~~European Journal of Plant Pathol.~~

107330

Detection of a foveavirus associated with the lethal 'ringspot' disease of African oil palm (*Elaeis guineensis* Jacq.) in South America

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Key words: Mancha anular, *Bactris* sp., West Africa

Abstract

A filamentous virus ca. 800 nm in length was consistently associated with a lethal disease of young African oil palms, referred to as "ringspot" in Colombia, Ecuador and Peru, in South America. This disease has also been reported from Ivory Coast in West Africa. Partial molecular characterization showed this virus to be distinct but related to tentative species of the *Foveavirus* genus, namely *Cherry green ring mottle virus* and *Cherry necrotic rusty mottle virus*, and more distantly to *Apple stem pitting virus*, the type foveavirus species.

Introduction

The 'ringspot' disease of African oil palm (*Elaeis guineensis* Jacq.) was first observed in 1969 (Dzido et al., 1978; Arévalo, 1988), in the north-western foothills of the Amazon region of San Martin, Peru. The disease affected mainly one year-old palms, which developed a systemic yellowing particularly noticeable in the younger leaves. Ringspots and elongated rings were observed on leaflets and rachis of affected leaves, which eventually turned brown and died within three months after initial symptom expression. A section of the stem of ringspot-affected oil palms showed a purplish colour in the vascular system. The roots and fruit bunches of diseased young palms often showed systemic necrosis. Five year-old and older oil palms were not affected. The lethal ringspot disease re-emerged around 1973 in the north-western oil palm-producing region of Ecuador, where it was referred to as 'lethal yellowing' and 'bud mottle' (Dzido et al., 1978, Chávez, 1988). Disease incidence varied between 3 and 40%, but some lots suffered the loss of up to 95% of the palms 18-24 months old. In 1985, a similar lethal disease reached the neighbouring oil palm production region of Tumaco in south-western Colombia (Jiménez, 1988). In this region, however, the characteristic symptoms consist of elongated yellow patches, which eventually appear as streaks on the basal leaflets and raquis of affected oil palm leaves. By 1988, the disease was affecting young plantations up to two and a half years old in an area of approximately 4.500 hectares. Disease incidence ranged from 2-45% and affected palms were usually found in foci (Jiménez, 1988). Previous investigations conducted in the United States, France and Latin America on the etiology of this disease failed to demonstrate the presence of any identifiable

pathogen (Dzido et al., 1978; Dollet et al., 1980; Renard and Quillec, 1984; Arévalo, 1988; Corley and Wood, 1990).

This investigation was conducted in response to the increasing incidence of the lethal 'ringspot' disease in south-western Colombia, and the threat it poses to the remaining 130.000 hectares of African oil palm grown in this country, the major producer of this crop in Latin America. This disease is also a major concern for other countries in Latin America, such as Brazil, Venezuela, Suriname, Costa Rica, Guatemala, Honduras, Mexico and Nicaragua, which cultivate approximately 160.000 additional hectares of African oil palm. Ecuador and Peru already have over 100.000 hectares of African oil palm at risk. This research may also benefit small-scale African oil palm producers in West Africa, the centre of origin of *E. guineensis* (Sauer, 1994), where similar symptoms have been observed to affect this important oil crop (Renard and Quillec, 1984).

Materials and methods

Virus isolates

All virus isolates used in this investigation were obtained from symptomatic spear leaves of 1-2 year old African oil palms showing the characteristic foliar yellowing (Figure 1) and streaking of the younger leaves and rachis in the municipality of Tumaco, Nariño, Colombia. The stems of all diseased palms selected for this investigation were dissected to observe the characteristic purplish rings associated with the lethal ringspot disease (Figure 2). Stem samples were also tested in this investigation. Selected tissue was processed fresh or conserved at -80 C° until needed. A leaf sample obtained from a wild palm belonging to the genus *Bactris* (tentatively identified as *B. setulosa*), was also

assayed here because the palm was found in the municipality of Tumaco, showing foliar yellowing symptoms similar to those described for 'ringspot' in African oil palm.

