Ann. appl. Biol. (1980), **96**, 193–200 n° 2
With 1 *plate*
Printed in Great Britain

Groundnut eyespot virus, a new member of the potyvirus group

BY J. DUBERN*

Laboratoire de Virologie, Centre ORSTOM d'Adiopodoumé, BP V51, Abidjan, Côte d'Ivoire

AND M. DOLLET

Département Virologie, IRHO, Station de Recherches de Pathologie Comparée INRA-CNRS 30380 Saint-Christol-lèz-Alès, France

(*Accepted 10 June 1980*)

SUMMARY

A virus causing 'eyespot' leaf symptoms in groundnut plants was transmitted by sap-inoculation and by *Aphis craccivora* in the non-persistent manner. It infected 16 of 72 species from five of 12 families and was easily propagated in *Arachis hypogaea* and *Physalis floridana*. The virus has particles *c.* 13 × 755 nm and is serologically closely related to soybean mosaic and pepper veinal mottle viruses, and more distantly to four other potyviruses. The virus differs in host range, *in vitro* properties and serological properties from previously described strains of soybean mosaic and pepper veinal mottle viruses. It seems to be a distinct member of the potyvirus group and we propose the name groundnut eyespot virus.

INTRODUCTION

Eyespot leaf symptoms on a local, unnamed cultivar of groundnut (*Arachis hypogaea*) were first observed near Korhogo, in the north of the Ivory Coast, in October 1976 (Dubern & Dollet, 1978). Infected leaves had dark green spots surrounded by a chlorotic halo within which was a dark green ring. Many leaflets also had dark green line patterns but there was little reduction in size of leaves or plants. The diseased plants flowered and produced seeds normally. Fewer than 0·1% of the plants seemed diseased and crop yield was not reduced.

In several groundnut diseases the leaves develop ringspot symptoms: these include the diseases caused by groundnut ringspot (Klesser, 1966; Kuhn, Hammons & Sowell, 1964), groundnut chlorotic spot (Haragopal & Nayudu, 1971), groundnut ring mottle (Sharma, 1966), groundnut mosaic (Nariani & Dhingra, 1963), peanut mottle (Bock, 1973; Kuhn, 1965), peanut clump (Thouvenel, Dollet & Fauquet, 1966) and tomato spotted wilt (Helms, Grylls & Purss, 1961) viruses. However none of these viruses induces typical eyespot symptoms consisting of concentric coloured rings.

This paper describes the host range, purification and some properties of the virus, isolated from infected plants in the Ivory Coast, which we now call groundnut eyespot virus (GEV).

MATERIALS AND METHODS

Virus. The virus was isolated from a naturally infected plant at Korhogo in the north of the Ivory Coast.

Plant growth conditions. All test plants were grown in sterilised soil in screen houses at day

* Present address: ORSTOM, Laboratoire de Virologie-Bactériologie des Plantes, BP 1386 Dakar, République du Sénégal.

temperatures ranging from 28 to 35 °C. Relative humidity was always 95–100% and day length *c.* 12 h. *Chenopodium* spp. and some European species were given 4 h extra light from fluorescent tubes.

Mechanical transmission. Inocula were prepared by grinding infected leaves of *Arachis hypogaea* cv. Te3 in 0·05 M potassium phosphate buffer (4 ml/g of tissue) at pH 7·3 containing 0·01 M sodium diethyldithiocarbamate and 12·5 mg/ml magnesium bentonite. The extract was rubbed manually on Carborundum-dusted leaves of test plants. All plants used in host range studies were inoculated when young and growing vigorously. Groundnut plants were used when they were approximately 10 days old and bore three leaves.

Infectivity assay. No suitable local lesion host was found for GEV so systemic hosts were used for infectivity assay. *A. hypogaea* cv. Te3 was used for determining the *in vitro* properties of the virus and for back inoculations in host range studies, and either *Physalis floridana* or *Arachis hypogaea* was used as a source for purification.

