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Groundnut Crinkle Virus, a New Member of the Carlavirus Group

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With 3 figures

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A disease causing leaf crinkling and stippling symptoms on groundnut (*Arachis hypogaea* L.) leaves was observed in the south of Ivory Coast, near Dabou, Abidjan and Bingerville, all along the lagoon, in 1976—1977 (DUBERN and DOLLET 1979). The symptoms were noted on local varieties: very slight crinkling on the leaves, as if the main vein was too short; this crinkling was often associated with a very delicate stippling. Little reduction in size of leaves or plants was noticed. The diseased plants flowered and produced seeds. Production seemed not to be reduced. These very slight symptoms, in comparison with those of groundnut chlorotic rosette or groundnut eyespot (DUBERN and DOLLET 1978), often passed unnoticed previously. However, in the fields near the lagoon, often more than 50% of the plants seemed diseased.

Of the groundnut diseases reported in the literature (DUBERN 1979), only groundnut rugose leaf curl (GRYLLS 1954) developed on the leaves similar symptoms. In Ivory Coast, symptoms resembling those of rugose leaf curl were observed by A. MONSARRAT (unpublished data 1976). However, in the affected plants, the shoots became increasingly distorted and no stippling was noticed.

This paper described the host range, purification and some properties of the new virus, named groundnut crinkle virus (GCV).

Materials and Methods

All test plants were grown in sterilized soil in greenhouses, where temperature varied from 28 to 35 °C during the day. Relative humidity was always 95—100 % and day-length C. 12 h per day. *Chenopodium* spp. and some European species, including *Trifolium repens* and *Pisum sativum*, were given extra-light provided by fluorescent tubes.

The original inoculum was obtained from naturally infected plants collected in the south of Ivory Coast.

Inocula were prepared by grinding infected leaves of groundnut 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. All test plants used in host range studies were inoculated when young and growing vigorously. Groundnut plants were used when they were approximately 10 days old, with 3 leaves. Attempts were made to recover virus from all inoculated test plants by back inoculation of sap to groundnut cv. Te3 seedlings.

No suitable local lesion host was found for the virus at the beginning of this study, and a systemic host was therefore used for infectivity assay. For testing stability of the virus in crude extracts and for back inoculations in host range studies, it was *A. hypogaea* L. cv. Te3. Later, many species in the Leguminosae were found to give necrotic local lesions, and might be used for infectivity assay: e. g. *Vigna unguiculata* cv. Black Eye and *Dolichos jacquinii*.

The standard procedures described by BOS *et al.* (1960) were used with crude extracts from young leaves of groundnut plants infected at least 5 weeks previously. Extracts for all studies were prepared 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 quantities of extracts, contained in 5 ml tubes, in a water bath at various temperatures for 10 min, and then placing them in an ice bath and inoculating immediately. To test ageing *in vitro*, the infectivity of extract kept at 25 °C and at 4 °C was checked every hour. Resistance to air-drying was determined by testing daily the infectivity from *Arachis hypogaea* leaves dried in boxes dehydrated by Silica gel. Effect of freezing and thawing was determined by testing infectivity from groundnut leaves alternately frozen at —20 °C and thawed once a day. Studies on the effect of pH were done by two methods: (a) by adjusting the pH values to pH 7.5 before inoculating, and (b) electrofocusing the purified virus in an ampholine column.

Aphid transmission experiments were done in the laboratory using *Aphis craccivora*, *A. citricola* (= *A. spiraecola*) and *A. gossypii* reared on groundnut plants. Adult and late instar apterous aphids were starved for 2—3 h, then allowed an acquisition access feed of various times on diseased groundnut seedlings, and inoculation access time of 24 h on healthy groundnut plants. Finally they were killed by applying an insecticide, systoate. In each experiment, 5, 10 or 20 aphids were used on each test plant.

