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Full Length Research Paper

Is there *Avocado sunblotch* Viroid in Ghana?

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The prevalence of *Avocado sunblotch* Viroid (ASBVd) among Ghanaian accessions was investigated. One hundred and eighty five (185) symptomatic and symptomless avocado trees were tested by DIG-dot blot hybridization for the presence of ASBVd. One (0.01%) accession tested positive, 158 (85.8%) tested negative, while the results of the remaining 26 (14.1%) were inconclusive (herein referred to as "possible carriers"). Only one true symptomless carrier of the viroid was identified. The viroid showed no geographical or topographical preferences. The positive and "possible carriers" were evenly distributed around the country. The incidence of the disease in Ghana was found to be very low and hence steps must be taken to eradicate it and maintain a clean industry.

Key words: Avocado sunblotch viroid, digoxygenin, dot blot hybridization.

INTRODUCTION

Avocado sunblotch, the only viroid disease of avocado, was first described by Horne and Parker (1931). Palukaitis et al. (1979) and Allen et al. (1981) identified avocado sunblotch viroid (ASBVd) as the causal agent of sunblotch. After Thomas and Mohamed (1979) established a viroid as the probable causal agent, Symons (1981) then established the primary and secondary structures of the viroid.

ASBVd, an infective, single-stranded, circular RNA molecule of between 246 and 251 nucleotides in length, and a member of the *Avsunviroidae* family, replicates and accumulates in the chloroplast of its host (Palukaitis et al., 1979; Pallas et al., 1988; Rakowski and Symons, 1989; Bonfiglioli et al., 1994; Lima et al., 1994; Navarro et al., 1999; 2000). It is transmitted mechanically (Desjardins et al., 1980; Desjardins and Drake, 1983; Desjardins et al., 1987) through pollen (Desjardins et al., 1979) infected scions (Suarez et al., 2005) or infected rootstocks by root-grafting (Whitsell, 1952), and also through seeds (Wallace and Drake, 1962a); with seed transmission rate varying from very low (0 - 5.5 %) in symptomatic trees to high (80 - 100%) in symptomless trees (Wallace and Drake, 1962b). No known animal vector of the viroid has yet been identified.

Asymptomatic cultivars, obviously, do not exhibit symptoms of the disease upon infection. Unlike symptomless trees, viroid concentrations in symptomatic trees vary greatly from branch to branch of the same tree (Semancik and Desjardins, 1980; Allen and Dale, 198i; Utermohlen and Ohr, 1981), and even greater between trees (Palukaitis et al., 1981). The varied symptoms of the disease, as described by Dodds et al. (2001), may be observed on the fruits, leaves, stem, and possibly, the roots, and are associated with infection by variants of ASBVd. Trunk symptoms include roughened or fissured barks, with yellow, orange or white, usually sunken, streaks on twigs and petioles. Fissured bark, a characteristic feature of most West Indian cultivars, should however not be misconstrued to mean ASBVd infection. Foliar symptoms usually appear as either chlorotic zones associated with vascular tissues, which commonly appear bleached, or as a general variegation of white, yellow or pink areas on deformed or distorted leaves (Horne and Parker, 1931; Dale et al., 1982; Desjardins et al., 1987). Fruit produced from infected trees usually develop sunken white, yellow or red blotches or streaks and are usually small, deformed and unmarketable. Severely affected trees are low-yielding (da Graca et al., 1983), stunted and develop a low, sprawling habit that leads to increased exposure and injury from the sun.

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Generally, symptoms of the disease develop quicker in warmer climates than colder conditions (da Graca, 1979). *Avocado sunblotch* accounts for 40.1% loss in revenue as a result of reduction in yield (>25%) and production of under-graded fruit (>25%) (da Graca et al., 1983).