Determination of in vitro *properties.* The standard procedures described by Bos, Hagedorn & Quantz (1960) were used; crude extracts from young leaves of goundnut plants infected for at least 3 wk were used as the source of virus. For all studies extracts were prepared in the same way as inocula for mechanical transmission. The dilution end-point was determined by serial dilutions in 0·05 M potassium phosphate buffer at pH 7·3. The thermal inactivation point was tested by immersing 2 ml samples of extracts, contained in 5 ml tubes, in a water bath at different temperatures for 10 min and then cooling the tubes in an ice bath before inoculation. To test ageing *in vitro*, the infectivity of extracts kept at 25 °C was checked hourly. Resistance to air-drying was determined by testing daily the infectivity of *A. hypogaea* leaves dried over silica gel.

Aphid transmission. Aphid transmission experiments were done in the laboratory using *Aphis craccivora* Koch. reared on groundnut plants. Adult and late instar apterous aphids were starved for 1–3 h, then allowed an acquisition access time of *c.* 30–60 s on diseased groundnut plants and an inoculation access period of 24 h on healthy groundnut seedlings. The aphids were then killed by applying an insecticide (systoate). Virus retention by aphids was studied by transferring aphids, after an acquisition access time of *c.* 30–60 s, to healthy *Vigna unguiculta*, a non-susceptible plant, for different times, and then transferring them to healthy groundnut seedlings for 24 h, before killing them with insecticide. Ten aphids/plant were used in each experiment.

Purification. GEV was purified from groundnut cv. Te3. Freshly collected leaves (1 g/4 ml) were ground for 1 min in a Waring Blendor with 0·5 M potassium phosphate buffer, pH 7·5, containing 1 M urea and 0·01 M sodium bisulphite; urea was used to disrupt virus aggregates and to solubilise virus particles (Damirdagh & Shepherd, 1970). The extract was strained through several layers of aseptic gauze, chilled in an ice bath and clarified by low-speed centrifugation in a Sorvall RC-2B refrigerated centrifuge (7000 ***g*** for 20 min). Chloroform was then added to 10% v/v and the mixture blended again for 10 s and then centrifuged for at least 10 min at 7000 ***g***. The aqueous phase was retained and, to disrupt residual chloroplast fragments, Triton X-100 detergent was added dropwise, while stirring, to a concentration of 5% (v/v) (van Oosten, 1972). The preparation was centrifuged in a Beckman L50 centrifuge at 105 000 ***g*** for 150 min and the pellets resuspended in 0·05 M potassium phosphate buffer, pH 7·5, containing 1 M urea and 0·01 M sodium bisulphite; urea was essential to resuspend the virus particles. In a second cycle of differential centrifugation the virus was sedimented through a 2 cm deep layer of 20% sucrose in the Beckman R30 rotor (100 000 ***g*** for 150 min). The pellets were resuspended in 0·05 M sodium borate buffer, pH 8·2. The virus was further purified by sucrose density gradient centrifugation. Density gradient columns (5–40% sucrose in 0·05 M borate buffer, pH 8·5) were prepared in 75 × 25 mm cellulose nitrate tubes. Gradients were centrifuged for 150 min at 51 000 ***g*** in a Beckman SW 25.1 rotor. They were fractionated by piercing the bottom of the tube and

collecting the drops in about 50 fractions. The optical density of each fraction, diluted with borate buffer, was determined at 260 nm in a Zeiss PMQ 11 spectrophotometer.

Electron microscopy. Virus preparations and sections were examined in a Philips EM 300 electron microscope after negative staining with 1% uranyl acetate. Electron micrographs were taken at magnifications of 11 000 and 40 000. To determine the dimensions of the particles, tobacco mosaic virus particles (18 × 300 nm) were added to the purified virus preparations as a standard.

For ultramicrotomy, leaf tissue was embedded in epon and ultrathin sections were stained with uranyl acetate and lead citrate.

Serology. Antiserum to GEV was prepared by injecting a rabbit intramuscularly with purified virus, emulsified with an equal volume of Freund's incomplete adjuvant, on four occasions at weekly intervals. Serum was stored at 4 °C with an equal volume of glycerol.