High-speed centrifugation was done in a Beckman L50 centrifuge. A Sorvall RC-2B refrigerated centrifuge was used for low-speed centrifugation. Density gradient columns, using 40—50 % sucrose in 0.05 M borate buffer, pH 8.5, were prepared in 3 × 1 inch cellulose nitrate tubes, and the 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 II spectrophotometer.

Leaf dip preparations from healthy and diseased plants were made using the method of HITCHBORN and HILLS (1965). Leaf dip and purified preparations were examined with a Philips EM 300 electron microscope after negative staining with 1 % uranyl acetate and 0.5 % sodium ethylenediamine tetraacetate, at pH 7.0. Electromicrographs were taken at magnifications of 4,000 and 11,000.

The microprecipitation reaction under paraffin oil in Petri dishes was used (VAN SLOGTEREN 1954). For testing, normal rabbit serum and plant protein preparations from healthy groundnut plants were used. Results were observed with a binocular microscope after

incubation for about 1 h, 3 h, 6 h and 12 h. No antiserum to GCV was prepared. Clarified virus preparations were tested against antisera to other groundnut viruses and other carlaviruses (HARRISON *et al.* 1971).

Results

Fifty-five species from 9 families were inoculated with the groundnut virus. The virus was not always recovered by back-tests from plants that developed symptoms but repeated inoculations gave the same results on these plant species.

Symptomatology on *Arachis hypogaea* L. cv. Te3

Approximately 20 days after inoculation, and frequently 30—40 days, the young terminal leaf developed a very delicate stippling; this consisted of very small streaks, 0.5 mm long, either covering the whole surface of the leaflets or occurring only along the main vein. Simultaneously this main vein did not grow and leaflets became crinkled. However, sometimes the crinkling symptom did not develop. Mechanical transmission was not easily accomplished, and usually only 10—20% of the inoculated seedlings were infected. These symptoms continued to develop on the newly-produced leaves for at least two months. No obvious stunting of leaves or plants was noticed and the diseased plants flowered and produced seed.

Species from which the virus was recovered

Leguminosae. *Arachis hypogaea* cv. Te3. *Canavalia ensiformis*: leaf curl, crinkling and faint mottle 3—5 weeks after inoculation. *Centrosema pubescens*: necrotic local lesions on inoculated primary leaves 8 days after inoculation; faint mottling after 3 weeks. *Dolichos jacquinii* necrotic local lesions on inoculated primary leaves 8—10 days after inoculation. *Phaseolus vulgaris*: necrotic local lesions on inoculated primary leaves 8—10 days after inoculation. *Phaseolus lathyroïdes*: necrotic local lesions on inoculated primary leaves 10 days after inoculation. *Psophocarpus tetragonolobus*: chlorosis on inoculated and newly-produced leaves. *Soja max*: necrotic local lesions 8—10 days after inoculation, then mottle after 3—4 weeks. *Vigna unguiculata* cv. Black Eye: necrotic local lesions on inoculated primary leaves 8—10 days after inoculation, the mosaic on the newly-produced leaves after 3—4 weeks.

Sensitive species from which the virus was not recovered

Leguminosae. *Cassia obtusifolia*: chlorotic local lesions, then faint ringspot on the young tip leaves. *Cassia occidentalis*: necrotic local lesions on inoculated primary leaves and mottle on the young tip leaves. *Desmodium polycarpum*: necrotic local lesions on primary leaves. *Pisum sativum*: complete necrosis of the plant.

Species not infected

Aizoaceae: *Tetragonia expansa.* Chenopodiaceae: *Beta vulgaris, Chenopodium album, C. amaranticolor, C. murale, C. quinoa.* Compositae: *Zinnia elegans.* Leguminosae: *Cassia atrorubens, Clitoria ternatea, Crotalaria atrorubens, C. juncea, C. pallida, C. usaramoensis, Kennedia* sp., *Medicago sativa, Melilotus alba, Phaseolus mungo, Stylosanthes gracilis, Trifolium repens, Vicia faba.* Malvaceae: *Gossypium hirsutum, Hibiscus esculentus.* Passifloraceae: *Passiflora coerulea, P. edulis.* Scrophulariaceae: *Anthirrium majus, Torenia fournieri.* Solanaceae: *Capsicum frutescens, Datura stramonium, Lycopersicon esculentum, Nicotiana clevelandii, N. clevelandii* × *N. glutinosa* cv. Christie, *N. glutinosa, N. megalosiphon, N. rustica, N. tabacum* (cv. Samsun, cv. White Burley, cv. Xanthi), *N. texana, N. tomentosa, Petunia rosea, Physalis alkekingie, P. floridana.*

