Journal of Agricultural Science; Vol. 6, No. 9; 2014
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

Histopathology of Avocado Fruit Infected by Avocado Sunblotch Viroid

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Received: June 9, 2014 Accepted: July 15, 2014 Online Published: August 15, 2014

doi:10.5539/jas.v6n9p158 URL: http://dx.doi.org/10.5539/jas.v6n9p158

Abstract

Avocado sunblotch viroid (ASBVd) was detected in the exocarp and mesocarp of symptomatic and asymptomatic 'Hass' avocado fruit. Macroscopic symptoms caused by the ASBVd in fruits are the result of anatomical and chemical changes in the structure of the exocarp and mesocarp cells, which are typified by marked cellular disorganization, accumulation of phenolic compounds in the cytoplasm and cell walls, and reduction of cytoplasmic content resulting in cell collapse and death. The mesocarp of symptomatic fruits showed a reduction of 28-38% in chlorophyll A and 23-28% in chlorophyll B content, as well as an increase of up to 50-62% in phenolic compounds compared to asymptomatic fruit. The phloem cells of symptomatic fruit showed hyperplasia and the xylem elements were occluded in the mesocarp. Additionally disorganization and cell collapse was observed leading to groove formation in symptomatic fruit. Chlorophyll reduction and increase in phenolic compounds probably leads to the development of yellow or red symptoms in the rind. Asymptomatic fruits did not show any anatomical change despite the presence of the ASBVd. The PCR and dot-blot hybridization membranes showed relatively higher signal intensity in symptomatic fruits in contrast to the asymptomatic ones.

Keywords: ASBVd, tissue anatomy, cellular changes

1. Introduction

Viroids classified in the families *Avsunviroidae* and *Pospiviroidae* are minimal infectious agents restricted to the plant kingdom. They are covalently-closed circular single-stranded RNAs of 246 to 401 bases that can fold and form secondary rod-like or ramified structures. Viroids, which do not code for any protein although some of them are endowed with ribozyme activity, replicate in an autonomous way inside a susceptible host by a rolling circle mechanism (Daròs et al., 2006; Flores et al., 1998).

The Avocado sunblotch viroid (ASBVd) belongs to the family *Avsunviroidae* and causes "avocado sunblotch disease", early associated with a transmissible agent (Horne & Parker, 1931). Subsequently in 1980, new evidence suggested than the causal agent of the disease was a viroid (Desjardins et al., 1980), and in 1981 the etiology (Allen et al., 1981) and molecular structure of the pathogen (Symons, 1981) was demonstrated. The symptoms of sunblotch on fruit consist of discolored depressions and the fruit may show overall distortion. The young shoots develop longitudinal white to yellowish streaks and sometimes the leaves show irregular white to yellow spots (Desjardins, 1987; De La Torre et al., 2009; GIIIA, 2013). Infected fruit are discarded for the international and domestic market (Saucedo-Carabez et al., 2014). Schroeder (1935) characterized the anatomical changes induced in avocado branches with symptoms of the disease, but there are no reports of the anatomical and cellular changes occurring in avocado fruit. The objective of this study was to characterize structural alterations and changes on pigment content in 'Hass' avocado fruit infected with the ASBVd.

2. Materials and Methods

2.1 Plant Material

Three symptomatic and three asymptomatic avocado trees (cv. 'Hass') previously diagnosed as ASBVd positive by RT-PCR (Schnell et al., 1997) were selected in a commercial orchard located in Tingambato, Michoacán, México (19.43729° LN, 101.87207° LW) and fifteen avocado fruits asymptomatic and/or symptomatic were harvested at physiological maturity (dry matter >22%) from each selected trees.

2.2 RNA Extraction and Molecular Analysis

RNA was obtained from 0.05 g of exocarp and mesocarp tissue from five symptomatic fruit and/or five asymptomatic fruit collected per tree selected, according to Mackenzie et al. (1997) and Ling-Wen et al. (2008). The RNA integrity was verified by electrophoresis in 1.5% agarose gel, stained with ethidium bromide (0.4 µl) and visualized on a photodocumenter 1000/26 MX Xpress® (VilberLourmat, Valley of Marne, France).