No known resistant cultivars have yet been identified. Hence, the disease is controlled and managed by using pathogen-free planting materials and pruning equipment, and removal of diseased branches and trees (Suarez et al., 2005). The available diagnostic techniques for the viroid are: RNA resolution (Dale and Allen, 1979; Palukaitis et al., 1979; Mohamed and Thomas, 1980; da Graca, 1981; da Graca and Mason, 1983), dot blot hybridization (Korsten et al., 1986), cDNA oligonucleotide probes (Allen and Dale, 1981; Palukaitis et al., 1981; Bar-Joseph et al., 1986), digoxigenin-labeled RNA probe (Manicom and Luttig, 1996); and reverse transcription-polymerase chain reaction (RT-PCR) (Semancik and Szychowski, 1994; Running et al., 1996; Mathews et al., 1997; Schnell et al., 1997; Luttig and Manicom, 1999). These techniques are now applied in place of the unreliable graft transmission and cross protection tests (Allen and Firth, 1980) previously employed. Even though detection by dot blot hybridization is less sensitive than RT-PCR, it provides a cheaper and faster means of screening for the viroid.

The prevalence of the disease among Ghanaian accessions has not been investigated. Our objective was to investigate the prevalence of ASBVd in Ghana by the dot-blot method and to identify any possible geographical and topographic preferences of the viroid.

MATERIALS AND METHODS

Plant materials and DNA extraction

Leaves were collected from 185 accessions from different topographic zones in the six forest regions of the country (Figure 1), as described by Mathews et al. (1997). Five fresh, moderately young leaves were sampled from each of the four compass points of selected trees.

RNA extraction and blotting

RNA was extracted by the CTAB method as described by Lodhi et al. (1994). Leaf discs (0.2 g), including sections of the midrib, were homogenized in liquid nitrogen, 1 mL CTAB extraction buffer [100 mM Tris-HCl (pH 8), 1.4 M NaCl, 20 mM EDTA (pH 9), 2% (w/v) CTAB, 2% (w/v) PVP, 1% (w/v) Na_2SO_3] was added and the mixture was incubated at 65°C for 30 min with intermittent vortexing. The sample was twice extracted with equal volumes of chloroform: isoamyl alcohol (24:1). The aqueous layer was clarified by high-speed centrifugation (14,000 rpm), and the nucleic acid precipitated by adding 0.5 volumes of 5 M NaCl (aq), and an equal volume of ice-cold isopropanol. The mixture was incubated at -20°C for 12 h, and nucleic acid was pelleted by high-speed centrifugation. The pellets were resuspended in 200 µL of TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] containing 1% SDS, then 100 µL of 5 M NaCl and 300 µL of ice cold isopropanol were added. The mixture was incubated at -20°C for 30 min and the RNA was pelleted by centrifugation. The resulting RNA pellets were washed in 400 µL ethanol (70%), air-dried, and resuspended in 100 µL of nuclease-free, ultra-pure water.

Preparation of the RNA membrane

The following were added in each well of a 96-well ELISA plate: 10 µL of the RNA extract and an equal volume of 20 mM NaOH (aq) containing 5 mM EDTA. The mixture was incubated at room temperature for 10 min, and 10 µL was spotted onto a positively-charged nylon membrane (Amersham plc, UK). All samples were spotted in duplicates. The membrane was air-dried at room temperature, and the RNA was fixed onto the membrane using the Stratalinker (Strategene, La Jolla, CA, USA). Membranes were stored at -20°C prior to hybridization.

DIG labeling, hybridization and detection of ASBVd

DIG labeling, pre-hybridization, hybridization and detection were carried out using a PCR DIG Probe Synthesis Kit (Roche and Molecular Biochemicals) as directed by the manufacturer. ASBVd probe, cloned in a pGEMTeasy plasmid, was DIG-labelled by PCR. The PCR labeling was performed in a total volume of 50 µL, containing 10 pg of plasmid DNA (containing an ASBVd insert), 1 X PCR buffer (with $MgCl_2$), 5 µL PCR DIG mix (Roche and Molecular Biochemicals), 0.5 µM of each of the ASBVd-specific primers (F:AAGTCGAAACTCAGAGTCGG; R: GTGAGAGAAGGAGGAGT), and 0.75 µL of PCR *Taq* Polymerase enzyme. The PCR profile consisted of 3 min denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 s, 1 min annealing at 50°C, 1 min extension at 72°C; with a final extension at 72°C for 10 min.