Properties *in vitro*

Dilution end-point: infectivity in *A. hypogaea* cv. Te3 extract decreased greatly between dilutions of 10^{-2} and 10^{-3}. No symptom infection was produced with extract diluted to 10^{-4}.

Thermal inactivation point: the infectivity of extract freshly expressed from inoculated groundnut leaves was much decreased after 10 min at 65 °C and was abolished after 10 min at 70 °C.

Resistance to air-drying: infectivity of *A. hypogaea* leaves was slowly decreased when they were dried, but it was not abolished two months after air-drying.

Longevity *in vitro*: the infectivity of *A. hypogaea* extract was lost after 6 h at about 25 °C, but was retained 3 days at 3—5 °C.

Effect of freezing and thawing: infectivity decreased little during one month when leaves of groundnut plants were stored at —20 °C, and was retained in leaves alternately frozen and thawed once each day for 5 days.

Effect of pH: extract freshly expressed from inoculated groundnut leaves was infective after exposure at pH 5.0 to 9.5, but not at pH 4.5 and 10.0. The most infectivity was retained at pH 7.5 to 8.5. When subjected to electrofocusing in the ampholine column, the virus was banded at about 4.70.

Aphid transmission

Aphids were allowed an acquisition access time of 1 min, 10 min, 1 h, 6 h, or 24 h. After an inoculation access time of 24 h on healthy groundnut seedlings, they were killed. Using six seedlings per time, and 5, 10 or 20 aphids per seedling, no transmission was obtained. The same negative results were obtained with the three species of aphids, *Aphis craccivora, A. citricola* and *A. gossypii.*

In another test, the three species of aphids were reared on infected groundnut plants. 150 late instar apterous aphids from each species, born on the diseased plants, were transferred to 15 healthy seedlings and cultured until they died naturally. No transmission was obtained.

Purification

GCV was purified from freshly collected groundnut cv. Te3 leaves. Leaves were ground for 1 min in a Waring Blendor with 0.5 M potassium phosphate buffer, at pH 7.5 (4 ml/g of tissue), containing 1 M urea and 0.01 M sodium bisulphite. Urea was used to disrupt virus aggregates and to solubilize virus particles (DAMIRDAGH and SHEPHERD 1970). The extract was strained through several layers of aseptic gauze, chilled in an ice bath and clarified by low-speed centrifugation (7,000 g for 20 min). Chloroform was then added to a concentration of 10 % and the mixture was emulsified for 10 s. The emulsion was centrifuged for at least 10 min at 7,000 g and the aqueous phase was retained. To disrupt residual chloroplast fragments, Triton X-100 detergent was added dropwise with stirring to a concentration of 5 % (VAN OOSTEN 1972). The preparation was centrifuged at 105,000 g for 150 min and the pellets resuspended in 0.05 M potassium phosphate buffer, at pH 7.5, containing 1 M urea and 0.01 M sodium bisulphite. Urea was essential to redissolve the virus particles. In a second cycle of differential centrifugation the virus was sedimented through a 2 cm deep layer of 20 % sucrose using a Beckman R 30 rotor (105,000 g for 150 min). The pellets were solubilized in 0.05 M sodium borate buffer, pH 8.2. The virus was further purified by sucrose density gradient centrifugation. An opalescent virus band was found at 25—30 mm below the meniscus (fractions 27—31) (Fig. 1). Virus yields,