The microprecipitation test, under paraffin oil in Petri dishes, was used (van Slogteren, 1954). As controls normal rabbit serum and healthy plant protein (purified preparations from healthy groundnut plants) were used. Results were observed with a binocular microscope after incubation for about 1, 3, 6 and 12 h.

Clarified virus preparations were tested against antisera of groundnut viruses and potyviruses (Brandes & Bercks, 1965).

RESULTS

Host range and symptomatology

Seventy-two plant species from 12 families were inoculated with GEV. The virus was not always recovered by back tests from plants that developed symptoms but repeated inoculations gave the same results with these species.

Approximately 5 days after inoculation small yellow spots 0·5 mm in diameter appeared on the young terminal leaf of *A. hypogaea* cv. Te3, these spots enlarged to 2 mm diameter and became slightly chlorotic. On leaves produced subsequently, characteristic eyespots up to 20 mm in diameter were observed; typically each had a very dark green centre surrounded by a chlorotic halo within which was a dark green ring. Line patterns were formed on many leaflets, and these always had a dark green centre (Plate, figs 1–4). These symptoms continued to develop on newly-produced leaves for at least 2 months. Leaves and plants were not noticeably stunted and the diseased plants flowered and set seed normally.

In addition to *A. hypogaea* cv. Te3 the virus produced symptoms in or was recovered from the following species:

Papilionaceae. *Canavalia ensiformis:* vein mosaic and crinkling.

Solanaceae. *Lycopersicon esculentum* cv. Marmande: mottle 15 days after inoculation. *Physalis floridana:* mottle 5 days after inoculation. *Physalis alkekengi:* faint systemic ringspot. *Petunia hybrida:* mosaic and dwarfing. *Petunia nana-compacta:* mosaic and dwarfing. *Nicotiana clevelandii:* stunt and mottle.

Aizoaceae. *Tetragonia expansa:* systemic yellow spots then ringspots.

Chenopodiaceae. *Beta vulgaris:* symptomless.

Scrophulariaceae. *Torenia fournieri:* faint systemic ringspots. *Antirrhinum majus:* mottle and dwarfing.

The following produced symptoms but virus was not recovered from them:

Papilionaceae. *Centrosema pubescens:* dotting on inoculated leaves 6 days after inoculation. *Pisum sativum:* necrotic lesions on inoculated leaves. *Psophocarpus tetragonolobus:* necrotic

lesions on inoculated leaves. *Vigna unguiculata* cv. Black Eye: necrotic lesions on inoculated leaves.

The following species showed no symptoms and virus was not recovered from them:

Chenopodiaceae. *Chenopodium amaranticolor, C. quinoa, C. murale.*

Papilionaceae. *Cajanus cajan, Cassia occidentalis, C. obtusifolius, Clitoria ternatea, Crotalaria atrorubens, C. juncea, C. mucronata, C. pallida, C. usuramoensis, Desmodium polycarpum, Dolichos jacquinii, Kennedia* sp., *Medicago sativa, Melilotus alba, Phaseolus lathyroïdes, P. mungo, P. vulgaris* cvs Bountiful and Triomphe de Farcy, *Stylosanthes gracilis, Trifolium repens, Vicia faba.*

Solanaceae. *Capsicum annuum, C. frutescens, Datura metel, D. stramonium, Nicotiana clevelandii* × *N. glutinosa* hybrid, *N. glutinosa, N. megalosiphon, N. paniculata, N. rustica, N. tabacum* (cvs White Burley, Samsun, Samsun NN, Xanthi), *N. tomentosa, N. texana,* and *Solanum nigrum.*

Amaranthaceae. *Gomphrena globosa.*

Compositae. *Helianthus annuus, Zinnia elegans.*

Dioscoreaceae. *Dioscorea alata, D. cayenensis.*

Euphorbiaceae. *Hevea brasiliensis, Manihot utilissima, Manihot glaziovii, Ricinus communis.*

Gramineae. *Oryza sativa, Panicum maximum, Pennisetum japonicum, Sorghum vulgare, Zea mays.*

Malvaceae. *Gossypium barbadense, G. hirsutum, Hibiscus esculentus.*

Passifloraceae. *Passiflora edulis, P. foetida, P. quadrangularis.*

Properties in vitro

Dilution end-point. Infectivity of *A. hypogaea* cv. Te3 extracts decreased greatly between dilutions of 10^{-2} and 10^{-3}, and was lost at 10^{-4}.

