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# Detection of Avocado Sunblotch Viroid in Spain by Double Polyacrylamide Gel Eletrophoresis

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*With one figure*

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## Abstract

Avocado sunblotch viroid (ASBV) was detected in samples of young leaves and flowers of the avocado cultivars 'Hass' and 'Fuerte' grown in southern Spain. Partially purified nucleic acid preparations, obtained by cellulose chromatography, were analyzed by double polyacrylamide gel electrophoresis. This technique, consisting of two consecutive electrophoresis cycles under non-denaturing and denaturing conditions, followed by silver staining of the gel, allowed the selective and sensitive identification of ASBV. This is the first report on the occurrence of the causal agent of avocado sunblotch in Spain.

## Zusammenfassung

### Feststellen des Avocado Sunblotch Viroid in Spanien durch zweifache Polyacrylamidgelelektrophorese

Das Avocado Sunblotch Viroid (ASBV) wurde in jungen Blättern- und Blütenproben der Avocadosorten 'Hass' und 'Fuerte', die in Süd-Spanien angebaut werden, festgestellt. Teilweise gereinigte Nukleinsäurepräparate, die durch Zellulosechromatographie erhalten wurden, wurden durch zweifache Polyacrylamidgelelektrophorese analysiert. Dieses Verfahren aus zwei aufeinanderfolgenden Elektrophoresezyklen unter nicht denaturierenden und denaturierenden Bedingungen mit anschließender Silberfärbung des Gels, ermöglicht die selektive und empfindliche Bestimmung von ASBV. Dies ist die erste Meldung über das Vorkommen des Avocado Sunblotch-Erregers in Spanien.

Avocado sunblotch is a widespread disease affecting avocado (*Persea americana* Miller). The presence of a small disease specific RNA has been reported in nucleic acid extracts of sunblotch-infected material (DALE and ALLEN 1979, PALUKAITIS *et al.* 1979, THOMAS and MOHAMED 1979). This RNA is the causal agent of the disease (ALLEN *et al.* 1981, UTERMOHLEN *et al.* 1981) and has been termed avocado sunblotch viroid (ASBV). It is not unusual for ASBV to infect avocados symptomlessly, and its detection by graft transmission to indicator avocado seedlings can take up to 2 years. This long indexing period has stimulated the development of rapid biochemical methods for detecting ASBV by polyacrylamide gel electrophoresis (DA GRAÇA 1981, UTERMOHLEN and OHR 1981) and molecular hybridization either in liquid (ALLEN and DALE 1981, PALUKAITIS *et al.* 1981) or in mixed phase (dot-blot) systems (ROSNER *et al.* 1983, BAR-JOSEPH *et al.* 1985, BARKER *et al.* 1985).

Avocado is becoming an important crop (with approximately 5000 ha in 1985) in an area of southern Spain comprising part of the coast of the provinces of Málaga and Granada. Almost simultaneously with its introduction in the early seventies, as a result of the use of non-index material, symptoms of the sunblotch disease were observed (FARRÉ, personal communication). In the present article we report the use of a double electrophoretic technique (SCHUMACHER *et al.* 1983), for the detection of ASBV in partially purified preparations of nucleic acids of young leaves and flowers from trees showing symptoms of sunblotch. This method combined with the sensitive staining of the gels with silver, has permitted the rapid detection of ASBV in Spain.

## Materials and Methods

### Extraction and partial purification of ASBV

Samples (5 g) of young leaves and flowers, collected from different parts of avocado trees with symptoms of sunblotch (longitudinal streaks on the bark of young stems, broad spots sunken in the outer skin of the fruits), were homogenized by means of a Willems Polytron (Kinematica GmbH, Kriens-Lucerne, Switzerland). The extraction medium was made by mixing: 20 ml water-saturated phenol, 4 ml 0.2 M Tris-HCl pH 8.9, 1 ml 0.1 M EDTA pH 7.0, 1 ml 5 % SDS and 0.5 ml mercaptoethanol (SEMANCIK and WEATHERS 1972). The extracts were centrifuged at 6000 g for 15 min and the upper aqueous phases were removed, adjusted to a final volume of 10 ml in STE buffer (50 mM Tris-HCl pH 7.2, 100 mM NaCl and 1 mM EDTA) and made to contain 35 % ethanol. After adding 1 g of cellulose powder (CF-11 Whatman) (ALLEN and DALE 1981, FRANKLIN 1966), the mixtures were gently shaken in centrifuge tubes for 10—15 min and centrifuged at 1000 g for 5 min. The supernatants were discarded and the pellets of cellulose powder were washed twice with 30 ml of 35 % ethanol in STE, and then with 10 ml of STE. The nucleic acids eluted in the last wash were precipitated with 2.5 vol of ethanol at −20 °C overnight. The final pellets were collected by centrifugation (6000 g for 15 min), dried and resuspended in 0.5 ml of distilled water. Extracts of leaves from healthy controls of 'Hass' and 'Fuerte' cultivars were processed in a similar way.