Pathogenicity tests

The following plant species were mechanically inoculated with extracts obtained from ringspot-affected leaf tissue diluted 1:4 or 1:10 (w/v) in sterile distilled water or buffers prepared as 0.5 or 0.1 M potassium phosphate buffer, pH 7.5, containing 0.5 g of sodium sulphite and 1 mM sodium diethyl-dithiocarbamate (DIECA). *Chenopodium album, C. amaranticolor, C. quinoa, C. murale, Elaeis guineensis, Nicotiana benthamiana, N. tabacum* 'Samsun', *Sorghum bicolor* 'Rio', and *Zea mays*. Infected African oil palm extracts were inoculated with sterile gauze, sterile pins and syringes to healthy seedlings of this species, using 10 seedlings per test.

Electron microscopy

Leaf extracts from symptomatic oil palms were negatively stained in 2% uranyl acetate, pH 3.7, and examined for the presence of virus particles using a JEOL JEM-1010 electron microscope. Leaf tissue of diseased oil palms, was prepared for cytology as described earlier (Morales et al., 1990). Thin sections were cut with a diamond knife using a MT 6000 Sorval ultramicrotome.

Electrophoresis

Double-stranded RNAs were extracted from healthy and ringspot-affected African oil palm plants according to the procedure of Dodds and Bar-Joseph (1993). Approximate ds-RNA weights were estimated using a 1 Kb ladder (Bethesda Research Laboratory). The *Bactris* sp. sample was processed following the same procedure.

Nucleic acid extraction and cDNA synthesis

Double stranded RNA extractions were performed as described above for the electrophoresis methodology. The ds-RNA species were separated in low melting point agarose gels (Gibco BRL) and then re-extracted using a RNeasy Kit (Qiagen) according to instructions. Approximately 3 µg of ds-RNA in a 13 µl volume, were denatured with an equal volume of 40 mM methylmercuric hydroxide, in the presence of 500 ng of random primers and 500 ng of an 18mer-oligo(dT) (Gibco), according to the method of Jelkman and co-workers (1989). cDNA synthesis was calculated for a final volume of 40 µl of 50 mM Tris-HCl, pH 8.3; 75 mM Mg Cl_2, 10 mM DTT, 0.5 mM each dATP, dCTP, dGTP, dTTP; 40 U RNasin (Promega), and 400 U of SuperScript II RT (Gibco, BRL). The mixture was incubated for 60 min at 37° C prior to adding 200 U of enzyme with 30 additional minutes of incubation. The reaction proceeded at 70° C for 1 min and finally stopped by immersion in ice-water. For the second strand reaction, the 40 µl of the first reaction, were taken up to 150 µl in a final mixture of 25 mM Tris-HCl, pH 7.5, 100 mM

KCl, 5 mM Mg Cl_2, 10 mM $(NH_4)_2SO_4$, 0.15 mM ß-NAD^+, 0.25 mM dATP, dCTP, dGTP, dTTP, 1.2 mM DTT, 25 U *Eschericia coli* Ligase, 40 U *E. coli* Polymerase, and 4 U *E. coli* RNase H. The mixture was incubated for 3 h at 16° C, adding 30 U T4 DNA Polymerase prior to further incubation at 16° C for 10 min. The reaction was stopped by adding 10 µl 0.5 M EDTA, ph 8.0, and then the nucleic acid was extracted with phenol:chloroform:isoamyl alcohol (25:24:1). The cDNA was precipitated with a 1/10 volume of 7 M Ammonium acetate and 2.5 volumes of ethanol.