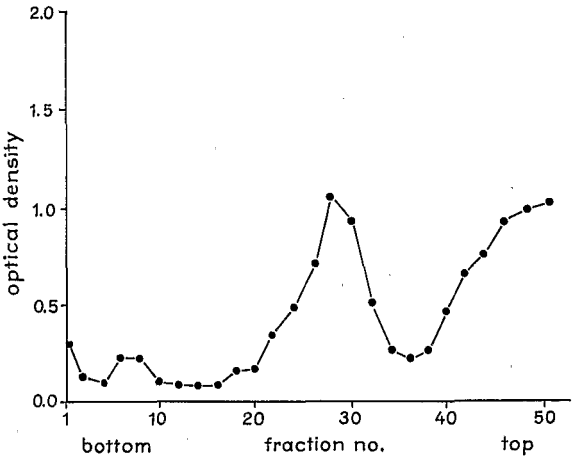

Fig. 1. Sucrose density gradient centrifugations of GCV. 2 ml of virus preparation was layered on a gradient of 5—40 % sucrose (26 ml total) in 0.05 M sodium phosphate buffer, pH 7.5, and centrifuged for 2 h at 25,000 rev/min in the SW 25.1 rotor

calculated on the basis of the virus collected from gradients and using the extinction coefficient $A_{260}^{1cm} = 2.4$ for 1 mg/ml of virus (PURCIFULL 1966), were between 20 and 40 mg of virus/kg leaf. Virus was collected, diluted with borate buffer and concentrated by ultracentrifugation (105,000 g for 150 min). The resulting pellets were resuspended in 0.05 M sodium borate buffer, at pH 8.2. These virus suspensions had the following ultraviolet absorption spectrum: maximum at 260 nm, minimum at 242 nm, ratio maximum/minimum of 1.15—1.20, ratio A_{260}/A_{280} of 1.28—1.32. This last ratio corresponds to a nucleoprotein containing 5.0—6.0 % RNA (LAYNE 1957).

Aggregation during the purification may be 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 ultracentrifugation and during the sucrose gradient centrifugation. The virus yield was twice that obtained using the first procedure but the preparation was less pure. In another procedure, instead of differential centrifugations, the virus was concentrated by precipitation for 4 h with 4 % polyethyleneglycol 20 000 together with 0.1 M potassium chloride (ALBRECHTOVA and KLIR 1970). The virus was then purified by centrifugation in a sucrose using the SW 25-1 rotor. However, the yield from this procedure was much smaller.

Electron Microscopy

Flexuous threads were found in infective virus preparations purified from groundnut leaves by differential and sucrose gradient centrifugations, and in dip preparations from infective leaves. 148 particles were measured. Measurements of the most important group gave average values of 650 ± 25 nm for length and 13 nm for diameter (Fig. 2). Polymeric forms were observed with lengths of 1300, 2000, 2600 and even 3200 nm.

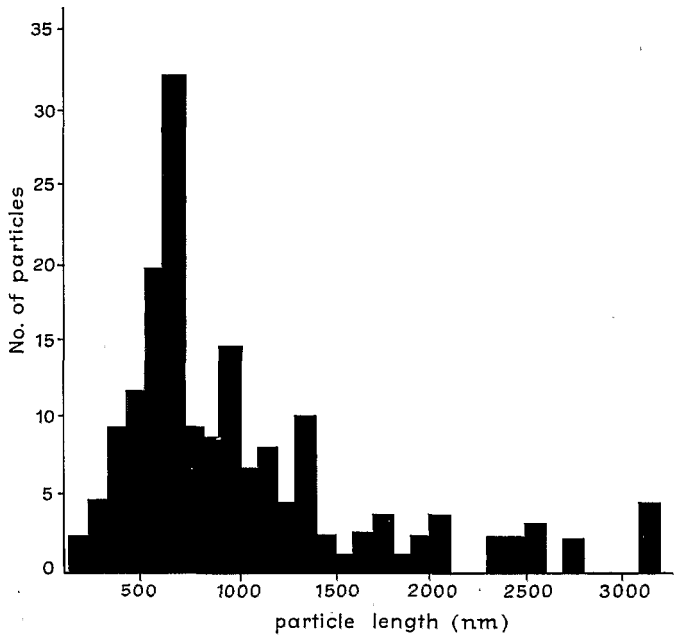

Fig. 2. Length distribution of GCV particles in samples from leaf dip preparations

