the remaining membranes assumed abnormal conformations. Figure 3.5 shows the membranes reduced to almost one granum, the presence of one starch grain indicating some photosynthetic activity, whilst the appearance of the two grana and the stroma lamellae shown in Figure 3.6 indicate that disintegration is under way. A few chloroplasts had their lamellae systems displaced to one side (Fig. 3.7). In some, one or two lamellae only were observed, lining the periphery of the organelle (Figs. 3.2 and 3.8). Some membranes appeared as concentric rings (Fig. 3.9), while in others the lamellae appeared to be connected in the form of loops (Fig. 3.10). Several of the chloroplasts were also swollen (Figs. 3.2 , 3.6 , 3.9 and 3.10).

Two different types of vesicles were observed. Only one instance was encountered of a chloroplast having several membrane-bound vesicles that lacked any internal structural organization (Fig. 3.11). However, a second type was

more common (Figs. 3.12 and 3.13), in which the vesicles appeared to be membrane-bound and to contain poorly defined particles and strands.

3.3.1.2 Other organelles

Other leaf cell organelles encountered during this study displayed no obvious ultrastructural changes. Figure 3.2 shows a normal, representative nucleus in a cell adjacent to another containing chloroplasts displaying defects. Mitochondria (Figs. 3.5 , 3.11 , 3.12 , 3.13 and 3.14) and dictyosomes (Fig. 3.14) similarly seemed to be structurally unaffected.

3.3.1.3 Paramural bodies

Numerous paramural bodies were observed in most cells in the yellow region of sunblotch-infected mature leaf tissue, whilst in the green regions and in young infected tissue there were far fewer. In contrast, in healthy mature or young leaf tissue they were observed only in consistently very small numbers and with different internal structure.

The paramural bodies in infected tissue were of two types. The one type was rare and was only seen in four cells of mature, yellow-infected tissue (Figs. 3.15 and 3.16). Each paramural body contained a few lightly staining scattered vesicles. Two of the bodies were close to the cell wall, but were membrane-bound within the cytoplasm. The latter could be ascribed to the plane of sectioning.

Those of the second type were all clearly located between the plasmalemma and the cell wall, and contained darkly-outlined vesicles (Figs. 3.17 to 3.21). In Figures 3.17 , 3.18 and 3.19 the vesicles are concentrated towards one side of the paramural body, the rest of the body containing granular or fibrillar material. Those shown in Figures 3.20 and 3.21 have a greater proportion of their space occupied by the darkly-outlined vesicles, with little evidence of other material.

The few paramural bodies encountered in healthy cells contained irregularly shaped, darkly-outlined vesicles and no granular or fibrillar material (Fig. 3.22). The one depicted is in close association with a plasmodesma.

3.3.2 Young Bark Phloem Tissue

Extensive nacreous wall thickening was observed in all sieve elements in the yellow areas of infected bark (Figs. 3.23 and 3.24). However,

examination of healthy tissue revealed that most sieve elements displayed the same nacreous thickening (Fig. 3.25), although in a few it appeared to be absent (Fig. 3.26). No other ultrastructural alterations were encountered.

3.4 DISCUSSION

Considering the macroscopic leaf symptoms (Fig. 3.1) it is not surprising that gross changes in the chloroplasts were encountered in the yellow tissues of leaves showing typical leaf symptoms. Alterations in chloroplast structure in several virus-infected plant species displaying chlorotic symptoms have been reported. In turnip yellow mosaic virus-infected Chinese cabbage the chloroplasts in the yellow areas are often rounded, have few grana, are devoid of starch grains and eventually lose their identity while those in the green areas are apparently normal (Chalcroft and Matthews, 1966; Rubio-Huertos, Vela and López-Abella, 1967). These observations are very similar to those presented here for sunblotch-infected avocado, except that total loss of organelle identity was not detected. However, examination of older tissue may have shown this.

Disappearance of grana and stroma lamellae has also been reported in beet western yellows virus-infected lettuce (Tomlinson and Wells, 1978), while barley infected with barley stripe mosaic virus also contains disorganized chloroplasts in the chlorotic areas, and apparently normal chloroplasts in the green (Gardner, 1967), the former chloroplasts being swollen, possessing few grana, and containing peripheral lamellae. In the present study of leaf yellow tissues, similar peripheral lamellae (Fig. 3.8) and swollen chloroplasts (Figs. 3.2 , 3.6 , 3.9 and 3.10) were encountered.

Small clear vesicles of unknown function, similar to those shown in Figure 3.11 have been reported in the chloroplasts of TYMV-infected Chinese cabbage (Rubio-Huertos *et al.*, 1967). The other major type of vesicle encountered in the yellow tissue of infected avocado leaves (Figs. 3.12 and 3.13) is similar to those observed in chloroplasts of TMV-infected Xanthi n.c. tobacco (Hršel and Brčák, 1964), in TMV-infected *Nicotiana glutinosa* and Samsun NN tobacco (da Graça and Martin, 1976) and in wild cucumber mosaic virus-infected *Marah oreganus* (Allen, 1972). Their rôle in the infection cycle is unknown, but in none of the cases have they been encountered in all or even the majority of chloroplasts and therefore probably do not play a direct rôle in replication of the causal agents.

There is now circumstantial evidence that sunblotch is caused by a viroid. Mohamed and Thomas (1980) reported the occurrence of a small RNA species in close association with the chloroplasts and endoplasmic reticulum. The effects on the chloroplasts reported in the present study could be considered to have some correlation with this finding except for

(i) the absence of ultrastructural aberrations in green symptomless areas of infected leaves; and

(ii) recent evidence that this particular RNA molecule may not be the infectious agent (Semancik and Desjardins, 1980).

The occurrence of paramural bodies in virus-infected plant cells is quite well documented (Tu and Hiruki, 1971; Kim and Fulton, 1973; Bassi, Favali and Conti, 1974; Gill, 1974; Wheeler, 1974; McMullen, Gardner and Myers, 1977). Kim and Fulton (1973) suggested that these structures may play a rôle in the transport of metabolites into or out of the cytoplasm, or that they may be a response to unfavourable or pathogenic conditions. Their study on bean pod mottle virus-infected cells led them to conclude that the latter was true.

Semancik and Vanderwoude (1976) reported the occurrence of paramural bodies in citrus exocortis viroid (CEV)-infected Gynura aurantiaca, and proposed that this was a major cytopathogenic effect since they were absent in healthy cells. However, Wahn, Rosenberg-de Gomez and Sänger (1980 a; 1980 b) working with the same system, found paramural bodies in equal numbers in healthy and diseased tissue, but those in infected cells were malformed, including changes in vesicle size and shape. These studies on CEV-infected plants are relevant to the present study in view of the mounting evidence that sunblotch disease is caused by a viroid. The findings in this study differ slightly from both of the above. While present in both healthy and infected tissues with symptoms, they were more numerous and apparently structurally altered in terms of vesicle shape and absence of fibrillar material, in the latter. Their increased manifestation in the diseased cells certainly seems to be associated with symptom expression, and it is feasible to conclude that metabolite flow in such tissue is altered.

Since nacreous wall thickening is present in healthy avocado sieve elements (Esau and Cheadle, 1958), its occurrence in sunblotch-infected cells is probably not a cytopathogenic effect. However, the occurrence of some sieve elements lacking nacreous thickening only in healthy samples may indicate an indirect relationship to infection. Evert (1963) reported that in

healthy apple sieve elements the nacreous wall thickening disappears as the phloem matures. Should a similar process occur in avocado, it is possible that disappearance of nacreous thickening is impeded in diseased tissue.

The lack of development and differentiation of vascular tissue reported by Schroeder (1935), in bark tissues underlying external grooving, was not confirmed in this study.

3.5 ABBREVIATIONS USED IN FIGURES

Cp	.....	Chloroplast
CW	.....	Cell wall
D	.....	Dictyosome
G	.....	Granum
M	.....	Mitochondrion
N	.....	Nucleus
NT	.....	Nacreous thickening
P	.....	Plasmalemma
PB	.....	Paramural body
Pd	.....	Plasmodesma
PP	.....	Phloem plastid
SP	.....	Sieve plate
St	.....	Starch grain

3.6 EXPLANATION OF FIGURES

FIGURE 3.2 Cells in the yellow region of a young sunblotch-infected avocado leaf, one containing an apparently normal nucleus, another possessing swollen, disorganized chloroplasts and paramural bodies.

FIGURE 3.3 Chloroplast in a mature healthy avocado leaf cell.

FIGURE 3.4 Apparently normal chloroplast in the green region of a mature sunblotch-infected avocado leaf.

FIGURE 3.5 Abnormal chloroplast in the yellow region of a mature sunblotch-infected cell with its membranes reduced to one granum. Adjacent mitochondria apparently normal.

FIGURE 3.6 Swollen and internally disorganized chloroplast in the yellow area of a mature sunblotch-infected avocado leaf.

FIGURE 3.7 Chloroplast in the yellow region of a mature sunblotch-infected avocado leaf showing only lateral displacement of the lamellae system.

FIGURE 3.8 Disorganized chloroplast in the yellow area of a young infected avocado leaf. Note the lamellae lining the periphery of the organelle.

FIGURE 3.9 Disorganized, swollen chloroplast in the yellow area of a young sunblotch-infected leaf with its lamellae in the form of rings.

FIGURE 3.10 Disorganized, swollen chloroplast in the yellow area of a mature sunblotch-infected leaf. Lamellae appear to be connected forming loops between the grana.

FIGURE 3.11 A mature leaf cell chloroplast in the yellow area of an infected leaf with large grana and vesicles (arrowed) lacking internal structure.

FIGURES 3.12 and 3.13 Chloroplasts in the yellow zone of a mature sunblotch-infected avocado leaf possessing membrane-bound vesicles (arrowed) with poorly defined internal organization.

FIGURE 3.14 Slightly disorganized chloroplast in the yellow region of a mature infected leaf with adjacent apparently normal mitochondria and dictyosome.

FIGURES 3.15 and 3.16 Paramural bodies in the yellow area of a sunblotch-infected young leaf containing lightly stained vesicles.