The molecular analysis of RNA extractions was carried out by RT-PCR according to Schnell et al. (1997) with some modification, using the SuperScript™ III One-Step RT-PCR (Invitrogen™). The components per reaction of 5 µl were: 2.5 µl of 2X PCR buffer, 0.25 µl of forward primer 10 µM (5'-AAGTCGAAACTCAGAGTCGG-3'), 0.25 µl of reverse primer 10 µM (5'-GTGAGAGAAGGAGGAGT-3'), 0.5 µl PVP-40 (10%), 0.2 µl of the enzyme SuperScript™ III RT/Platinum® *Taq*Mix*, 0.3 µl of total RNA extracted and 1 µl of nuclease free water. The amplification program included an initial retrotranscription (50 °C 32 min, 94 °C 2 min), followed by 30 cycles of amplification (94 °C 15 s, 53 °C 15 s y 68 °C 30 s) with a final extension (68 °C 5 min). PCR products were examined in 1% agarose gel electrophoresis stained with 0.5% ethidium bromide.

Additionally, the RNA extracted from all fruits was analyzed by dot-blot hybridization using a non-radioactive Dig-High Prime-UTP-T7 riboprobe (Roche®, Mannheim, Germany) specific to the ASBVd (De La Torre et al., 2009). The samples were applied on positively charged nylon membrane (Immobilon® NY+, Millipore Corporation™) and processed following the manufacturer instructions for chromogenic detection with the DIG High Prime DNA Labeling and Detection Starter Kit I, Roche®. Fluorescent detection was carried out using the DIG High Prime DNA Labeling and Detection Starter Kit II, (Roche®) and RX films (Kodak®), which was placed inside an autoradiography cassette (Amersham Hypercassette™) for 30 min and processed according to the manufacturer's instructions (Kodak®).

2.3 Tissue Processing for Histology

Exocarp and mesocarp tissue sections (1 cm^2) were cut from symptomatic and/or asymptomatic fruit and the comparison of severity levels included yellowish slightly sunken spot (SS), yellowish sunken crack (CS) and yellowish sunken crack with epidermal necrosis (CN). The material was fixed in Craf III, dehydrated in an ethanol, ethanol-xylene series, and embedded in Paraplast® according to the methodology proposed by Johansen (1940). Subsequently, samples were cut at 10 µm of thickness and mounted on glass slides. Sections were de-waxed in xylene and stained with safranine and fast green (Curtis, 1986). All observations were conducted on a microscope III Carl Zeiss® and images recorded using a Pax Cam® 3 camera system. The image analysis of samples was carried out using the Image Tool© 3.0 program.

Figure 1. Symptomatic and asymptomatic avocado fruits showing different symptoms: asymptomatic (A) yellowish slightly sunken spot (SS), yellowish sunken crack (CS) and yellowish sunken crack with epidermal necrosis (CN)

2.4 Phenols and Chlorophyll Extraction and Quantification

Chlorophyll content was determined by extraction with 100% acetone (Lichtenthaler, 1987) and measurement of absorbance (A) at 662 and 645 nm. Chlorophyll quantification was obtained using the following formulas (AOAC, 1998):

$$\text{Chlorophyll A} = 11.24A_{662} - 2.04\,A_{645}$$

$$\text{Chlorophyll B} = 20.13A_{645} - 4.19A_{662}$$

$$\text{Chlorophyll A+B} = 7.05A_{662} + 18.09\,A_{645}$$

Extraction of mesocarp phenols was performed according to the method of Stewart (1989) and their quantification by the method of Folin–Ciocalteau (Waterman & Mole, 1994).

2.5 Statistical Analysis

The statistical analysis of cell size was based on 100 measurements of each symptom studied, and the phenol and chlorophyll content was evaluated per duplicate in five fruits from each selected tree using a completely randomized design. Analysis of variance and means comparison by the Tukey test were performed with the SAS®V9.0 statistical package.

3. Results and Discussion

3.1 Molecular Analysis

PCR products of 250 and 500 base pairs (pb) corresponded to the ASBVd (Schnell et al., 1997; De La Torre et al., 2009). The PCR signal intensity and the dot-blot hybridization indicated that the viroid RNA concentration probably was higher in symptomatic than in asymptomatic fruits (Figure 2). According to Semancik (2003) the viroid can be broadly distributed in different tissues and organs of infected trees and its concentration is highly variable among the different tissues, but it is higher in symptomatic ones (Semancik & Szychowski, 1994; Markarian et al., 2004).