Pre-hybridization and hybridization were carried out according to the manufacturer's protocol at 50°C for 30 min and 16 h respectively in DIG Easy Hyb [containing 50 % deionised formamide, 5X SSC, 0.1% (w/v) N-lauroylsarcosine, 0.02 (w/v) SDS, 2 % blocking reagent] using a hybridization oven. Membranes were washed twice with 200 mL of pre-warmed Low Stringency Buffer (2X SSC containing 0.1 % SDS) for 20 min each at 65°C, and again twice with pre-warmed High Stringency Buffer (0.1X SSC containing 0.1% SDS) for 15 min each. Membranes were then incubated at room temperature successively in the following solutions: 100 mL of washing buffer (0.1 M Maleic acid, 0.15 M NaCl; pH 7.5, 0.3% (v/v) Tween 20) for 2 min, 100 mL Blocking solution [1 X Blocking solution and Maleic acid (1:10)] for 30 min, and 20 mL Antibody solution (containing Anti-Digoxigenin-AP diluted 1:10,000 with Blocking Solution) for 30 min.

Finally, the membranes were washed twice in 100 mL of Washing Buffer for 15 min each, equilibrated for 3 min in Detection Buffer (0.1 M Tris-HCl, 0.1 M NaCl pH 9.5), incubated for 5 min in 1 mL CDP-*Star* at room temperature, and exposed to Lumi-Film X-ray film for 15 min. Film was developed using a Curix 60 X-ray Developer, and the ASBVd-infection status of the accession was determined by the presence or absence of dark spots on the film. Similar results from both duplicates were scored as such, while duplicates returning different results were scored as negative (absence of ASBVd). The positive control (purified ASBVd extract) and a negative control (sterile nuclease-free, double distilled water) were spotted on the membrane prior to pre-hybridization. Spots with intensities greater than 10%, but 50% less than that of the positive control were assumed to be “possible carriers”.

RESULTS AND DISCUSSION

The results of the diagnostic assay (the dot hybridization) as well as the exact geographic locations of the positive