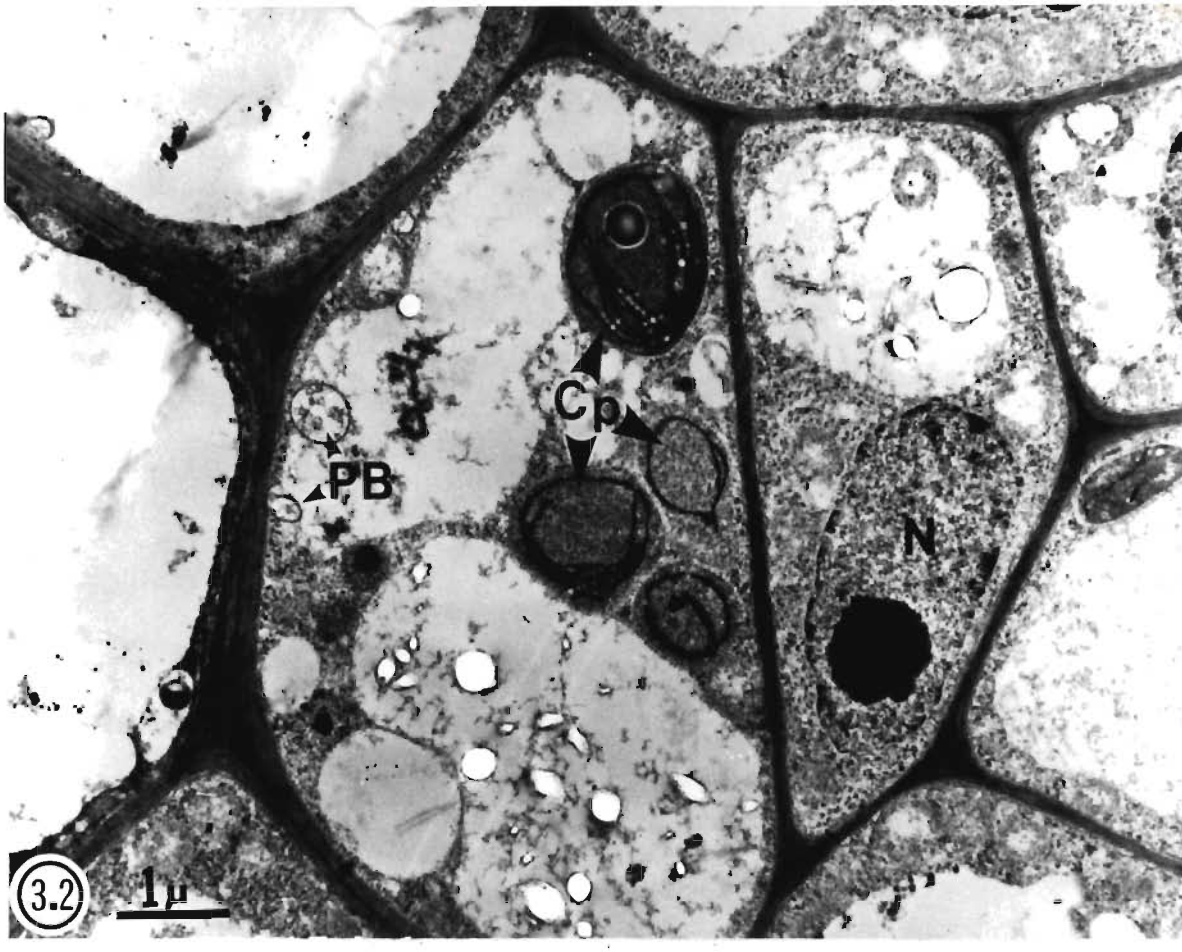
FIGURES 3.17, 3.18, 3.19, 3.20 and 3.21 Paramural bodies containing darkly outlined vesicles and fibrillar and granular material in the yellow region of sunblotch infected mature leaf.

FIGURE 3.22 Paramural body in a healthy mature avocado leaf cell in association with a plasmodesma.

FIGURES 3.23 and 3.24 Nacreous wall thickening in sieve elements of young avocado bark with sunblotch symptoms.

FIGURE 3.25 Nacreous wall thickening in sieve elements of healthy young avocado bark.

FIGURE 3.26 Healthy avocado sieve element without nacreous thickening.


Cp
PB
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3.2
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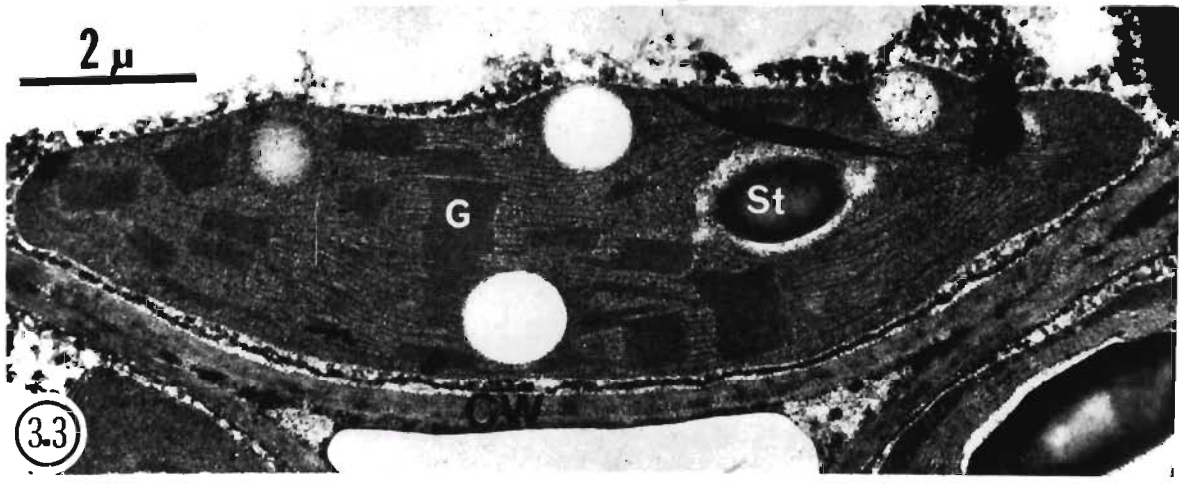
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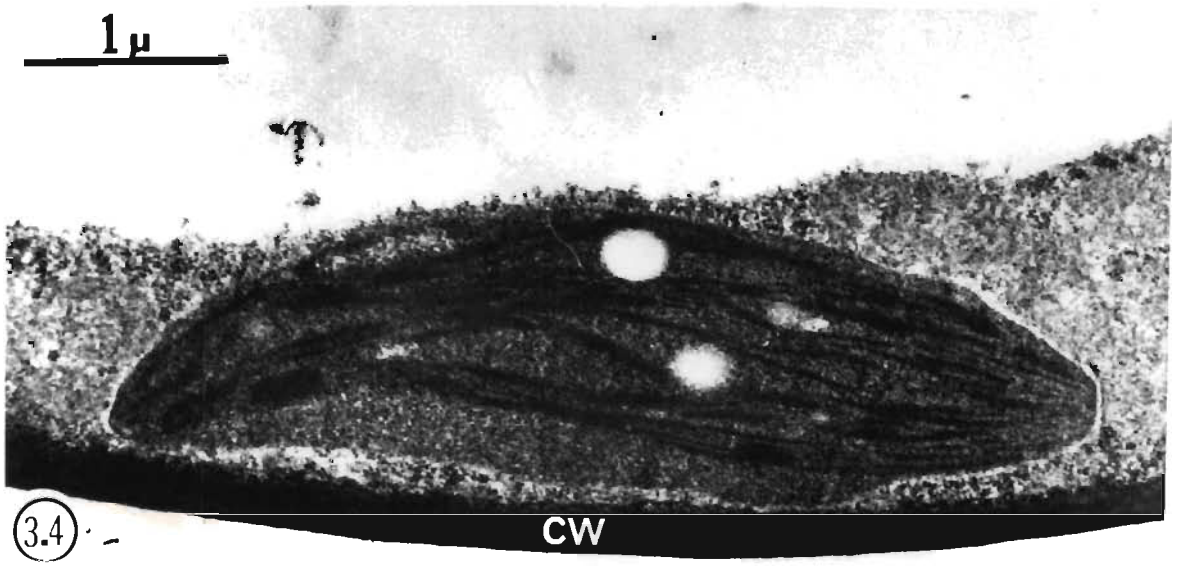
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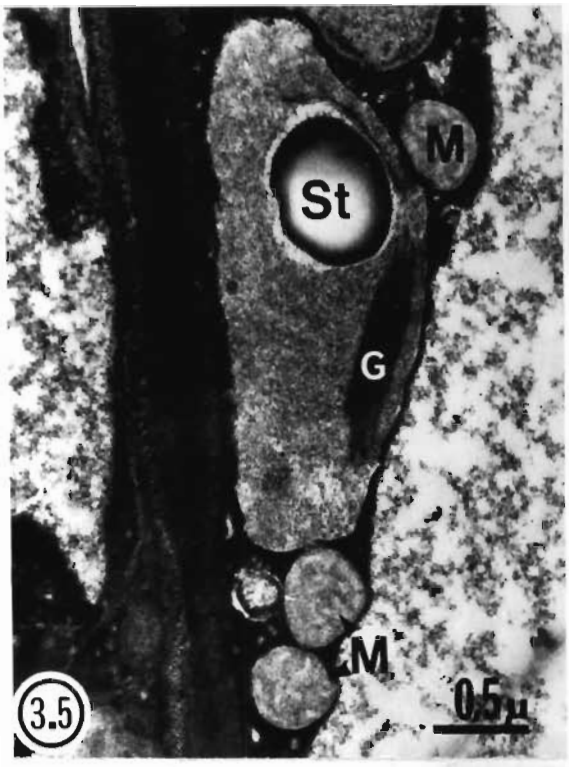