Thermal inactivation point. The infectivity of freshly expressed sap from inoculated groundnut leaves was much decreased after 10 min at 42 °C and was lost after 10 min at 44 °C.

Resistance to air-drying. Infectivity was lost when infected groundnut leaves were dried.

Longevity in vitro. The infectivity of *A. hypogaea* extracts was lost after 3 h at 25 °C.

Effect of freezing. Infectivity decreased little during 1 month when leaves of groundnut where stored at −20 °C. Infectivity was retained in leaves alternately frozen and thawed every day for 10 days.

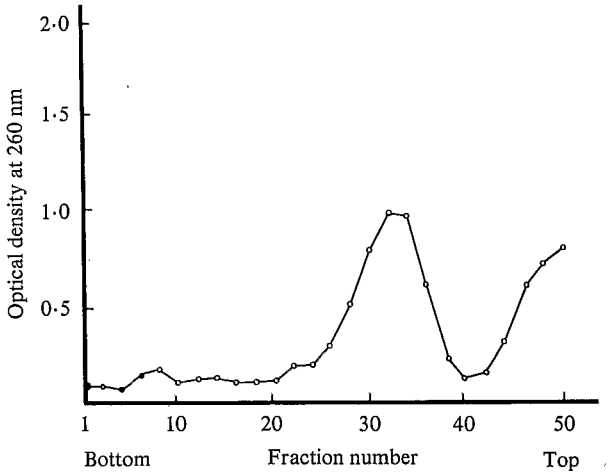
Effect of pH. The infectivity of extracts, freshly expressed from inoculated groundnut leaves, depended on pH; at pH 5·0 to 9·0, extracts were infective. Most infectivity was obtained at pH 7·5 to 8·5. At pH 4·5 and 9·5 no infectivity was detected.

Aphid transmission

After an acquisition access time of *c.* 30–60 s, GEV was transmitted by *Aphis craccivora* in three experiments to 3/5, 23/36 and 20/24 plants, respectively. Using the formula of Gibbs & Gower (1960), it was calculated that 17, 19 and 30% of the aphids had transmitted virus. In retention studies *A. craccivora*, that had previously been on infected plants for *c.* 30–60 s, transmitted after being kept for 10 h, but not after 12 h, on *Vigna unguiculata.*

Purification

Opalescent virus bands were found at 20–24 mm beneath the meniscus (fractions 30–33) in sucrose density gradients (Text-fig. 1). Yields of virus from the gradients, calculated from the extinction coefficient $OD_{260}^{1\,cm} = 2{\cdot}4$ for 1 mg/ml of the morphologically-similar tobacco etch virus (Purcifull, 1966), were between 25 and 35 mg/kg leaf. Virus was collected, diluted with borate buffer, and concentrated by ultracentrifugation (105 000 *g* for 150 min). The resulting



Text-fig. 1. Sucrose density gradient centrifugation of GEV. 2 ml virus preparation was layered on a gradient of 5–40% sucrose (26 ml total) in 0·05 M sodium phosphate buffer, pH 7·5, and centrifuged for 2 h at 25 000 rev/min in a SW 25.1 rotor.

pellets, after resuspension in 0·05 M borate buffer at pH 8·2, had the following u.v. properties: maximum absorption at 260–262 nm, minimum at 247 nm, ratio maximum/minimum of 1·10–1·15; the $A_{260/280}$ ratio was 1·22–1·24. This last ratio corresponds to a nucleoprotein containing 5·5–6·5% RNA (Layne, 1957).

Aggregation during purification was partially prevented by using 0·1% Triton X-100 detergent in sodium borate buffer at pH 8·2 for resuspending the virus after the last R30 ultracentrifugation and during sucrose density-gradient centrifugation. The yield of virus was twice that of the first procedure but the preparation was less pure.