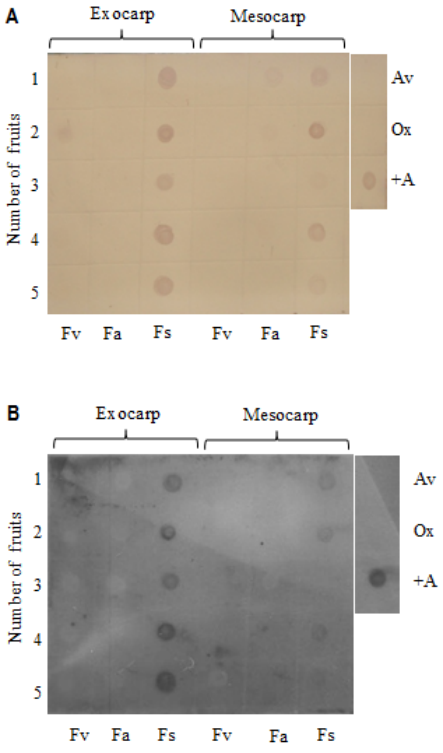

Figure 2. Dot-blot hybridization with a non-radioactive riboprobe for chromogenic detection (A) and fluorescent detection (B) of the ASBVd in avocado fruits (*Persea americana*) cv. Hass. Asymptomatic fruits harvested from different vigorous asymptomatic trees (Fv), asymptomatic fruits harvested from different symptomatic trees (Fa) and symptomatic fruits (Fs). Av: negative control, RNA from healthy avocado. Ox: negative control, RNA from Oxalis sp., +A: RNA from avocado infected with ASBVd

3.2 Histology

The structure of the exocarp, mesocarp and vascular system tissues of asymptomatic fruit (Figure 3A) did not show any alterations. The exocarp had a 8.1 μm thick cuticle with anticlinal projections toward the epidermal cells, and contained dense brown and reddish ergastic compounds of possible phenolic origin, followed by parenchyma tissue composed of various layers of isodiametric cells of 33.9 μm with abundant chloroplasts. The mesocarp was made up of isodiametric parenchyma cells of an average of 44.5 μm. The vascular system did not show any alterations in phloem and xylem (Figure 3A) (Cummings & Schroeder, 1942; Barrientos-Priego et al., 1996).

The slightly sunken yellowish spot (SS) on the exocarp did not show alterations on the cuticle, nor on the epidermal cell thickness compared to the asymptomatic fruits (Tukey, $p = 0.05$). However, the parenchyma cell walls exhibited a higher accumulation of reddish polyphenols, similar to those described by Zamora-Magdaleno et al. (2001). Additionally, this parenchyma tissue showed an apparent lower chloroplast content (Figure 3B). The mesocarp cells were slightly disorganized, their size was significantly reduced to 41.7 μm (Tukey, $p = 0.05$) and the number of cells with phenolic content was higher. The vascular system did not show any alterations (Figure 3B).

The sunken, yellowish crack symptom (CS) on the exocarp presented no-altered cuticle, but the epidermal and parenchyma cells showed a higher accumulation of polyphenols in cell walls. The CS mesocarp was disorganized and showed more phenolic compounds. Cells became smaller (36.5 μm) and collapsed. The vascular tissue presented hyperplasia, phloem cells collapsed and xylem vessels were probably occluded with phenolic compounds (Figure 3C).

Finally, the sunken yellowish crack with epidermal necrosis fruits (CN) presented a cuticle of 4.4 μm in thickness corresponding to a 45 - 50% reduction compared to the asymptomatic LS and CS cuticles (Tukey, $p = 0.05$). Parenchyma cells were significantly reduced in size (Tukey, $p = 0.05$) with an average of 22.5 μm. The exocarp cells became necrotic, collapsed, and showed polyphenols in cell walls (Figure 3D). The vascular tissue showed similar characteristics to the alterations observed in CS fruits (Figure 3D).