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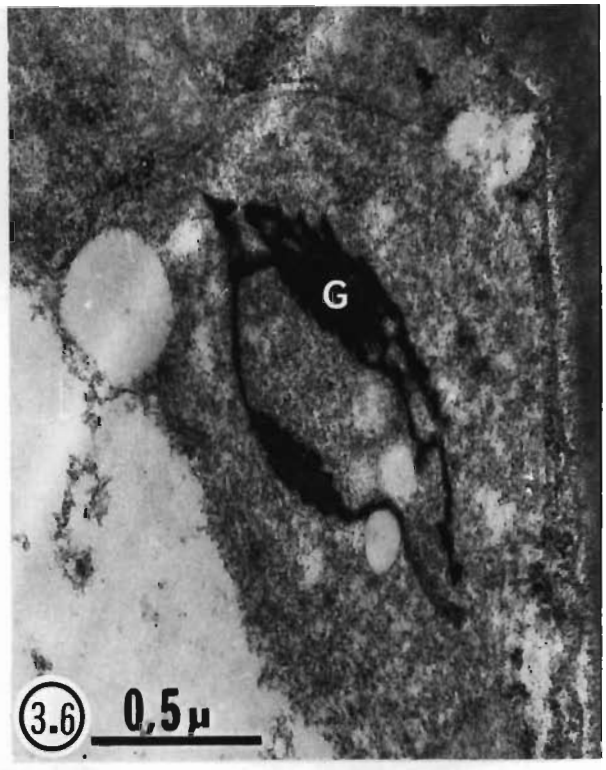

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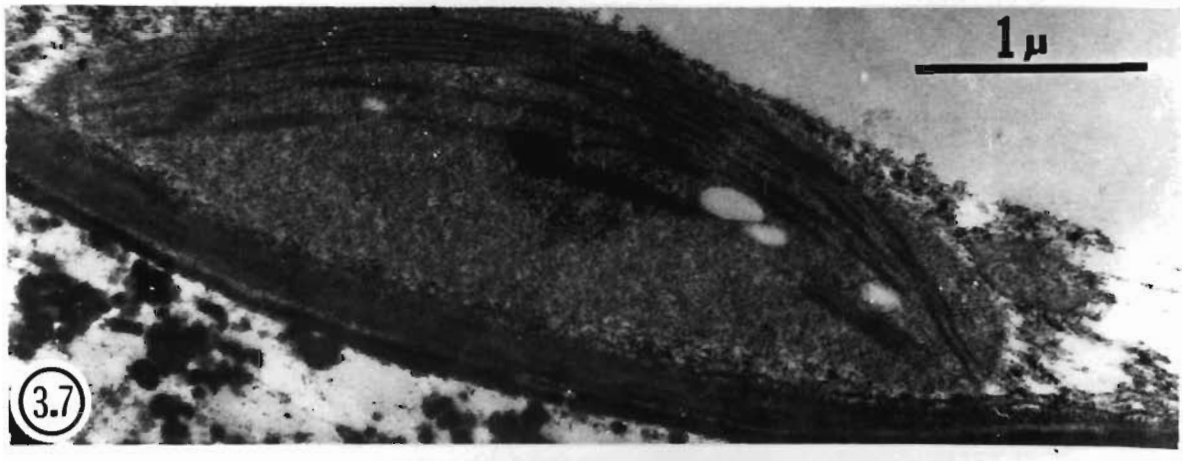

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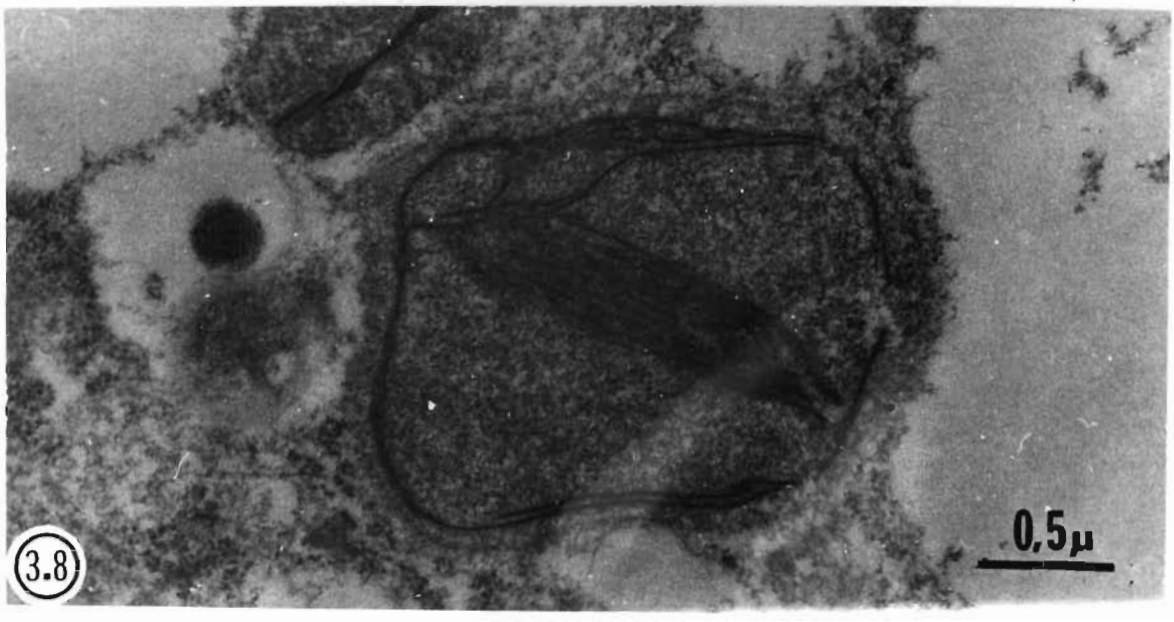

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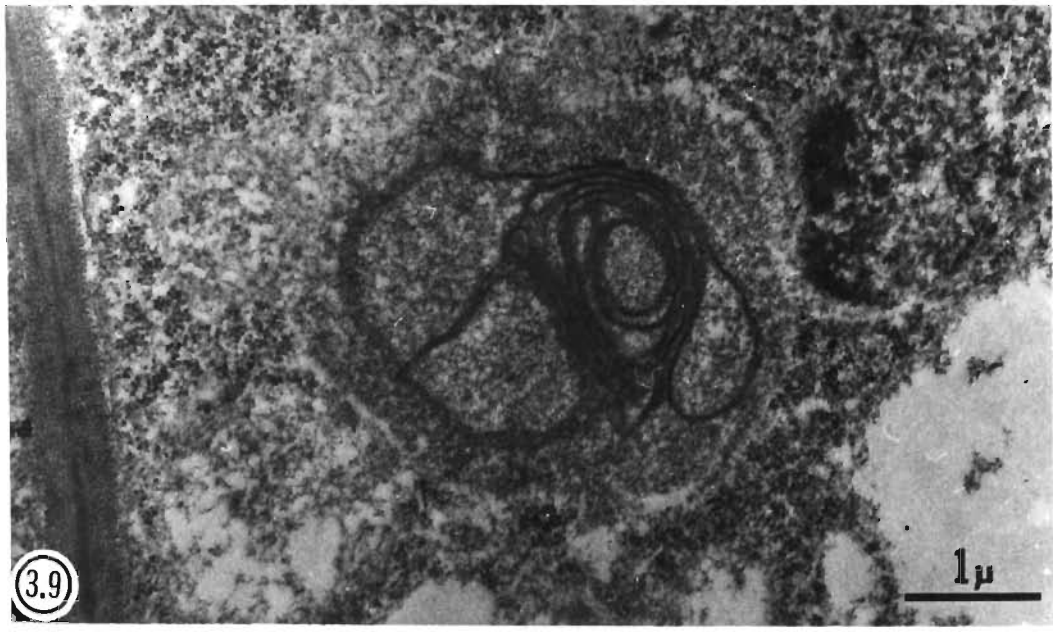
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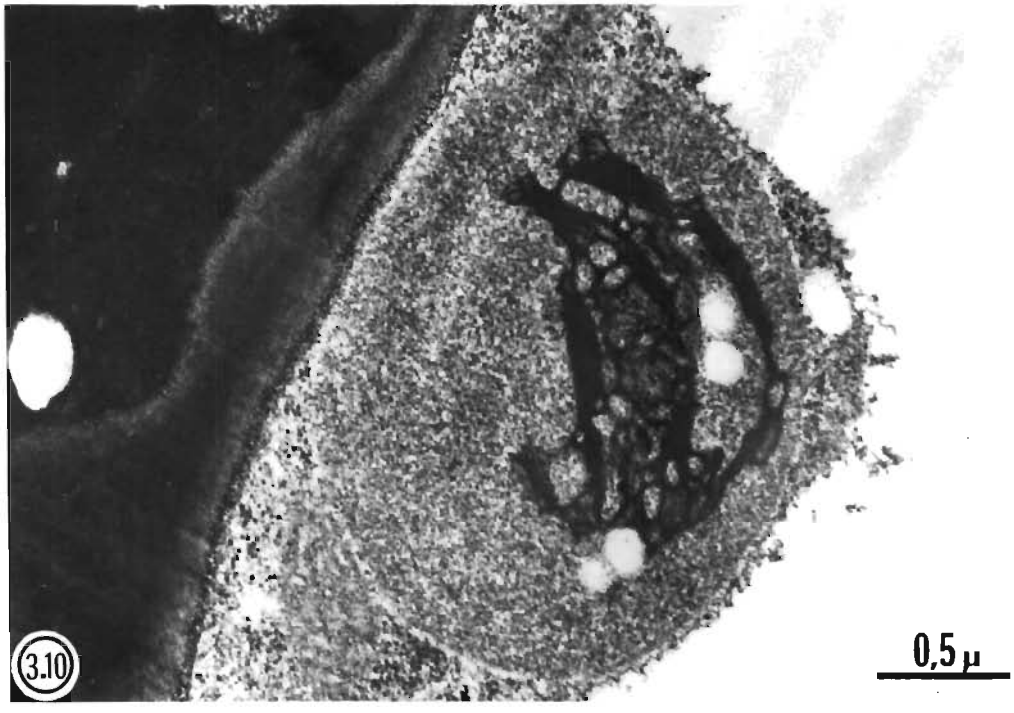
1µ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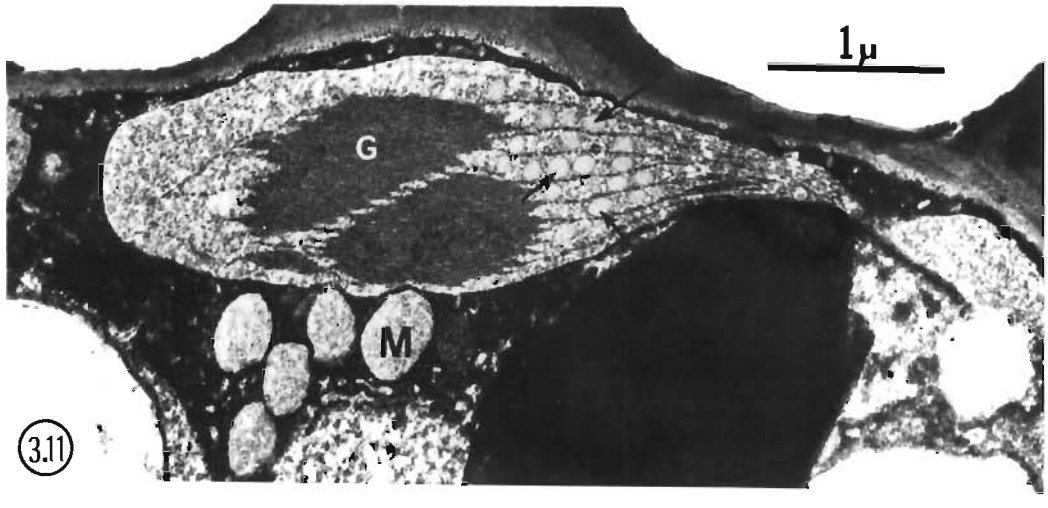
1µ
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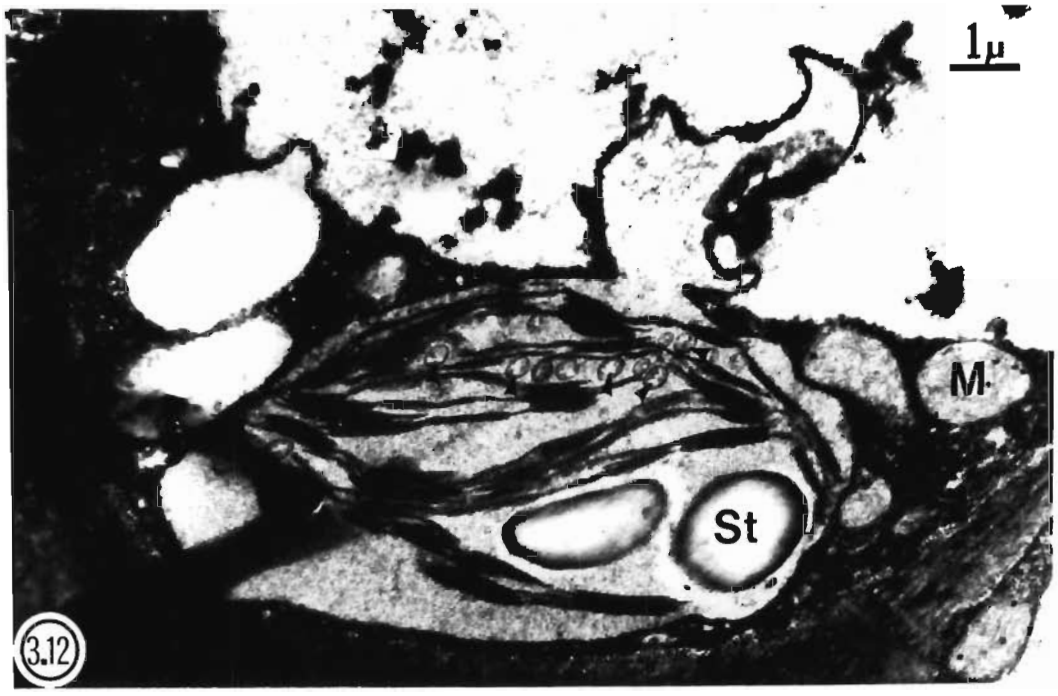