The virus was also concentrated by precipitation for 4 h with 4% polyethylene glycol, mol. wt 20 000, with 0·1 M potassium chloride (Albrechtova & Klir, 1970). The virus was then purified by centrifugation on a sucrose gradient in the SW 25-1 rotor. However, the yield from this procedure was the poorest of the methods used.

Electron microscopy

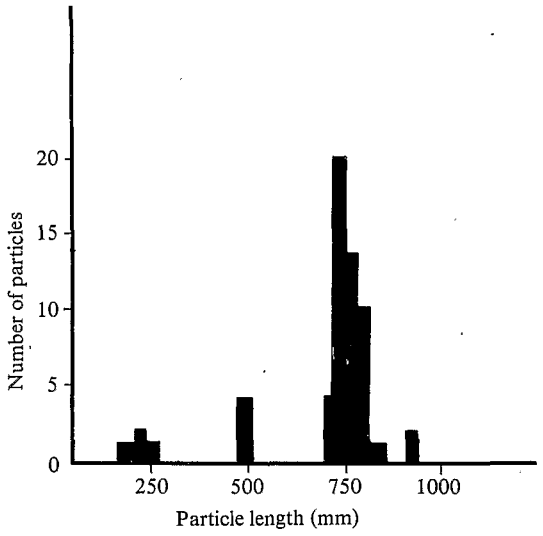
Flexuous, filamentous particles were found in infective virus preparations purified from groundnut plants by differential and sucrose density-gradient centrifugations (Plate, fig. 5). Measurements of 106 particles gave average values of 755 ± 25 nm for length and 12·5 nm for width (Text-fig. 2).

Observations on ultrathin sections of epon-embedded leaf tissue showed cytoplasmic inclusions typical of potyviruses (Plate, fig. 6).

Serology

The homologous antiserum, after absorption with clarified sap of healthy plants, reacted up to dilutions of 1/256 with GEV.

GEV failed to react with antisera (donors of sera and reciprocals of homologous titres in parentheses) to: tobacco mosaic virus (Waterworth, 64; Mink, 256) and peanut clump virus (Thouvenel, 1024), and to the following potyviruses: peanut mottle (Bock, 256; Kuhn, 256), sugarcane mosaic (Gillaspie, 256; Pirone, 512), Colombian datura virus (Bartels, 256), cowpea aphid-borne mosaic (Conti, 128), yam mosaic (Thouvenel, 512), potato A (Bartels, 128), henbane mosaic (Bartels, 128), parsnip mosaic (Murant, 64), pea seed-borne mosaic (Mink, 400), clover yellow vein (Bartels, 1024; Barnett, 2048), potato Y (Bartels, 126 and 256; Luisoni



Text-fig. 2. Length distribution of GEV particles in samples from purified preparations.

& Lovisolo, 2048), tobacco etch (Bartels; Purcifull; Maat, 512), bean common mosaic (Luisoni & Lovisolo, 2048; Maat & Bos, 512), bean yellow mosaic (Maat & Bos, 512; Luisoni & Lovisolo, 128), lettuce mosaic (Tomlinson, 64), turnip mosaic (Tomlinson, 16), bidens mottle (Purcifull, 256), Arizona pepper virus (Zitter, 512), ryegrass mosaic (ATCC) and hippeastrum mosaic (Brunt, 1024).

However, with GEV as the antigen, antisera to guinea-grass mosaic virus (Thouvenel, 2048) reacted up to dilutions of 1/256, passion-fruit ringspot virus (de Wijs, 2048) up to 1/256, wisteria vein mosaic virus (Luisoni & Lovisolo, 1024) up to 1/64 and iris fulva mosaic virus (Barnett, 256) up to 1/64. In similar tests antisera to soybean mosaic virus (Barnett, 128) reacted strongly up to 1/64, pepper veinal mottle virus (Ivory Coast strain) (de Wijs, 4096) up to 1/2048 and pepper veinal mottle virus (Ghana strain) (Brunt, 8192) up to 1/8192.