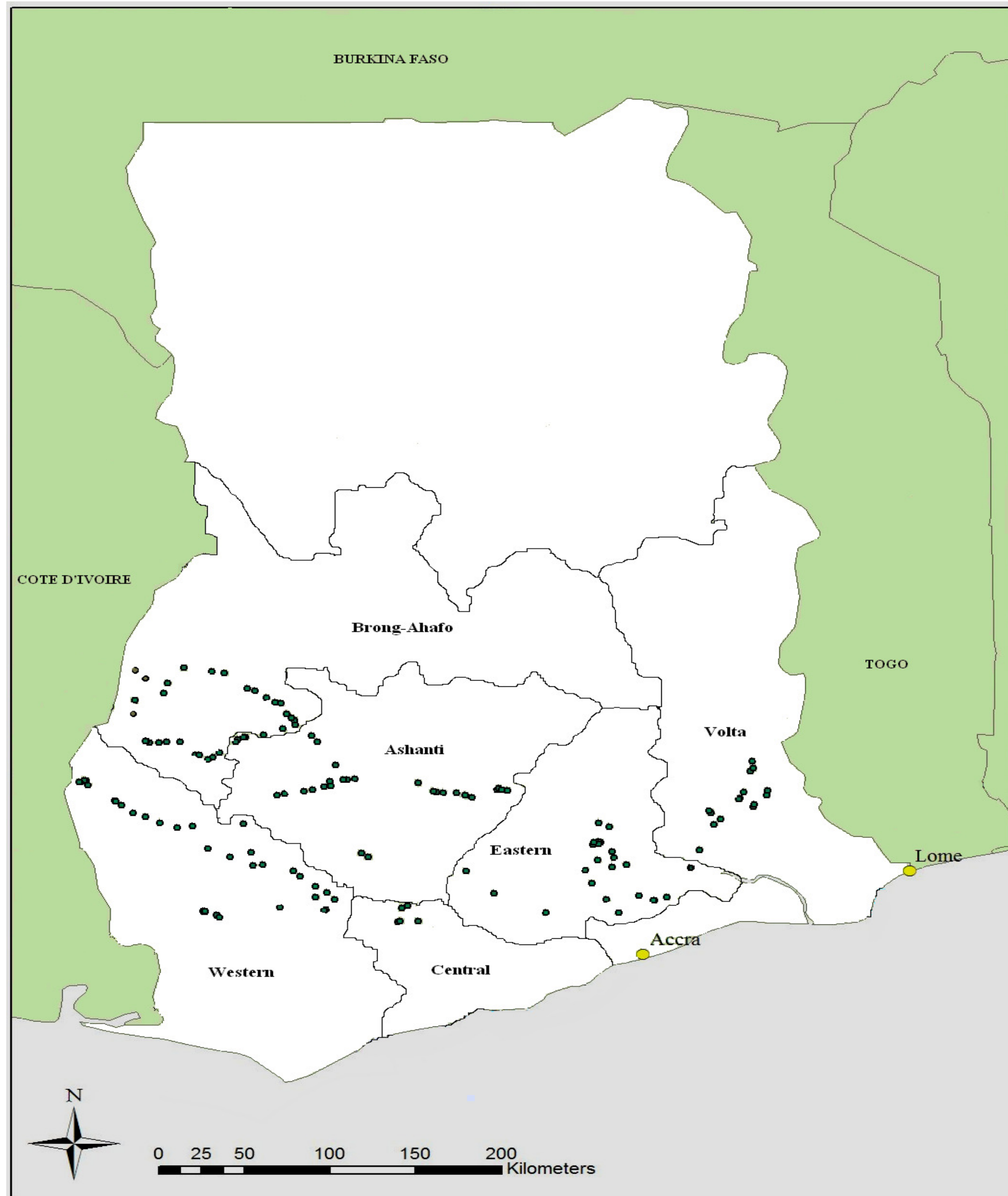

Figure 1. Map of Ghana, showing points of sample collection. ● Sample collection point.

accession and "possible carriers" are summarized in Table 1. Only one of the 185 Ghanaian accessions, representing 0.54%, tested positive for ASBVd (Sample #1; membrane position 7 L, Figure 1). This accession, located at Kuren in the Brong-Ahafo Region, exhibited no symptoms of ASBVd. We suspect this accession to be a symptomless carrier of the viroid. The more sensitive and highly reliable RT-PCR may confirm this assertion. This accession should be quarantined and extensively examined to gain more insight into the unique physiological and morphological adaptations of the symptomless carriers.

Spots having intensities greater than 10%, but 50% less than that of the positive control (X) were assumed to be 'possible carriers' of the viroid. A total of 26 (14.1%) "possible carriers" were detected (Figure 2). The low sensitivity of the dot-blot hybridization technique, the uneven distribution of the viroid RNA within the plant, and

Table 1. Geographical locations and coordinates of samples as well as sample positions of infected and "possible carriers" of ASBVd on the micro-titre well.