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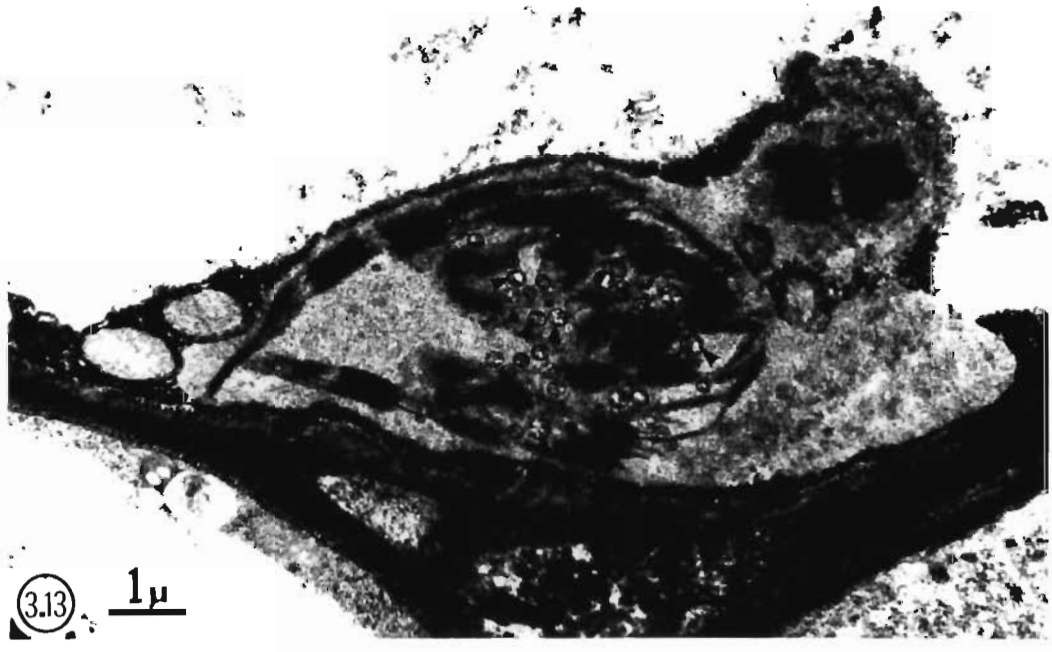

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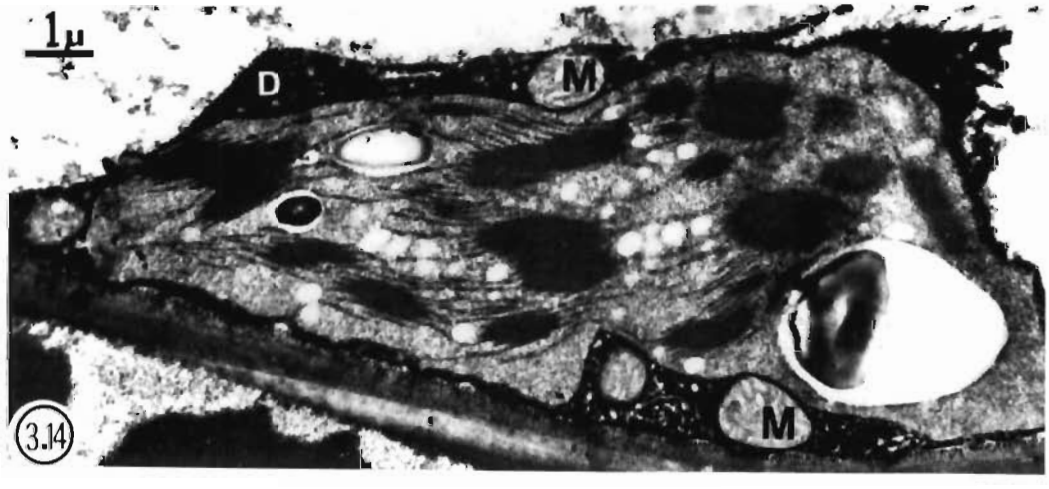