Nucleic acid hybridisation

Random-primed c-DNA was labelled with [α^{32} P]dATP. First strand cDNA was purified through Sephacryl S-400 (Pharmacia). Dot blot hybridizations were performed using 100 mg of young oil palm tissue were homogenized in liquid nitrogen. Total RNA extraction was performed according to the instructions provided for the RNeasy kit (Qiagen). One µg of total RNA per sample was applied onto Hybond-N+ nylon membranes (Amersham, Pharmacia Biotech). The membranes with the samples were treated with 0.05 N NaOH-0.15 M NaCl for 5 min and, then, another 5 min with 0.1 M Tris, pH 7.5-0.15 M NaCl, prior to being fixed in a UV-Stratalinker (Stratagene). The hybridization was carried out for 16 h at 50° C in 40% formamide, 5X SSPE, 6X Denhardt's and 100 µ/ml salmon DNA. Membranes were washed twice at 52° C with 0.5% SDS and 0.5X SSC. Besides the ringspot-affected and disease-free African oil palm controls, two potyviruses, *Sugarcane mosaic virus* and *Bean common mosaic virus*, maintained in African oil palm and common bean, respectively, were also used as controls.

Molecular cloning

The cDNA was ligated into the SmaI site of pBlueScript II (+) plasmids (Stratagene), and then used to heat shock transform *E. coli* cells DH5α (Stratagene). Transformed *E. coli* cells were selected using agar-ampicillin, X-Gal, and IPTG (Stratagene). To identify bacterial colonies containing transformed plasmids, a sample of every white colony observed was re-suspended in 50 µl of a PCR mix containing 1 µl 10 mM dNTPs, 5 µl 25 mM Mg Cl_2, 0.5 µl T3 primer (10 pmol, Stratagene), 1 U Taq DNA polymerase (Promega), 5 µl PCR 10X buffer (Promega) and distilled water to reach the final volume. Samples were amplified in a PTC 100 thermocycler (MJ Research) following a 30 cycle program (94 C°/3 min, 94 C°/30 s, 72 C°/2 min, and a final extension at 72 C°/5 min. The products obtained were analysed by electrophoresis in a 1.3% agarose gel.

Sequencing

Sequencing was performed with an ABI PRISM 377 DNA automated sequencer (Perkin Elmer). Sequences obtained were analysed using the BLAST program of the National Center for Biotechnology Information (National Institute of Health, Bethesda, MD).

Results

Pathogenicity tests

None of the test plants mechanically inoculated in this study developed symptoms or was shown by electron microscopy to contain virus-like particles. The inoculated African oil palm seedlings were maintained under observation for over a year, but none of the 10 seedlings inoculated by the standard, pricking or injection methods expressed any symptoms.

Electron microscopy

Direct observation of negatively stained spear leaf tissue extracts prepared from ringspot-affected African oil palms consistently revealed the presence of flexuous, filamentous virus-like particles approximately 800 nm in length and 15 nm in diameter (Figure 2), albeit at a very low concentration. Longer particles of similar morphology were occasionally observed in these tissue extracts, suggesting the occurrence of end-to-end aggregation. Similar filamentous virus-like particles were observed in total disarray in the cytoplasm of leaf cells obtained from ringspot-affected African oil palms (Figure 3). These particles were localised in particular areas of the mesophyll, but did not form any particular type of inclusion or viroplasm.

Electrophoresis

The extraction and electrophoresis of ds-RNA from ringspot-affected African oil palms, revealed the presence of up to three bands approximately 8034, 7171 and 5854 bp in size (average of nine electrophoresis tests for an equal number of ringspot-affected palms, over a three year period). The 8034 band predominated in most tests (Figure 4), whereas the concentration of the two lower bands varied appreciably, suggesting the existence of only one ribonucleic acid species and two degraded sub-products. The leaf sample of the symptomatic *Bactris* sp. palm tested here, yielded a similar band pattern, with a predominant ds-RNA species of approximately 8135 bp.

Nucleic acid hybridisation

Positive hybridisation reactions were observed with leaf and stem tissue extracts prepared from ringspot-affected African oil palms, but not with similar extracts prepared from disease-free palms. Very mild background reactions were observed with a potyvirus that infects African oil palm (Figure 5).