Sample No.	Source/Town	Region	Longitude (North)	Latitude	Elevation (m)	Sample Position (on membrane)
1	Kuren	Brong-Ahafo	07°13.206'	002°52.422'W	277	7L
2	Addonkwanta	Eastern	06°10.619'	000°26.332'W	191	Not Shown
3	Addonkwanta	Eastern	06°10.564'	000°26.355'W	211	Not Shown
4	Addonkwanta	Eastern	06°10.574'	000°26.395'W	220	1C
5	Kukurantumi	Eastern	06°11.468'	000°21.405'W	190	1I
6	Nyagbo Akofafa	Volta	06°49.288'	000°22.384'E	142	Not Shown
7	Abutia Teti	Volta	06°32.455'	000°23.085'E	145	Not Shown
8	Ziavi Dzogbe	Volta	06°37.827'	000°27.315'E	225	Not Shown
9	Nyagbo Sroe	Volta	06°46.471'	000°22.616'E	625	Not Shown
10	Asikuma Junction	Volta	06°24.535'	000°10.335'W	104	3A
11	Sika Akabi	Western	06°27.536'	002°49.287'W	175	Not Shown
12	Asankrangua	Western	05°48.758'	002°26.418'W	107	Not Shown
13	Asankrangua	Western	05°48.145'	002°25.827'W	106	Not Shown
14	Asankran-Moseaso	Western	05°50.329'	002°30.322'W	84	Not Shown
15	Kwahu Praso	Eastern	06°37.717'	000°54.892'W	207	3G
16	Bomfa	Ashanti	06°37.232'	001°17.254'W	193	4G
17	Nyinahin	Ashanti	06°35.975'	002°07.548'W	194	5G
18	Acherensua	Brong-Ahafo	06°58.660'	002°18.330'W	199	6G
19	Mim (Kokoboso)	Brong-Ahafo	06°51.750'	002°33.401'W	208	6H
20	Maabang	Brong-Ahafo	06°59.672'	002°11.971'W	250	6I
21	Abesin-Sunyani	Brong-Ahafo	07°17.678'	002°17.082'W	224	7F
22	Tanoso	Brong-Ahafo	07°16.890'	002°14.627'W	246	7G
23	Kuren	Brong-Ahafo	07°13.210'	002°52.410'W	276	7H
24	Kuren	Brong-Ahafo	07°13.205'	002°52.405'W	276	7I
25	PGRRI, Bunso	Eastern	06°16.718'	000°27.904'W	199	8K
26	PGRRI, Bunso	Eastern	06°16.723'	000°27.906'W	191	Not Shown
27	PGRRI, Bunso	Eastern	06°16.723'	000°27.911'W	191	Not Shown

Sample exhibited no morphological symptoms of the ASBVd but tested positive for disease using dot-blot hybridization

the low viroid load or concentration in the analyzed tissues may have accounted for the above mentioned results. Another reason which can be adduced, even though far-fetched, is that these accessions may be carrying new strains of the ASBVd, which do not hybridize well with the probes. Using the same technique, Korsten et al. (1986) identified 83% negatives, 8% "possible carriers", and 3% positives from 91 South African avocado accessions tested. Only one accession, maintained at Kukurantumi, in the Eastern Region of Ghana, exhibited symptoms of ASBVd infection. Samples collected from this accession (Sample # 4; membrane position 1I) however, returned a "possible" result after the screening test. The low sensitivity of the dot blot, may have accounted for this. Again, the more sensitive and reliable RT-PCR may be more informative.

This apparent low sensitivity of the technique notwithstanding, 25 accessions which did not exhibit morphological symptoms of the disease were screened as "possible carriers". Can these accessions be safely classified as the so-called symptomless carriers? Hybridization of the ASBVd probe to avocado DNA sequences similar or identical to those of ASBVd could also have accounted for these "probable carriers". It is also probable that these accessions may have been in the early stages of infection, with low viroid titres, and hence exhibited no symptoms. Monitoring the said accessions over a period of time will help establish whether or not they are indeed infected and whether they are symptomless. The dot blot hybridization is a cheaper, simpler and quicker technique for screening. However, it is less sensitive and the results could be interpreted as false positives.

Among the "possible carriers", there was however no correlation between the prevalence of the viroid and the geographic and the climatic parameters, at which the accessions were maintained, since they were identified in all six Regions studied (Table 1).