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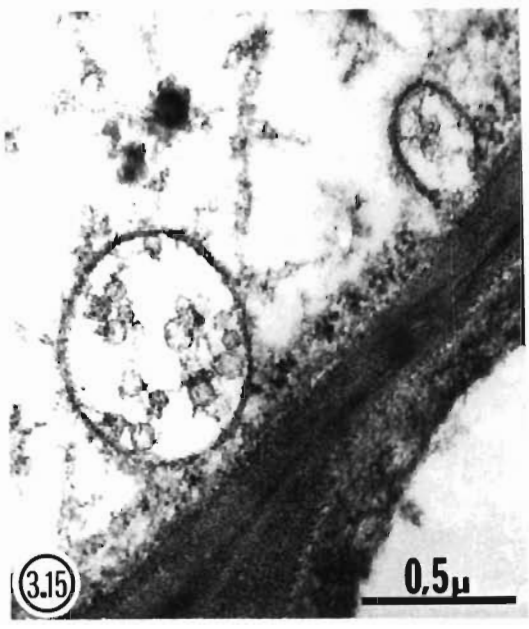
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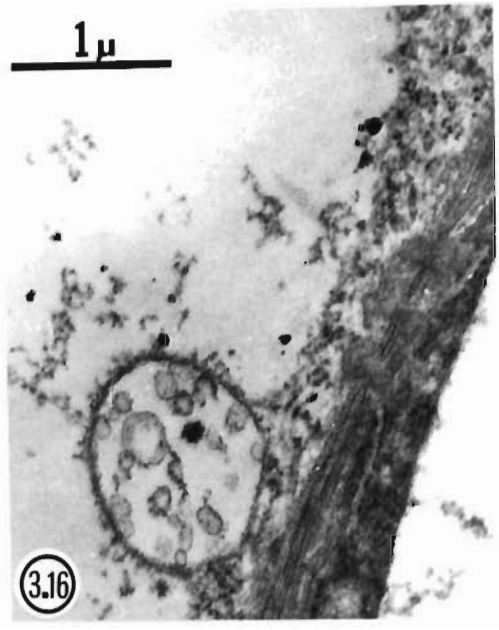
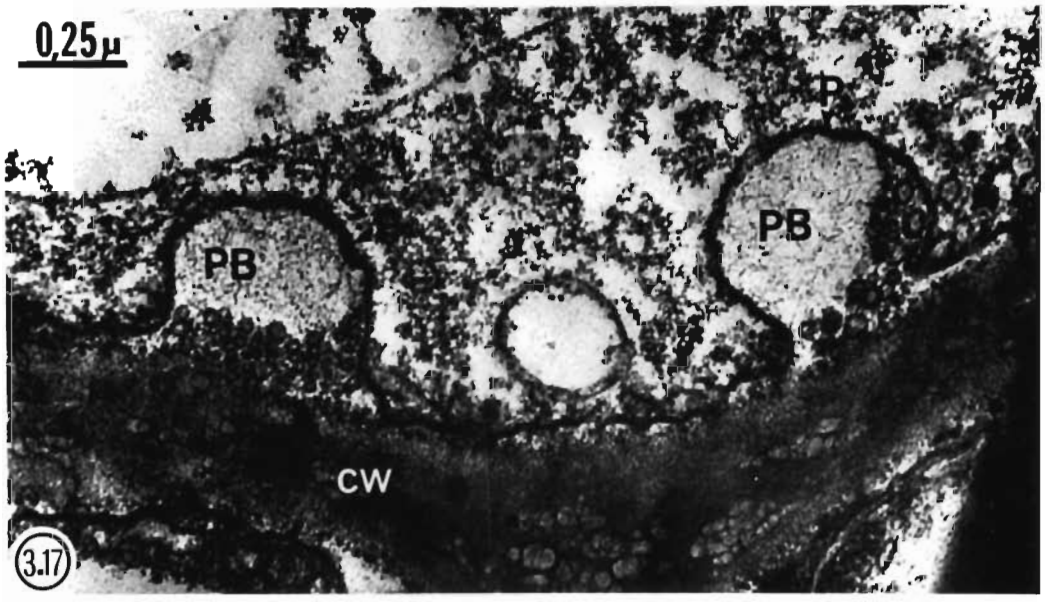
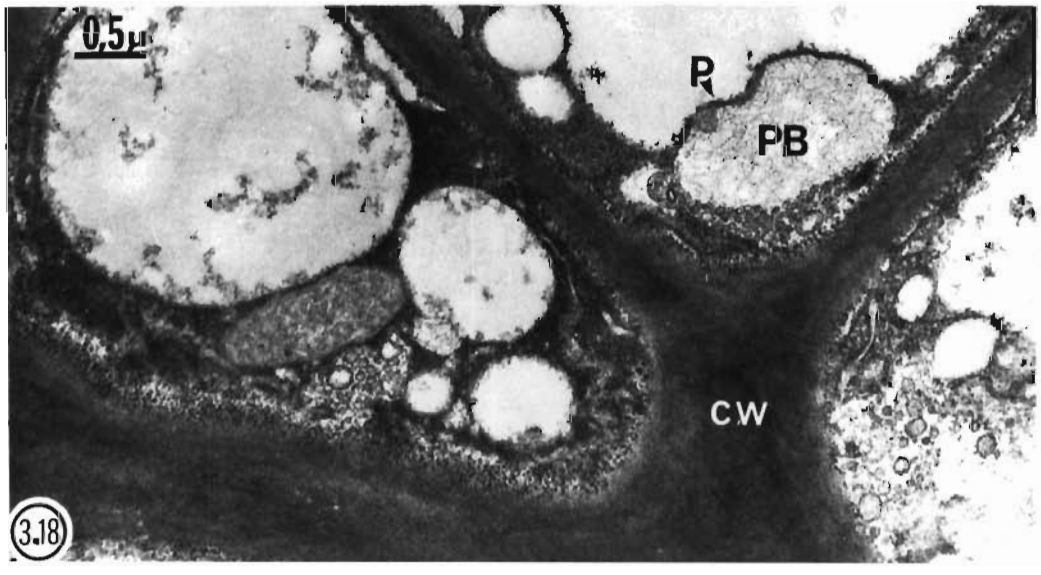
3.15


1µ
3.16


0,25µ
PB
PB
CW
3.17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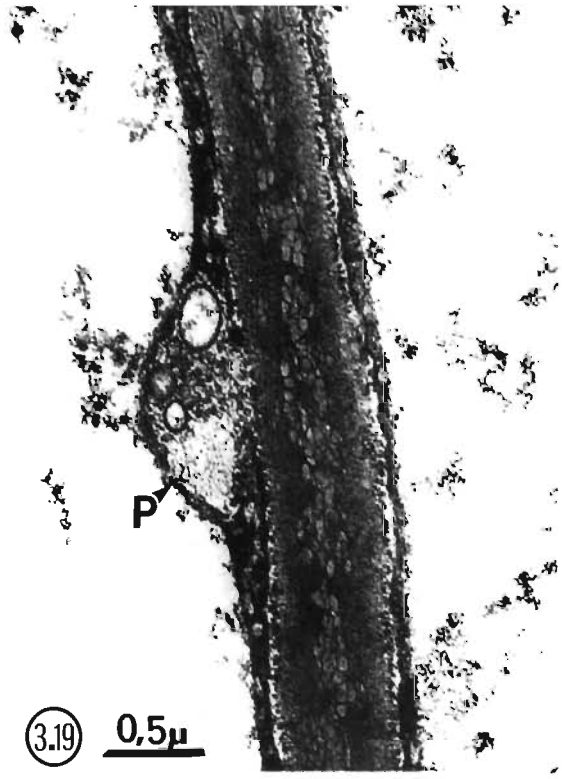
P
PB
CW
3.18



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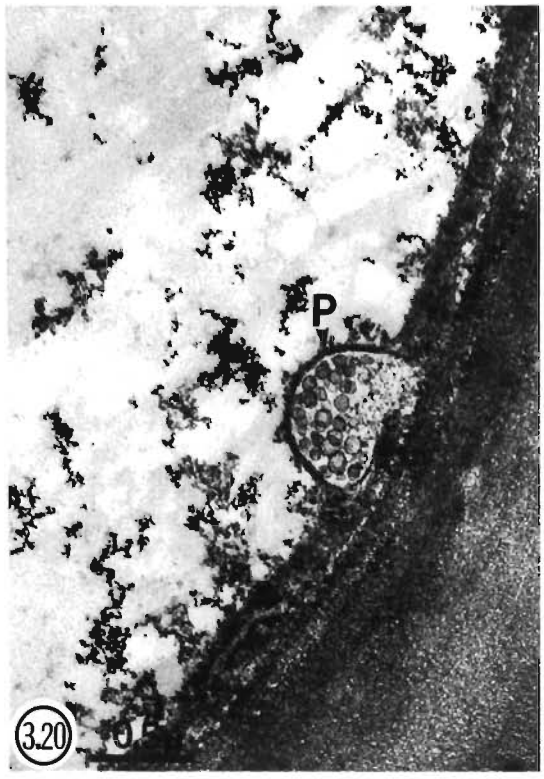

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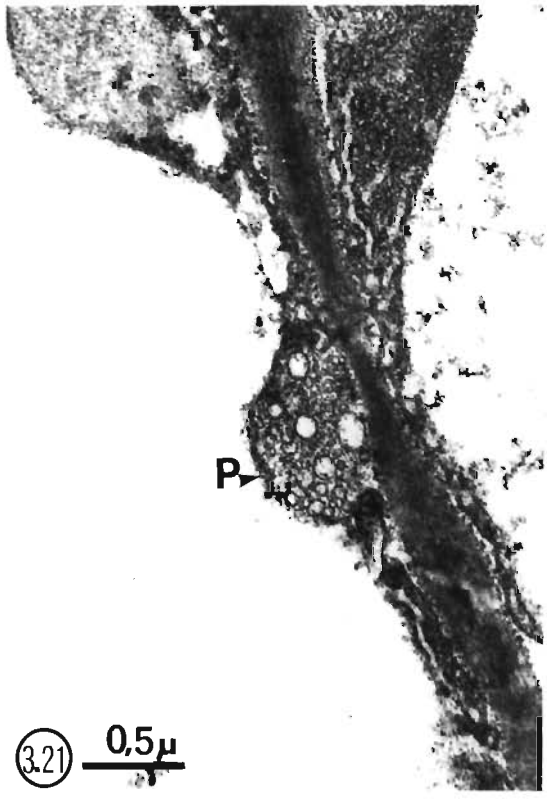

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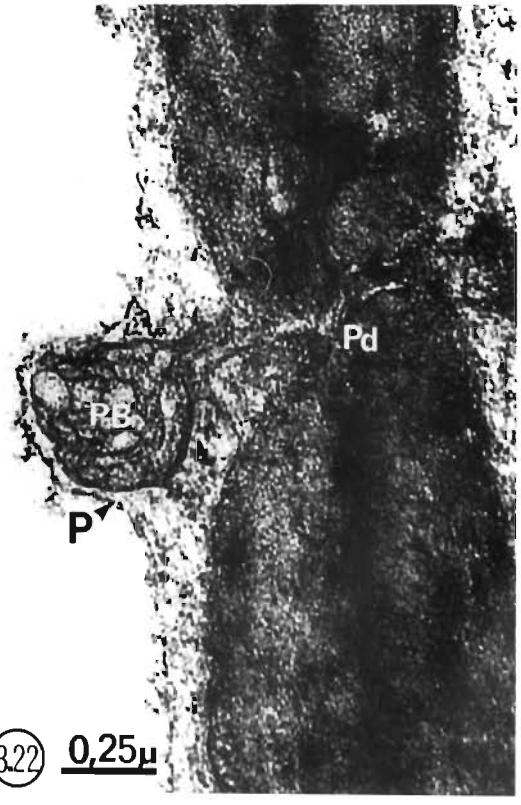