Serology

There was no reaction between GCV and the following antisera to groundnut viruses (donors and reciprocals of homologous titres given by donors in parentheses): peanut stunt (WATERWORTH, 64; MINK, 256), peanut

clump (THOUVENEL, 1024), peanut mottle (BOCK, 256; KUHN, 256), groundnut eyespot (DUBERN, 256), groundnut chlorotic rosette virus (DUBERN, 128).

No reaction was obtained with other viruses that might infect groundnut: tobacco mosaic (ORSTOM, 2048) and okra mosaic virus (GIVORD, 4096).

GCV reacted strongly with passiflora latent virus antiserum (WETTER, 1024) up to a dilution of 1024, likewise with potato virus S antiserum (WETTER, 1024) up to 1/512 and carnation latent virus antiserum (WETTER, 16 384) up to 1/8192. It reacted moderately with potato virus M antiserum (WETTER, 1096) up to 1/128. However, no reaction was obtained with cactus virus 2 antiserum (WETTER, 8192) and hyppeastrum latent virus antiserum (WETTER, 4096). For all these antisera, homologous and heterologous titres were not determined by the same procedure and might not be compared; homologous titres, given by authors, had unknown procedures of determination; heterologous titres were determined by VAN SLOGTEREN's procedure (1954).

Discussion

Many viruses infect groundnut plants, but are different from groundnut crinkle disease. Some are not sap-transmissible: groundnut ringspot (KUHN *et al.* 1964), groundnut green rosette (KLESSER 1968), groundnut ring mottle (SHARMA 1966), groundnut mosaic (NARIANI and DHINGRA 1963, BERGMAN 1956), groundnut bunchy top (SHARMA 1966), groundnut witches' broom (BERGMAN 1956), groundnut marginal chlorosis (VAN VELSEN 1961) and groundnut rugose leaf curl (GRYLLS 1954, MONSARRAT 1976). Some diseases are sap-transmissible but have different particle morphology: groundnut eyespot (DUBERN and DOLLET 1978, 1980), groundnut rosette (OKUSANYA and WATSON 1966), peanut clump (THOUVENEL *et al.* 1976), tomato spotted wilt (HELMS 1961), tobacco mosaic (NIAZI 1973), okra mosaic (GIVORD and HIRTH 1973), passionfruit woodiness (TAYLOR and GRABER 1973), and clitoria yellow vein (BOCK and GUTHRIE 1977). Moreover, some sap-transmissible viruses have unknown particle morphology: groundnut chlorotic spot (HARAGOPAL and NAYUDU 1971), groundnut ringspot (KLESSER 1966), groundnut veinbanding (KLESSER 1967) and groundnut chlorosis (SHARMA 1966); all these diseases are different in their symptomatology, host range and *in vitro* properties, and are insect transmissible.

Only one virus occurring in groundnut plants is sap-transmissible and has similar particle morphology: cowpea mild mottle virus (BRUNT and KENTEN 1974); however, its properties are different: symptoms on grounut leaves (few necrotic lesions, chlorotic rings ore line patterns are developed on inoculated leaves, followed by systemic leaf chlorosis, leaf rolling and some veinal chlorosis, and finally severe stunting of plants), host range (five families infected including the species *Beta vulgaris, Nicotiana clevelandii* and *Chenopodium quinoa*) and serological properties (not related to potato M, potato S and passionfruit latent viruses, and only distantly related to carnation latent virus). Although GCV was not compared serologically with cowpea mild mottle virus, GCV would seem to be distinct from cowpea mild mottle virus.