### Analysis by polyacrylamide gel electrophoresis

The double electrophoretic technique reported by SCHUMACHER *et al.* (1983), was followed with some modifications. Aliquots of 50 µl of the nucleic acid solutions obtained after the cellulose fractionation, were made to contain 20 % glycerol and subjected to polyacrylamide slab gel electrophoresis (70 mA constant current at 4 °C for 2.5 h in a gel of 130 × 100 × 2 mm) under non-

denaturing conditions (MORRIS and WRIGHT 1975). A preparation of apparently circular molecules of ASBV ($ASBV_c$), corresponding to the slowest migrating band of the two which purified viroids give rise in denaturing gels (SCHUMACHER *et al.* 1983), was applied in the two outside lanes. Gels were stained with ethidium bromide (0.5 µg per ml in water for 10 min), and a 0.75—1 cm segment of the gel containing the ASBV band was excised and applied directly on top (SEMANCIK and HARPER 1984) of a 5 % fully denaturing urea gel (SÄNGER *et al.* 1979). Following electrophoresis (17 mA constant current at room temperature for 3 h in a gel of the same size as the previous one) the denaturing gel was stained with ethidium bromide, photographed and subsequently silver-stained with the procedure of SAMMONS *et al.* (1981) as modified by IGLOI (1983). The same experimental approach has been used recently for the detection of viroid and viroid-like RNAs from grapevine (FLORES *et al.* 1985).

## Results

The method used in the present work, which combined the extraction and cellulose fractionation of the nucleic acids from avocado with their subsequent analysis by two cycles of electrophoresis and final staining with silver, allowed the rapid and sensitive detection of $ASBV_c$. Some representative results of this analysis are shown in Fig. 1. From these data it can be concluded that polyacrylamide gel electrophoresis under non-denaturing conditions followed by ethidium bromide staining, which is the base of two previous methods to detect ASBV (DA GRAÇA 1981, UTERMOHLEN and OHR 1981), permitted in some cases (Fig. 1A, lanes *b*, *c*, *f*, *g*, *h* and *i*) the observation of a band with the same mobility as the standard of pure ASBV; nevertheless in the healthy controls of 'Hass' and 'Fuerte' cultivars (lanes *a* and *j*), a band migrating in the same area that could be confused with the viroid band, was also visible. A second electrophoresis under denaturing conditions of the nucleic acids migrating in the first gel in the zone of ASBV provided, after ethidium bromide staining, the clear distinction in some samples (Fig. 1B, lanes *b*, *c*, *f*, *g*, *h* and *i*) of bands co-migrating with the $ASBV_c$ standard. A further staining of the same gel with silver revealed the presence of the $ASBV_c$ band in two additional lanes where it was not detected after ethidium bromide staining (Fig. 1C, lanes *d* and *e*). Under denaturing conditions the healthy controls did not show any band migrating in the zone of $ASBV_c$ (Fig. 1B and C, lanes *a* and *j*).

The application of this method to Spanish samples led to the detection of ASBV in young leaves in 7 of the 11 trees analyzed of the cultivar 'Hass' (the most important commercially in Spain) and in the 3 trees investigated of the cultivar 'Fuerte'. All the samples were taken from trees showing symptoms of sunblotch of different severity, coming from one and three different locations in the case of 'Fuerte' and 'Hass' cultivars respectively. The $ASBV_c$ band was always observed in samples from trees showing fruit symptoms, whereas the 4 'Hass' trees where this band could not be detected, were exhibiting only mild streaks on the stems that may not be true ASBV-induced lesions. Alternatively, ASBV could be present in these trees at a very low concentration not detectable with the method used. The analysis of flower samples gave results totally coincidental with those obtained with young leaves. Therefore flower buds, as indicated previously (UTERMOHLEN and OHR 1981, DA GRAÇA and MANSON 1983) are an appropriate