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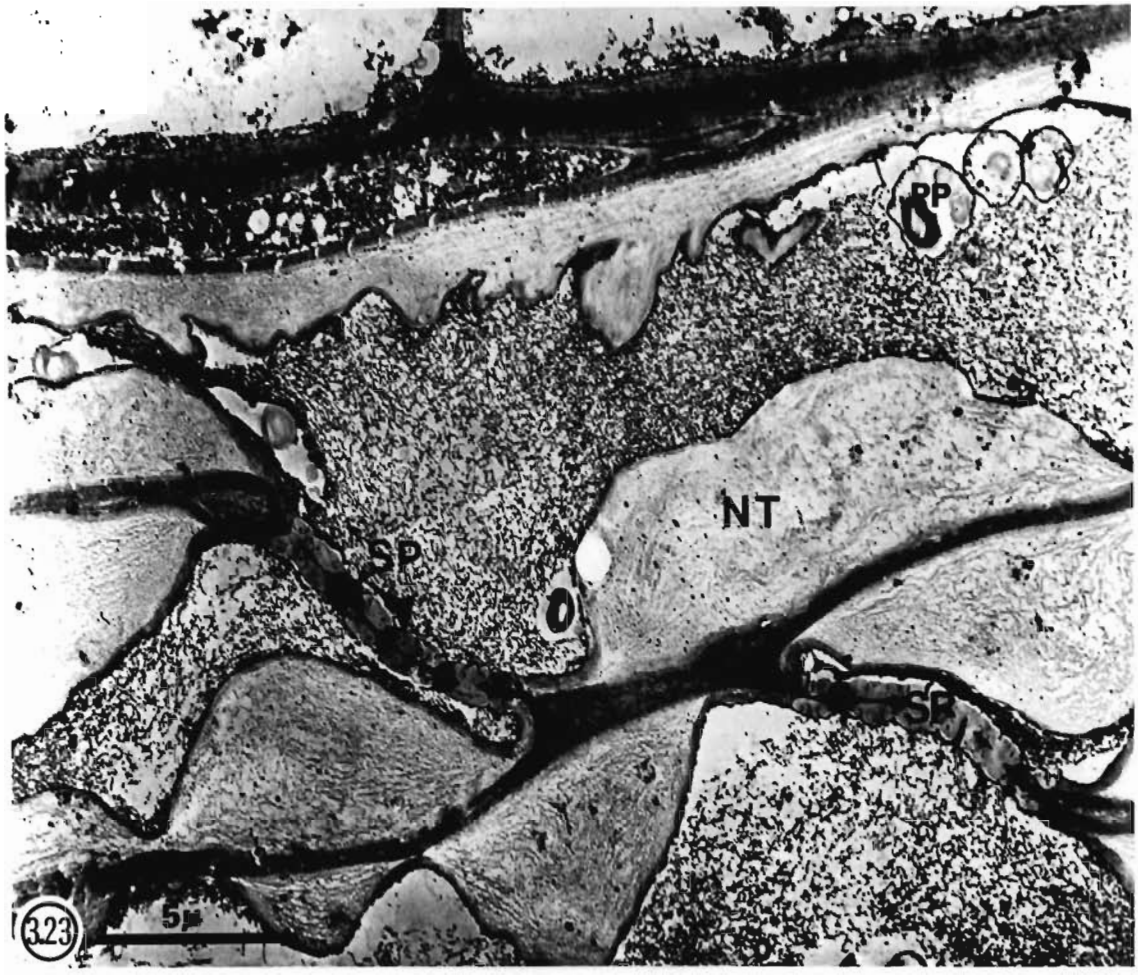

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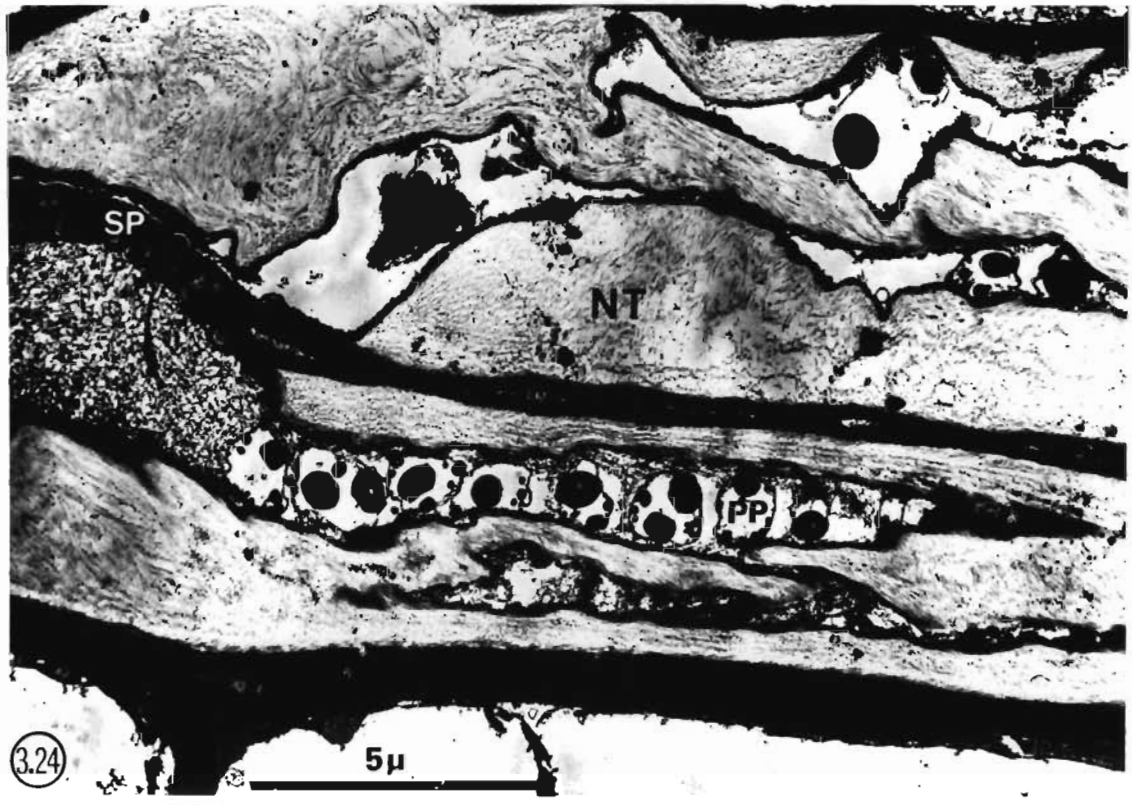
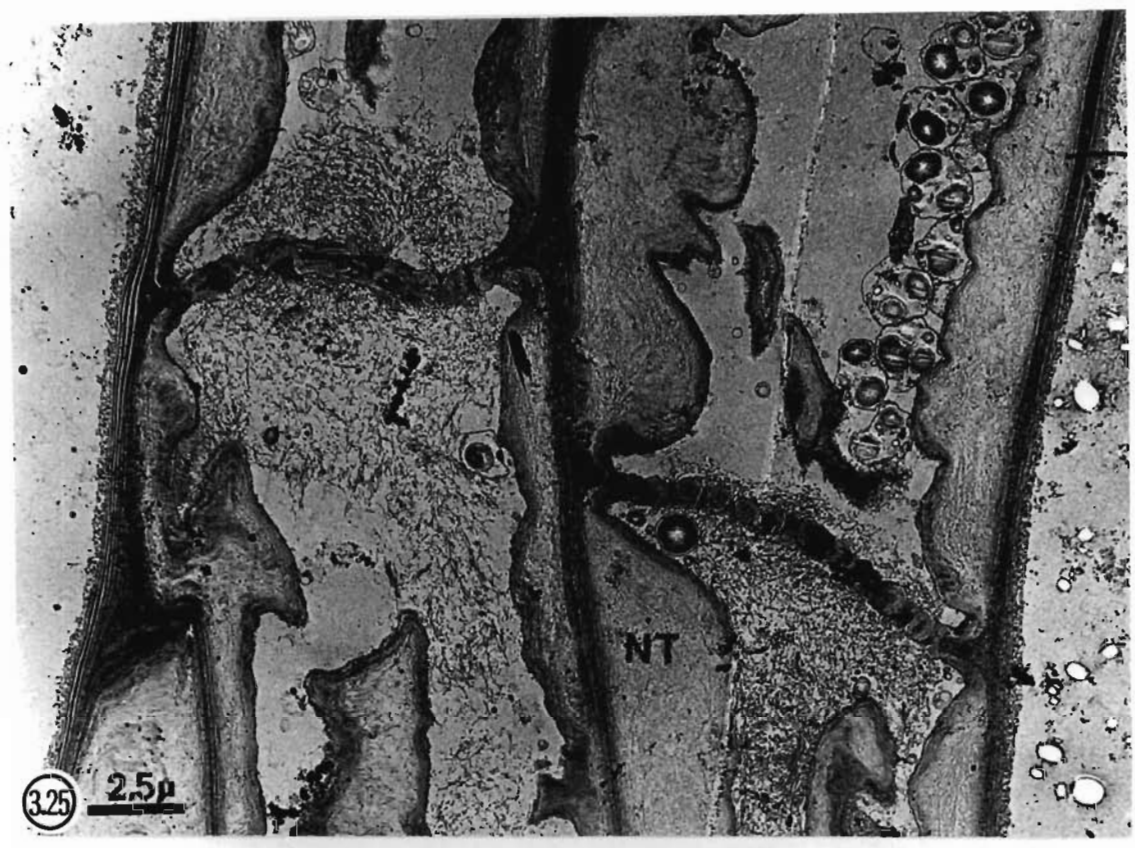


3.24


NT
3.25
2,5µ


SP
PP
3.26
2,5µ

CHAPTER 4

STUDIES ON THE VIROID HYPOTHESIS OF AVOCADO SUNBLOTCH DISEASE

4.1 INTRODUCTION

Thomas and Mohamed (1979) and Dale and Allen (1979) simultaneously presented evidence from PAGE studies that there is a small RNA species associated with avocado sunblotch disease, and suggested that the disease is caused by a viroid. This RNA has since been shown to be a covalently closed circular molecule smaller than known viroids (Palukaitis et al., 1979) with a molecular weight of 65 000 daltons (Mohamed and Thomas, 1980).