The homologous and heterologous titres of these sera were not determined by the same procedure; quoted homologous titres are as given by the donors and heterologous titres were determined by the microprecipitin test (van Slogteren, 1954). Thus the homologous titres of the antisera to guinea-grass mosaic virus, passion-fruit ringspot virus and pepper veinal mottle virus (Ivory Coast strain) were determined by microprecipitin tests, and that of the antiserum to pepper veinal mottle virus (Ghana strain) by tube precipitin tests (Brunt, Kenten & Phillips, 1978). In comparative studies on the sensitivity of these two procedures, passion-fruit ringspot virus and pepper veinal mottle virus (Ivory Coast strain) had homologous titres of 2048 and 4096 respectively in microprecipitin tests but 512 and 1024 in tube precipitin tests. This suggests that the homologous titre of the antiserum to pepper veinal mottle virus (Ghana strain) would probably be about 32 000 in microprecipitin tests.

DISCUSSION

Of the numerous viruses reported to infect groundnuts throughout the world, only groundnut latent (Bock *et al.*, 1968) and peanut mottle (Kuhn, 1965; Bock, 1973) have particles of similar morphology and size to those of GEV. However, GEV seems to be distinct from both these viruses because it is serologically unrelated to peanut mottle and, unlike groundnut latent, it is aphid-transmissible. Although GEV is serologically related to guinea-grass mosaic, passion-fruit ringspot, iris fulva mosaic and wisteria vein mosaic viruses, none of these infects groundnut and

they also differ from GEV in other ways. However, as GEV is serologically so closely related to soybean mosaic (SMV) and pepper veinal mottle (PVMV) viruses, we have considered whether it is a strain of either or both viruses. Neither PVMV nor SMV infects groundnut (de Wijs, 1973; Bos, 1972), and both have host ranges differing markedly from GEV. In particular, SMV infects only leguminous species and some *Chenopodium* species, while PVMV does not infect leguminous species. Both viruses also differ serologically from GEV. Thus, unlike GEV, SMV is related to bean yellow and bean common mosaic viruses, and PVMV to potato virus Y. Moreover, our comparative tests indicate that GEV, SMV and PVMV are serologically related but not identical. For these reasons, GEV is probably best considered as a distinct member of the potyvirus group rather than as a strain of either SMV or PVMV.

We thank Dr Remaudières (Institut Pasteur) for identifying the aphids, Mr J.-C. Renard for providing groundnut cv. Te3, Mrs A. Dubern, Mr G. Petiot (Photography), Aho Kouakou, Drs A. Monsarrat and C. Fauquet for technical assistance, and Drs Barnett, Bartels, Bock, Bos, Brunt, Conti, de Wijs, Fauquet, Gillaspie, Kuhn, Lovisolo, Luisoni, Maat, Mink, Murant, Pirone, Purcifull, Thouvenel, Tomlinson, Waterworth and Zitter for supplying antisera.

We also thank Drs Vuittenez and Fauquet for helpful discussions and, Drs B. D. Harrison and A. A. Brunt for criticism of this manuscript.

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(*Received 10 March 1980*)

EXPLANATION OF PLATE

Fig. 1. Symptoms of groundnut eyespot virus in *Arachis hypogaea* cv. Te3, mechanically systemically infected leaf.

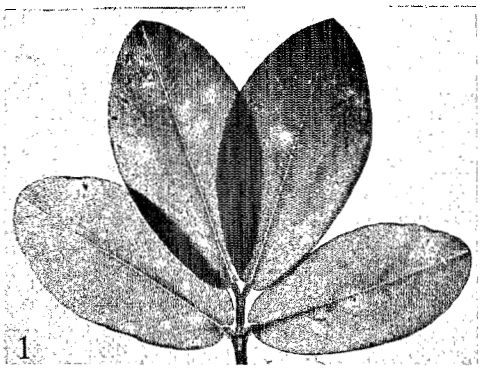
Fig. 2. Eyespots on the second systemically infected leaf, 8 days after inoculation.

Fig. 3. Line pattern on old systemically infected leaf of a 3-month-old plant.

Fig. 4. Higher magnification view of eyespots, each showing a very dark green centre surrounded by a chlorotic halo which contains a dotted dark green ring.