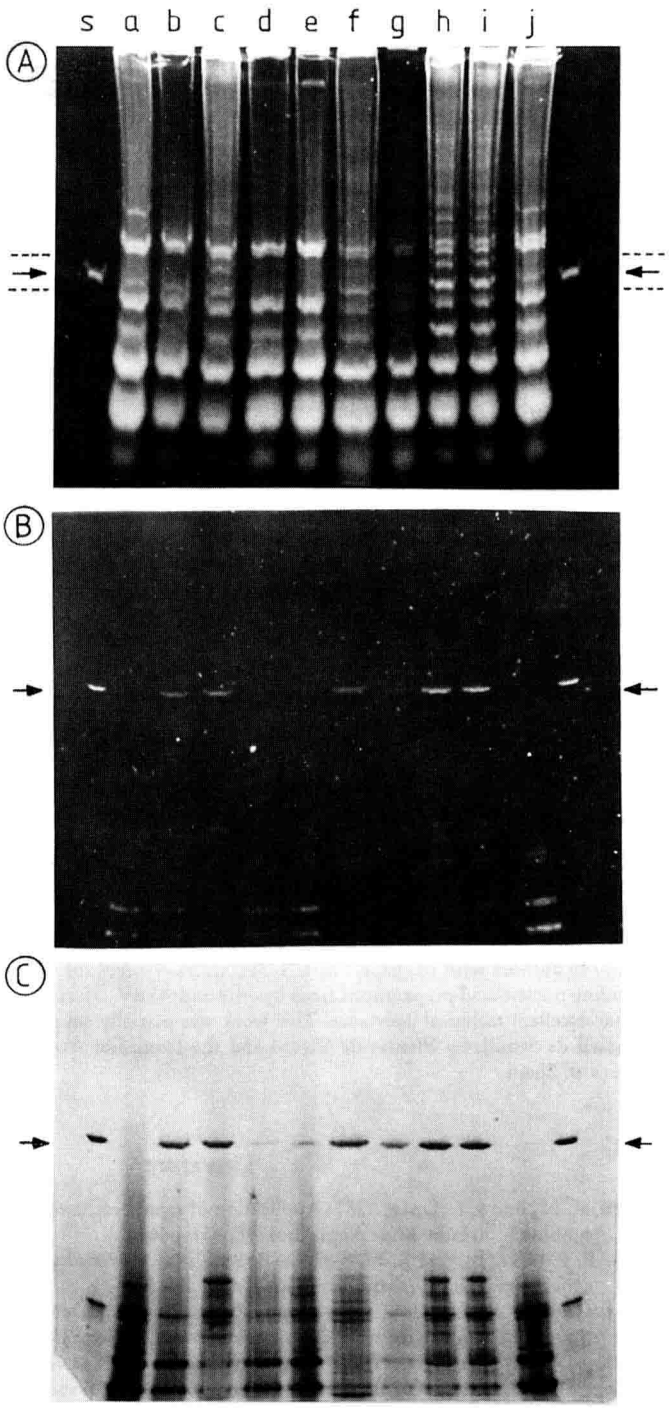


Fig. 1. Polyacrylamide gel electrophoresis under non-denaturing (A) and denaturing conditions (B and C) of nucleic acid preparations from avocado tissue. Lanes *s*, standard purified circular ASBV-RNA whose position is indicated by arrows. Lanes *a* and *j*, foliar extracts from healthy controls of 'Hass' and 'Fuerte' cultivars respectively. Lanes *b* and *c*, extracts of leaves and flowers respectively from a 'Hass' tree. Lanes *d*, *e*, *f* and *g*, extracts of leaves from four additional 'Hass' trees. Lanes *h* and *i*, extracts of leaves and flowers respectively from a 'Fuerte' tree. The non-denaturing gel (A) was stained with ethidium bromide and the area indicated within dashed lines was cut and applied on top of a denaturing gel that was stained with ethidium bromide (B) and then with silver (C). In this last case a band in the lower part of lanes *s* can be observed, that should correspond to linear forms of ASBV contaminating the $ASBV_c$ preparation

plant material for the detection of ASBV, although in our hands they were similar to young foliar tissue in terms of the presence and intensity of the viroid band.

As the present work was restricted to plants showing symptoms, the existence of symptomless trees in Spain remains to be studied. The identification of this type of trees does not appear to be a problem, since it has been reported previously (DA GRAÇA and MASON 1983) that successful detection of ASBV by polyacrylamide gel electrophoresis under non-denaturing conditions, is higher in leaves from symptomless carriers than in leaves from branches with symptoms.

## Discussion

Although the new diagnostic tests for ASBV, based on dot-blot hybridizations to probes of $^{32}$P-labelled sequences of ASBV-RNA (ROSNER *et al.* 1983) and ASBV-cDNA (BAR-JOSEPH *et al.* 1985, BARKER *et al.* 1985), are more sensitive than polyacrylamide electrophoresis techniques, the latter are still a useful alternative since they avoid problems involved with the manipulation of radioisotopes. We think that the double electrophoresis method used here for the detection of ASBV is more reliable than the electrophoretic techniques applied previously for two reasons. First, the ASBV is unambigously identified due to the low mobility of the apparently circular viroid molecules in the denaturing gel. Second, an increased sensitivity is obtained with the final silver staining, which we have estimated in the nanogram amounts of ASBV per band (data not shown), a value in accordance with those reported in the cases of tRNAs (IGLOI 1983) and other viroids (SCHUMACHER *et al.* 1983). This increased sensitivity is particularly important when the high variation in the levels of ASBV in different trees observed previously (PALUKAITIS *et al.* 1981) is taken into account. We have confirmed this variability in the present work (compare in Fig. 1 B and C, lanes *b*, *c*, *f*, *h* and *i* with lanes *d*, *e* and *g*).

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