Recently, Semancik and Desjardins (1980) detected five new small RNA species in infected avocado which they termed ASV_{1-5}, one of which (ASV_5) appears to correspond to that previously described by the other groups mentioned above. This result was achieved using ethidium bromide instead of toluidine blue to stain the gels. The authors concluded that ASV_4 is probably the pathogenic species, although final proof will have to await successful infectivity studies.

ASV_5 appears most strongly in extracts of symptomless carrier material. If it proves to be a specific sunblotch-associated molecule, it could provide a useful marker for detecting sunblotch in symptomless carriers.

To complete this study on sunblotch, attempts were made to develop a rapid PAGE test for sunblotch-associated RNA's in leaf tissue, suitable for routine application in a proposed avocado improvement programme.

4.2 MATERIALS AND METHODS

Two methods were used, the first being more protracted requiring approximately 30 to 50 hours for execution, while the second requires less than 6 hours.

METHOD A:

Features of several reported techniques for extracting viroid RNA's were incorporated into the following method.

Fifteen grams of mature healthy avocado leaves, or mature symptomless leaves from symptom-bearing Collinson seedlings, or from symptomless carrier Edranol seedlings, were washed and dried. Each sample was homogenized for

2 minutes with an Ultra Turrax macerator in 50 mℓ buffer [0,2M glycine, 0,1M Na_2HPO_4 , 0,6M NaCl , 0,1M Na_2SO_3 , 0,1% sodium diethyldithiocarbamate (DEDTC), 1% sodium lauryl sulphate - pH adjusted to 9,5 with NaOH], 12 mℓ chloroform, 12 mℓ n-butanol, 10 mℓ water-saturated phenol (with 0,1% 8-hydroxyquinoline) and 2,5 g polyvinyl pyrrolidone (PVP-40). This extraction method is similar to that used by Morris and Wright (1975) and Morris and Smith (1977) for potato spindle tuber viroid (PSTV), except that Na_2SO_3 and DEDTC were used instead of mercaptoethanol, PVP-40 was included to absorb tannins (Mohamed and Thomas, 1980) and chloroform-butanol replaced chloroform-pentanol (Schumann, Thurston, Horst, Kawamoto and Nemoto, 1978).

The homogenate was centrifuged at 10 000 g for 20 minutes at 4° C , and the aqueous supernatant collected. To this was added $\frac{1}{5}$ volumes of 10M LiCl before placing on ice for 2 hours to precipitate large nucleic acids and centrifuging at 8 000 g for 15 minutes at 4° C . To reduce the volume of the supernatant all the remaining small nucleic acids were precipitated by adding to volumes of 95% ethyl alcohol, placing on ice for 2 hours, and then centrifuging and resuspending the pellet in 5 mℓ distilled water. Insoluble matter was pelleted at 10 000 g for 20 minutes and the supernatant was dialysed overnight in the cold against distilled water. The nucleic acids were again precipitated on ice for 2 hours with two volumes of 95% ethyl alcohol plus a few drops of 4M sodium acetate (Morris and Smith, 1977), centrifuged at 10 000 g for 20 minutes and resuspended in 0,1 mℓ distilled water. A drop each of 0,05% bromophenol blue and glycerol were then added.

Electrophoretic separation was performed on 5% polyacrylamide gels (Adesnik, 1971). The gels were prepared as follows:

4 mℓ acrylamide-MBA (15 g acrylamide, 0,375 g MBA per 100 mℓ)
4 mℓ 3X buffer (0,12M tris, 0,06M sodium acetate, 0,003M disodium EDTA, pH adjusted to 7,2 with acetic acid)
3,78 mℓ distilled water
0,23 mℓ 0,1% ammonium persulphate (freshly prepared)
0,02 mℓ TEMED.

The reservoir buffer used was 40 mM tris, 20 mM sodium acetate, 1 mM disodium EDTA (pH 7,2).

The gels were pre-run for 30 minutes at 3,5 mA per tube. After loading with the 0,1 mℓ nucleic acid extract they were run at room temperature at 3,5 mA per tube for 15 minutes and then at 6 mA per tube until the tracer dye was 1 cm from the bottom of the gel (Mosch, Huttinga, Hakkaart and Bokx, 1978).

The gels were either

(i) stained overnight with 0,1% (Mosch et al., 1978) or 0,01% (Morris and Smith, 1977) toluidine blue, and destained with several changes of 5% acetic acid (Mohamed and Thomas, 1980); or

(ii) were stained with ethidium bromide in 0,001M EDTA for 15 minutes and destained for 15 minutes in 0,001M EDTA, and then viewed under ultraviolet light using a UVL-22 Blak-Ray longwave lamp.

Two concentrations of ethidium bromide used by other workers, viz. 0,75 µg/mℓ (Semancik and Desjardins, 1980) and 20 µg/mℓ (Pfannenstiel, M. A., personal communication), were compared with an intermediate concentration of 7,5 µg/mℓ.

METHOD B:

In order to speed up RNA extraction, the LiCl fractionation was included in the maceration step and dialysis was omitted (Pfannenstiel, Slack and Lane, 1979). The method used for PSTV (Pfannenstiel, personal communication) was slightly modified for avocado sunblotch. Five grams of each of healthy and symptomless sunblotch-infected avocado leaves, the latter from plants with symptoms or symptomless carriers, were macerated on ice in 5 mℓ distilled water, 2 mℓ 4M NH_4OH , 2 mℓ 0,1M EDTA, 6 mℓ 10M LiCl , 20 mℓ water-saturated phenol (with 0,1% 8-hydroxyquinoline) and 0,7 g PVP-40. Each homogenate was centrifuged at 10 000 g for 15 minutes at 4° C and the aqueous supernatant withdrawn and mixed with two volumes of 95% ethyl alcohol. The mixture was then incubated at -10° C for 30 minutes. After centrifugation at 10 000 g for 20 minutes, the pellet was resuspended in 5 mℓ distilled water and again precipitated by adding ethyl alcohol, this time in the presence of a few drops of 4M sodium acetate. After centrifugation at 10 000 g for 15 minutes the resultant pellet was resuspended in 0,1 mℓ distilled water. Each sample was divided into two on a 2 : 1 basis, and both placed on 5% polyacrylamide gels. Electrophoretic separation was performed as described above and the gels were stained with 7,5 µg/mℓ ethidium bromide in EDTA, destained in EDTA and viewed under ultraviolet light.

4.3 RESULTS

Using toluidine blue to stain the gels, one additional RNA band appeared about halfway down the gels of infected symptomless carrier samples but not in gels similarly prepared with extracts of plants with symptoms or healthy plants. In some cases the host 9S RNA appeared in healthy and diseased

samples above the position of the new species (Fig. 4.1), but more frequently it was not visible (Fig. 4.2). Toluidine blue used at 0,01% was found to be more satisfactory than 0,1% because destaining was more rapid.

Five additional bands were visible in gels of infected symptomless carrier samples stained with 7,5 µg/mℓ ethidium bromide (Fig. 4.3). The position of the smallest species corresponded to that of the single additional band shown as ASV in Figures 4.1 and 4.2. Staining with 0,75 µg/mℓ was found to understain the gels, while 20 µg/mℓ stained the entire gel and no bands could be distinguished.

Applying the rapid extraction method only two of the new species, ASV_3 and ASV_5, were detected in samples both from plants with symptoms and symptomless carriers (Fig. 4.4). Better band separation was achieved with the smaller volume.

4.4 DISCUSSION

The results obtained in this study using toluidine blue gel stain confirm Thomas and Mohamed's (1979) observation that the new sunblotch-associated RNA species could be detected only in extracts of symptomless carrier plants. The detection of five sunblotch-associated RNA species using ethidium bromide (Semancik and Desjardins, 1980) was also confirmed in this study. Infectivity studies by both these groups on these species have been initiated (Thomas and Mohamed, 1979; Mohamed and Thomas, 1980; Semancik and Desjardins, 1980). The lower sensitivity of toluidine blue was also evident in this study in its inability always to detect the host 9S RNA (Fig. 4.2), whereas ethidium bromide always showed its presence.

PAGE offers a rapid and sensitive technique for the detection of viroids, and the strong evidence available indicating the viroid nature of the sunblotch agent means that indexing which previously took 18 months or more to complete (Burns *et al.*, 1968; 1969) can now be done rapidly. Thomas and Mohamed (1979) state that their method takes four days to complete, whilst methods for PSTV and chrysanthemum stunt viroid detection take three days (Morris and Wright, 1975; Morris and Smith, 1977; Mosch *et al.*, 1978). Use of ethidium bromide not only increases the sensitivity of the method, it also reduces the time to two days since the overnight staining step is eliminated.

Results presented in this study show that Method 2, which is a modified version of that recently developed for PSTV (Pfannenstiel *et al.*, 1979), achieves a further reduction in time reducing the entire test to less than 6 hours. It should now be possible to test large numbers of avocado trees in

a very short period.

According to Palukaitis *et al.* (1979) hybridization analysis with ^{32}P-labelled complementary DNA is more sensitive than PAGE, and was able to detect the sunblotch-associated RNA (ASV_5) in three cases from which no band was visible in toluidine blue stained gels. However, it has been shown by both Semancik and Desjardins (1980) and the present author that ethidium bromide is far more sensitive than toluidine blue, and it may not therefore be necessary to conduct hybridization analysis for sunblotch indexing.