Fig. 5. Electron micrograph of a partially purified preparation of GEV. Particles are about 755 nm long.

Fig. 6. Electron micrograph of a parenchyma cell from a systemically infected leaf 8 days after the plant was inoculated. Numerous pinwheels are present in the cytoplasm.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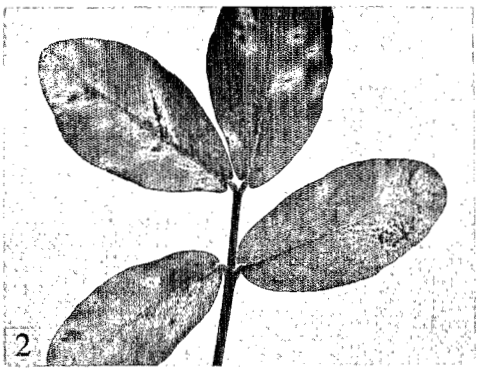
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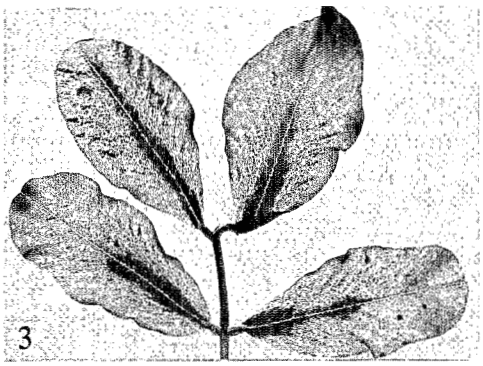
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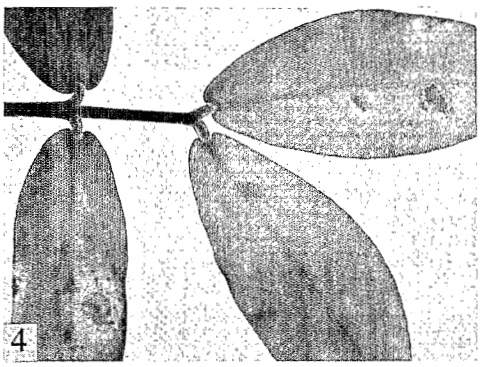
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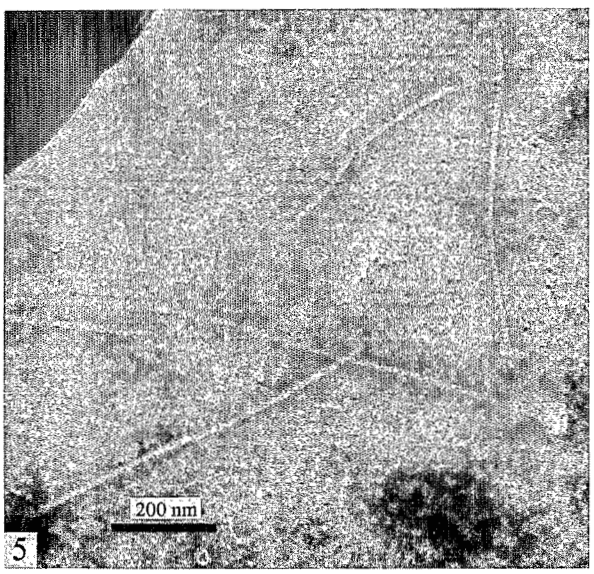

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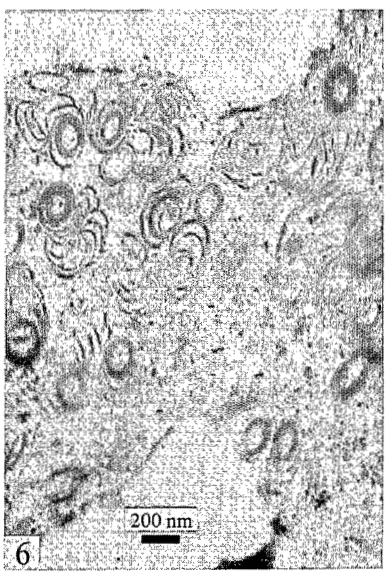

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