According to the observations, the symptom severity is a function of the cellular changes, which were absent in asymptomatic fruits. The macroscopic symptoms are the result of the gradual reduction in cell size of the exocarp and mesocarp tissues, which could be associated with the tissue depression.

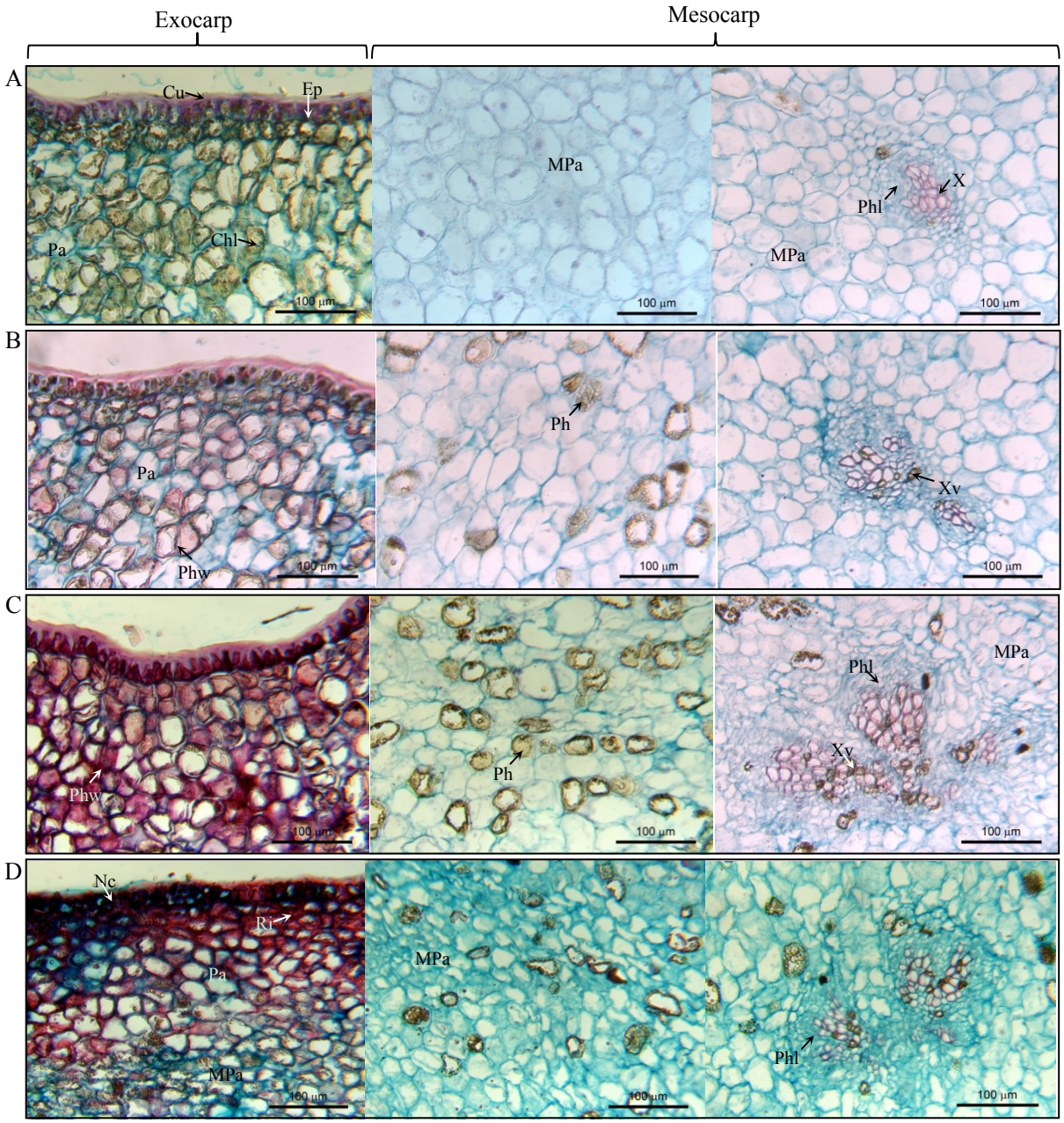

Figure 3. Cross section micrographs of the exocarp and mesocarp tissues of avocado fruits showing different classes of ASBVd symptoms: (A) asymptomatic, (B) yellowish slightly sunken spot, (C) yellowish sunken crack and (D) yellowish sunken crack with epidermal necrosis. Parenchyma (Pa), phloem (Phl), xylem (X), xylem vessels (Xv), accumulation of red inclusions (Ri), cuticle (Cu), epidermal cells (Ep), chloroplasts (Chl), phenols (Ph), phenols accumulation in cell wall (Phw), mesocarp parenchyma (Mpa) and necrotic cells (Nc)