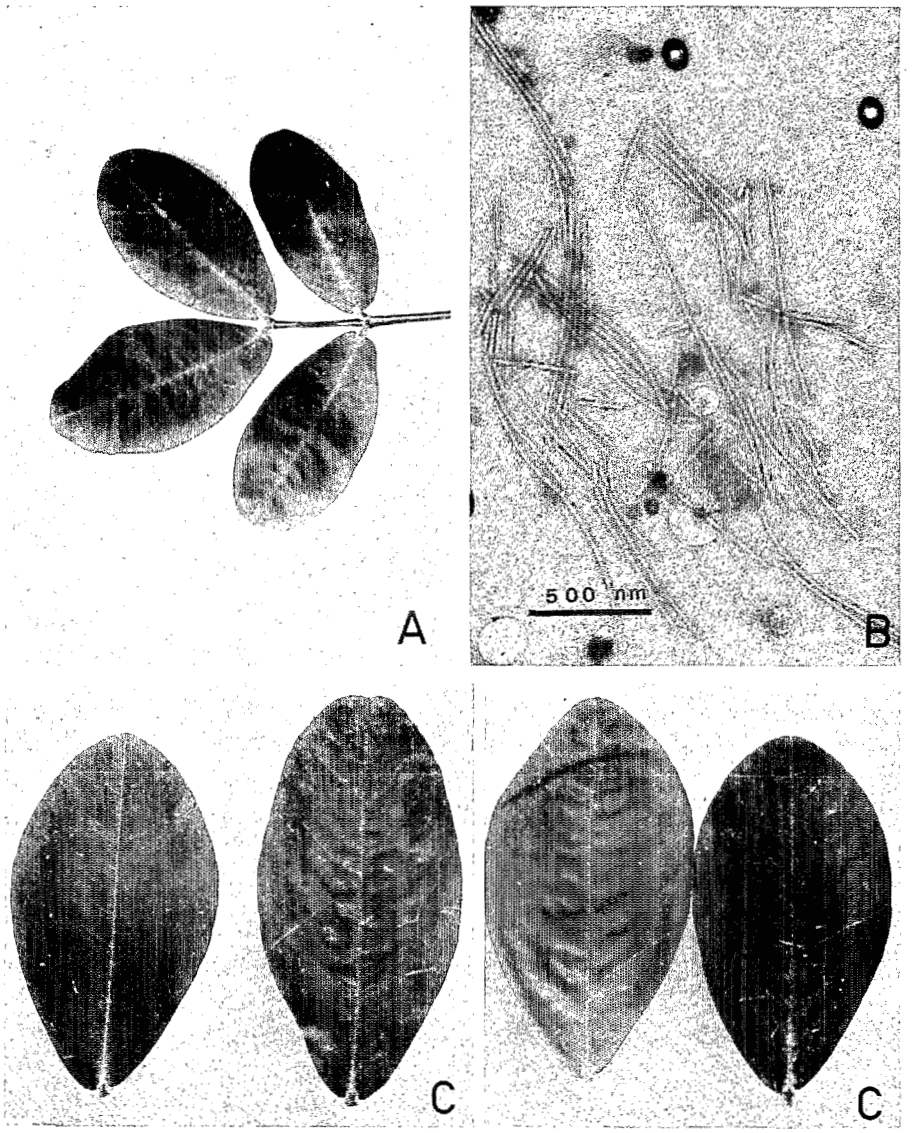

Fig. 3. Symptoms developed by *A. hypogaea* L. cv. Te3, mechanically inoculated with GCV at the four-leaf stage. A. Diseased leaf. B. Electron micrograph of a suspension of purified GCV. Particles about 650 nm long (× 60 000). C. Different strains observed on groundnut plant cv. Te3: helathy leaf, crinkling and stippling, stipping without crinkling, crinkling without stippling (left to right)

In our investigation, GCV reacted strongly with antisera to passiflora latent, potato S and carnation latent viruses. GCV did not infect passionfruit and none of these viruses is reported to infect groundnut plant. Moreover, these viruses have different host ranges and *in vitro* properties, and are aphid-transmissible. For these reasons it seems that groundnut crinkle virus may not

be considered as a strain of passionfruit latent, potato S and carnation latent viruses.