Semancik and Desjardins (1980) have shown that ASV_{1-3} are probably aggregates of ASV_5, and that ASV_4, which most resembles known viroids, is probably the causal agent of sunblotch. ASV_5 is probably a non-infecting viroid-associated molecule, similar to CEV_x of exocortis (Semancik and Desjardins, 1980) and $ccRNA_1$ of cadang-cadang (Randles, 1975). The differences in ASV_5 concentrations in extracts of tissue from plants with symptoms and symptomless carriers (Fig. 4.1) indicate that it may play a rôle in symptom expression. Semancik and Desjardins (1980) suggest that ASV_5, CEV_x and $ccRNA_1$ may all be involved in this, possibly in an analogous manner to the small RNA molecule associated with cucumber mosaic virus (CMV), CARNA 5 (Waterworth, Kaper and Tousignant, 1979) which increases symptom severity in tomatoes, but causes a drastic reduction of symptoms in *Zea mays* and *Capsicum frutescens*. The apparently higher levels of ASV_5 in symptomless carrier avocado trees may similarly have a symptom suppressing effect.

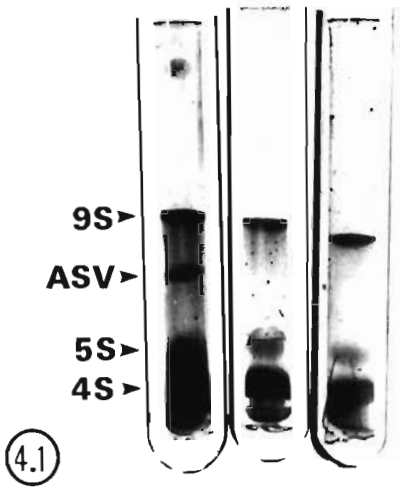
4.5 EXPLANATION OF FIGURES

FIGURE 4.1 PAGE of avocado leaf RNA's stained with 0,01% toluidine blue. From left to right: sunblotch-infected (symptomless carrier), infected (with symptoms), healthy.

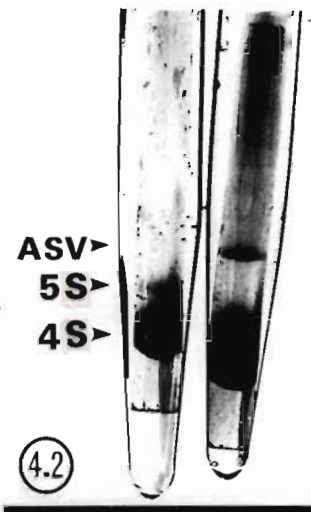
FIGURE 4.2 PAGE of avocado leaf RNA's stained with 0,01% toluidine blue. No 9S RNA is visible.
Left: healthy;
Right: sunblotch-infected (symptomless carrier).

FIGURE 4.3 PAGE of avocado leaf RNA's stained with ethidium bromide (two-day method).
Left: sunblotch-infected (symptomless carrier);
Right: healthy.

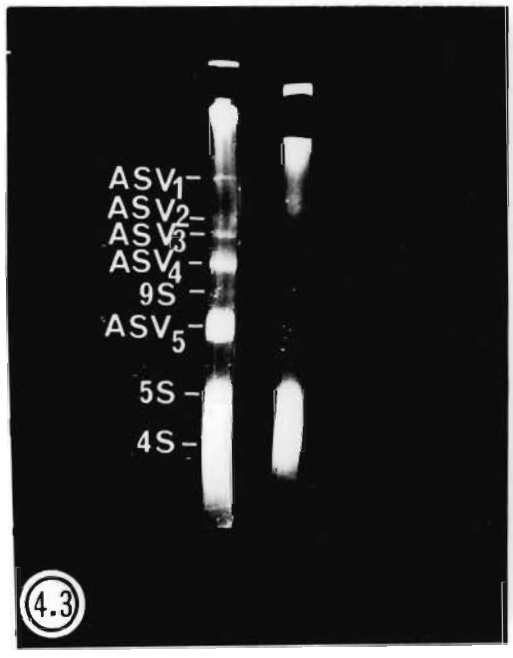
FIGURE 4.4 PAGE of avocado leaf RNA's stained with ethidium bromide (6-hour method).
From left to right: sunblotch-infected (symptomless carrier), infected (with symptoms), healthy.


9S
ASV
5S
4S

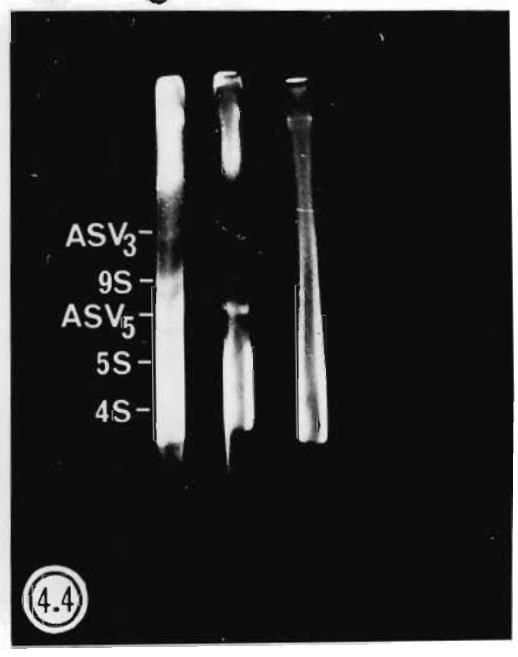
4.1


ASV
5S
4S

4.2


ASV1
ASV2
ASV3
ASV4
9S
ASV5
5S
4S

4.3


ASV3
9S
ASV5
5S
4S

4.4

SUMMARY

Avocado sunblotch disease is a graft-transmissible disorder known for over 60 years and has now been recorded in at least eight countries around the world. Affected trees develop yellow, depressed streaks on young stems and fruit, marked rectangular cracking of the mature bark and a decumbent style of growth. Often a tree with symptoms produces completely symptomless shoots, termed 'recovery' growth, which are latently infected. There is a reported 95 to 100% transmission of sunblotch through the seed of such branches, and the resultant seedlings are themselves symptomless.

Indexing for sunblotch to ensure that scion and, in view of seed transmission, especially rootstock material is free of the disease is very important. The standard method used for many years has been to graft tissue onto healthy indicator seedlings and observe for symptom development for 18 months to two years.

One aim of the study presented in this thesis was to develop more rapid methods for detecting the sunblotch agent. By conducting the standard indexing method in a glasshouse at controlled high temperatures of 30/28° C (day/night) and by cutting back the indicator plants every three months, the time was reduced from two years to eight months.

While this represents a considerable saving in time, the ideal must be to develop a laboratory diagnostic test that requires no more than a few days, at most, to complete. A comparative study was therefore initiated on the phenol metabolism of healthy and sunblotch-infected avocados to determine whether infection causes any major change that may reliably serve as a marker for diagnostic purposes. Significantly increased peroxidase (PO) and phenylalanine ammonia-lyase (PAL) activities, decreased indoleacetic acid (IAA) oxidase activity and higher sinapic acid levels were detected in bark tissue showing sunblotch symptoms, but not in symptomless 'recovery' growth. In contrast, increased polyphenoloxidase (PPO) activity and isoenzymes, total soluble protein levels, water soluble phenols and reduced ferulic acid levels were found in the bark of all infected trees tested, both with symptoms and symptomless. However, these latter changes have been associated with other plant-virus systems and are therefore not necessarily specific for sunblotch. Neither is any sufficiently large to be definitive as a diagnostic test. Two unidentified phenols were detected in infected, mature bark, but not in infected young bark and leaves.

Recent evidence indicating that the sunblotch agent is probably a viroid has introduced the possibility of rapid disease detection by polyacrylamide gel electrophoresis (PAGE) of extracted RNA's as used for known viroids. In this study the presence of previously reported small molecular weight sunblotch-associated RNA's was confirmed using PAGE methods requiring two to four days to complete. This thesis presents as a further development a more rapid method of PAGE detection of RNA's enabling indexing for sunblotch to be completed in under six hours.

Whilst the biochemical studies did not reveal diagnostically meaningful differences between healthy and infected avocados, there were tendencies towards differences between healthy and symptomless carrier tissues, further investigation of which may lead to a future understanding of symptom development and the symptomless condition. These include apparent higher PO and lower PAL activities in symptomless carrier tissue, as well as higher PO isoenzyme a_1 and lower IAA oxidase isoenzyme a_1 activities.

General studies on sunblotch-infected avocados showed that fruit from symptomless 'recovery' growth branches are significantly larger and have a higher oil content than those from healthy or diseased branches, the latter finding possibly indicating a more advanced state of maturity of 'recovery' growth fruit due to earlier flowering.

The avocado sunblotch agent was shown to have an *in vivo* thermal inactivation point of 55° C , a temperature higher than the avocado tissue can withstand thereby eliminating the possibility of thermotherapy of infected twigs.

In a host range study four lauraceous plant species, *Persea Schiedeana*, *Cinnamomum zeylanicum*, *C. camphora* and *Ocotea bullata*, were successfully infected with sunblotch by grafting from infected avocado. This is the first demonstration of any host other than avocado. A phanerogametic member of the same family, *Cassytha filiformis*, was shown to be able to transmit the disease from avocado to avocado. No hosts from other families were found.

During an electron microscope study of sunblotch-infected avocado leaf tissue, gross alterations of the chloroplasts in the yellow areas were observed. These changes included organelle swelling, loss of grana and stroma lamellae, rearrangement of remaining membranes and presence of vesicles. Also in the yellow areas paramural bodies were encountered in higher numbers and displaying altered structure than in healthy and symptomless infected leaf tissue.

This study on avocado sunblotch disease was successful in both of its aims. Firstly with regard to quicker indexing techniques, the standard method

using indicator plants was shortened from two years to eight months, while a rapid, six-hour test based on PAGE analysis, was developed. Secondly, more light has been shed on the biochemical and ultrastructural effects of sunblotch on its host, the avocado, as well as providing information regarding the thermal sensitivity and the host range of the agent.

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APPENDIX

PUBLICATIONS FROM THIS THESIS

Results presented in this thesis have been, or are being, published as follows:

CHAPTER 1: Da Graça (1978; 1979), da Graça and van Vuuren (1977; 1980 a; 1980 b; 1980 c; 1980 d).

CHAPTER 2: Da Graça (1978), da Graça and van Lelyveld (1978 a; 1978 b).

CHAPTER 3: Da Graça (1978), da Graça and Martin (submitted for publication).

CHAPTER 4: Da Graça (1980).

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