The mesocarp from symptomatic fruits presented 103.64 µg/g in Chlorophyll *A* and 52.6 µg/g in Chlorophyll *B* content, but the sunken yellowish tissue (Fss) showed the lowest values of chlorophyll *A* and *B* (Table 1). Those values were lower compared to the concentration determined in the asymptomatic fruits harvested from vigorous tress with 145.81 and 69.03 µg/g respectively, corresponding to a reduction of 29 to 65% in chlorophyll *A* and 24 to 52% of chlorophyll *B* compared with symptomatic ones (Tukey, $p = 0.05$). The ASBVd is replicated in the chloroplasts (Navarro et al., 1999) and has been located mainly in the thylakoids and stroma (Bonfiglioli et al., 1994; Lima et al., 1994). The alterations in the chloroplast structure cause the reduction in chlorophyll content

due to its synthesis inhibition or degradation (Bailiss, 1970; Šutić & Sinclair, 1991).This is probably a factor leading to the development of white to yellow coloration of rind in avocado fruits infected by ASBVd (Šutić & Sinclair 1991). Schroeder (1935) reported similar results on symptomatic branches (yellow stripes) of trees infected by the ASBVd. The alterations were characterized by a reduction in chloroplast content and changes in the development and differentiation of phloem and xylem, reducing the number and size of cells, and also cellular collapse.

Table 1. Phenols and chlorophyll content from mesocarp of avocado 'Hass' fruits infected with the ASBVd

	Chlorophyll *A*	Chlorophyll *B*	Chlorophyll *A*+*B*	Phenols
	μg/g	μg/g	μg/g	μg/g
Fv[a]	145.81 a[e]	69.03 a	214.85 a	0.42 b
Fa[b]	167.45 a	73.53 a	240.98 a	0.39 b
Fs[c]	103.64 b	52.60 b	156.25 b	0.63 a
Fss[d]	50.37 c	33.12 c	83.50 c	
Pr>F	<0.0001	<0.0001	<0.0001	0.0081

[a]Asymptomatic fruits harvested from vigorous asymptomatic trees (Fv). [b]Asymptomatic fruits harvested from symptomatic trees (Fa). [cd]Symptomatic fruits harvested from symptomatic trees: asymptomatic sections (Fs), and sunken yellowish tissue section (Fss). [e]Different letters indicate significant differences between means (Tukey, p = 0.05).

Phenolic compounds are considered secondary metabolites and perform different function in plants including pigmentation, growth, reproduction and resistance to pathogens, and apparently act triggering signaling cascades to activate defense responses (Lattanzio et al., 2006). The results showed an increase in the number of cells with polyphenols in symptomatic tissues (0.63 μg/g of phenols) (Tukey, p = 0.05), wherein the dot-blot hybridization signal was higher, while the asymptomatic tissues presented a lower content of polyphenols (0.42 μg/g) and a weak viroid signal (Figure 2).

The inability of viroids to codify proteins is characteristic and induces them to interact with host proteins and nucleic acids. This interaction triggers a series of events that eventually results in the macroscopic symptoms. The expression and severity of the symptoms caused by viroids depend on the plant host, cultivar, environmental conditions and variants predominating in the populations (Flores et al., 2005). Navarro et al. (2012) suggests a hypothesis of how the viroid incites the disease, which implies that the small viroid-derived RNAs (vd-sRNAs; 21–24 nt) resulting from the host defensive response, via RNA silencing, target for cleavage cell mRNAs and trigger a signal cascade eventually leading to symptoms.

Acnowledgements

We are grateful for grants from the Asociación de Productores y Empacadores Exportadores de Aguacate de Michoacán (APEAM) and Servicio Nacional de Sanidad Inocuidad y Calidad Agroalimentaria (SENASICA-DGSV).

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