Even though the incidence of the disease is low, the risk of the disease significantly affecting the industry re-

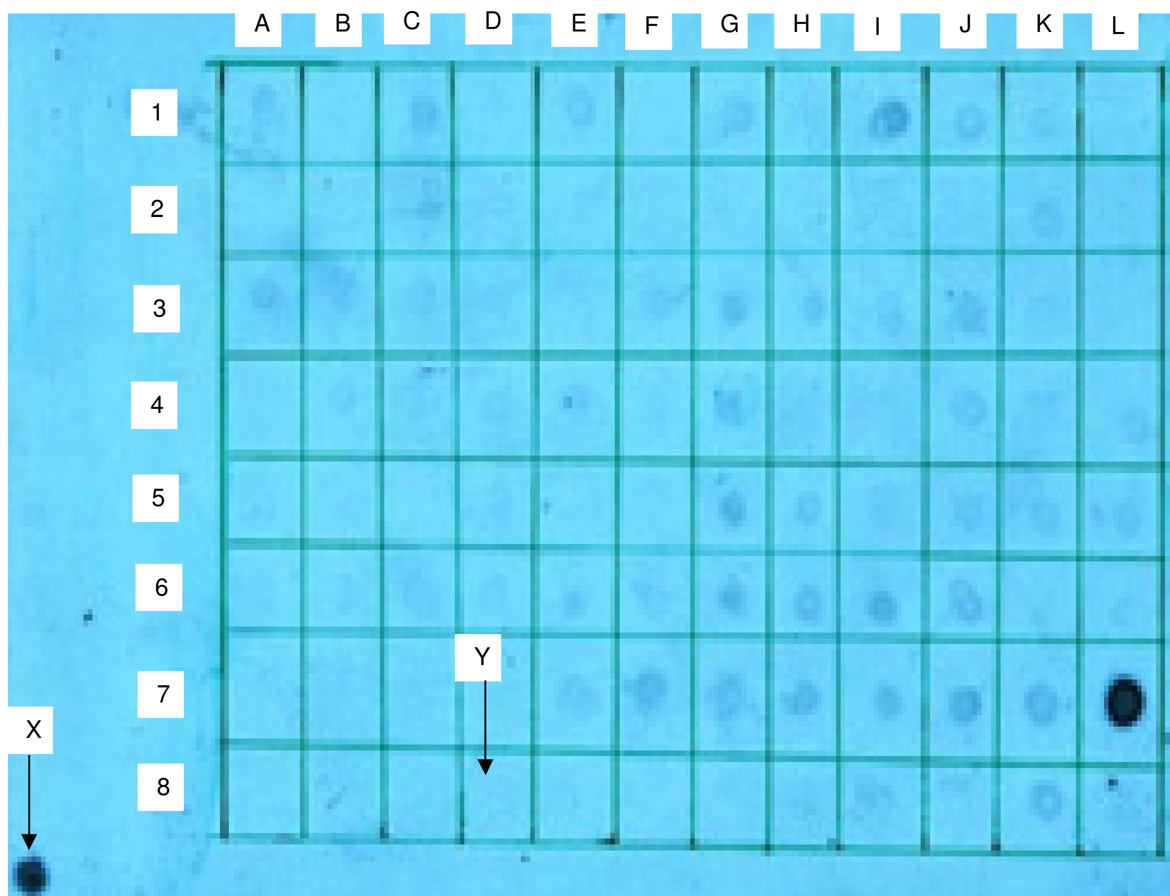

Figure 2. *Avocado sunblotch* viroid detection by dot-blot hybridization. Dark spot depict viroid infection. The intensity of spots generated by the "possible carriers" are >10% but < 50% that of positive control (X). Y = Negative control.

mains high if farmers continue collecting planting materials (mostly seeds) from uncertified sources (Acheampong et al., unpublished).

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