Molecular cloning and sequencing

A total of 10 clones were sequenced and selected for further analysis. Eight overlapping clones with a continuous sequence of 1667 bases, showed nucleotide sequence identities of 59.8% and 59.7% with corresponding terminal regions in ORF 1 (RNA-dependent

RNA polymerase) of *Cherry green ring mottle virus* (CGRMV) and *Cherry necrotic rusty mottle virus* (CNRMV), respectively. This clone shows a 49% nucleotide sequence identity in this genomic region with *Apple stem pitting virus* (ASPV). An 816 nucleotide long section of this clone, shows a 67% amino acid sequence identity with the corresponding sequences of CGRMV and CNRMV. An additional clone of 916 bp located in the central portion of ORF 1, had nucleotide sequence identities of 50.7% and 47.5%, and amino acid sequence identities of 52% and 39%, with the corresponding regions of CGRMV and ASPV, respectively.

Finally, a 671 bp clone was shown to contain a 469 bp fragment with a 43.7% and 48% nucleotide and amino acid sequence identity, respectively, to the corresponding region in ORF 4 (putative movement protein 3 of the triple-gene block) of CGRMV. The remaining 202 bp fragment had a 43% and 35% amino acid sequence identities with corresponding regions of the coat protein gene (ORF 5) of CNRMV and CGRMV.

Discussion

Results obtained in this investigation showed that the 'ringspot' disease of African oil palm in Colombia is consistently associated with a filamentous virus related to species in the genus *Foveavirus* (Regenmortel et al., 2000; Rott and Jelkmann, 2001; Zhang et al., 1998). The African oil palm virus, hereafter referred to as African oil palm ringspot virus (AOPRV), would be the first foveavirus found in the tropics. Similar tropical, filamentous viruses possessing genomic affinities to allexi-, carla-, fovea- and potexviruses, namely, Indian citrus ringspot virus (Rustici et al., 2000) and Banana mild

mosaic virus (Gambley and Thomas, 2001), have been recently described, but remain unassigned to any virus genus. The various clones sequenced in this investigation consistently showed a closer affinity between AOPRV and recognized or tentative foveaviruses, than to species belonging to other filamentous virus genera. Whereas it is possible that further molecular work on AOPRV and future taxonomic developments might require the re-classification of this virus, we propose it as a tentative member of the *Foveavirus* genus.

One important aspect of AOPRV is its epidemiology, considering that foveaviruses, such as ASPV, CGRMV and CNRMV, do not have known biological vector (Regenmortel et al., 2000). African oil palm does not require any cultural treatments, such as pruning, during its susceptible period (first three years of age) that might explain the transmission the causal virus. The ringspot disease of African oil palm disseminates rapidly during seasons of relatively low precipitation, when some arthropod vectors are more active. The disease also spreads among adjacent palms either in the nursery or in young plantations.

Acknowledgements

This research was possible thanks to the financial and logistic support of the Colombian Oil Palm Research Centre (CENIPALMA). We are grateful to Mr. Guillermo Guzmán for technical support with the graphic material included in this publication.

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Figure Legends

Figure 1. Young African oil palm showing initial foliar yellowing associated with the 'ringspot' disease.

Figure 2. Filamentous particle observed in African oil palms affected by the 'ringspot' disease.

Figure 3. Filamentous particles observed in the cytoplasm of 'ringspot'-affected African oil palm leaf cells.

Figure 4. Electrophoresis of double-stranded RNA. Lane 1: healthy oil palm; Lane 2: oil palm infected by a potyvirus; Lane 3: 1 Kb markers; Lane 4: oil palm affected by 'ringspot'.

Figure 5: Dot blot of: column 1: Leaf extracts from 'ringspot'-affected oil palm; Column 2: Extracts from necrotic oil palm stem tissue affected by 'ringspot'.; Column 3: healthy oil palm leaf extract; Column 4: Leaf extracts from potyvirus-infected oil palm.

Clones de cDNA obtenidos a partir de ds-RNA de tejido de palma de aceite con síntomas de Mancha Anular. Se empleó como agente desnaturalizante Hidróxido de Metil Mercurio 20 mM.