From this study, we concluded that the crinkle disease of groundnut is due to a newly-described virus. It is named groundnut crinkle virus (GCV) and its proposed cryptogram (GIBBS 1967) is */* : */(5) : E/E : S/*, a member of the carlavirus group.

Summary

A virus causing leaf crinkling and stippling symptoms on groundnut plants (*Arachis hypogaea* L.) is described. It was transmitted by inoculation with sap, but not by *Aphis craccivora, A. citricola* or *A. gossypii.* Its host range is apparently confined to species of Leguminosae. Infectivity in extracts of groundnut plant was lost after 10 min at 70 °C, dilution to 10^{-4} and storage at 25 °C for 6 h. The particles which are c. 650 × 13 nm were purified and were serologically related to carnation latent, potato S and passiflora latent viruses. However, the virus differs in host range, *in vitro* and serological properties from previously described groundnut viruses.

The virus is named groundnut crinkle virus, and seems to be a new member of the carlavirus group, with a cryptogram: */* : */(5) : E/E : S/*.

Résumé

Un nouveau membre du groupe des carlavirus: le virus de la Frisolée de l'Arachide

Un virus provoquant une frisolée et des mouchetures sur les feuilles d'Arachide (*Arachis hypogaea* L.) est décrit. Il a été transmis par inoculation mécanique d'extrait brut, mais non par *Aphis craccivora, A. citricola* ou *A. gossypii.* Seules quelques espèces de la famille des Léguminosae ont pu être contaminées. Les extraits bruts d'Arachide ne sont plus infectieux aprés 10 min à 70 °C, dilution à 10^{-4} ou stockage 6 h à 25 °C. Les particules virales, dont les dimentions sont d'environ 650 nm de longueur et 13 nm de diamètre, ont été purifiées. Elles sont reliées sérologiquement au virus latent de l'Oeillet, au virus S de la Pomme de terre et au virus latent de la Grenadille. Cependant, ce virus diffère par sa liste de plantes hôtes et par ses propriétés physico-chimiques et sérologiques des virus préalablement décrits dans la littérature.

Ce virus a été appelé virus de la Frisolée de l'Arachide et paraît être un nouveau membre du groupe des carlavirus. Son cryptogramme est: */* : */(5) : E/E : S/*.

Zusammenfassung

Das „groundnut crinkle virus", ein neues Mitglied in der Gruppe der Carlaviren

Es wird ein Virus, das Symptome der Blattkräuselung sowie punktartige Blattflecken auf Erdnußpflanzen (*Arachis hypogaea* L.) verursacht, beschrieben. Es wird durch die Inokulation mit Saft übertragen, nicht jedoch durch

Aphis craccivora, A. citricola oder *A. gossypii*. Sein Wirtsspektrum ist offenbar auf Leguminosenarten beschränkt. Die Infektiosität in Extrakten aus Erdnußpflanzen ging nach 10 Minuten bei 70 °C, bei einer Verdünnung von 10^{-4} sowie nach sechsstündiger Aufbewahrung bei 25 °C verloren. Die Partikel, die etwa 650 × 13 nm groß sind, wurden gereinigt und waren serologisch verwandt mit dem latenten Nelkenvirus, dem Kartoffel-S-Virus und dem latenten Passifloravirus. Das Virus unterscheidet sich jedoch im Wirtsspektrum, *in vitro* und serologischen Eigenschaften von den vorher beschriebenen Erdnußviren.

Das Virus wurde Erdnußkräuselvirus genannt und scheint ein neues Mitglied in der Carlavirusgruppe zu sein. Das Cryptogramm lautet: */* : */(5) : E/E : S/*.

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