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Some properties of peanut clump, a newly discovered virus

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SUMMARY

A mechanically transmissible soil-borne virus causing peanut clump disease in Upper Volta is described. It infected mainly species of Chenopodiaceae and was propagated in *Chenopodium amaranticolor*. Infectivity was lost from sap of *C. amaranticolor* after 10 min at 64 °C, and after dilution to 10^{-5} but not 10^{-4}. A purification procedure is described. The particles are rod-shaped and of two predominant lengths, 190 and 245 nm. The virus is not serologically related to tobacco rattle, pea early-browning, or soil-borne wheat mosaic viruses, or to a virus associated with a rhizomania-like disease of beet.

INTRODUCTION

During the summer of 1969, disease symptoms were observed in peanut at the agricultural station of Saria, in Upper Volta (Germani & Dhery, 1973), where about 6 out of 10 ha were affected. The symptoms seemed to be similar to those of the disease called 'clump' (Plate, fig. 1), previously described in Senegal (Bouhot, 1967). In both countries the disease reappears in the same places in succeeding crops; this and the fact that treatment of contaminated soils with DBCP (1,2-dibromide, 3-chloropropane) temporarily suppressed the disease (Germani & Dhery, 1973) suggests that it is soil-borne. Bouhot (1967), showed that it was not seed borne, Thouvenel, Germani & Pfeiffer (1974) and Germani, Thouvenel & Dhery (1975) showed that the disease is caused by a virus, which was named peanut clump virus (PCV). This paper describes the host range, purification and some properties of PCV.

MATERIALS AND METHODS

The original inoculum consisted of naturally infected leaves collected from Saria in Upper Volta, and then kept at −20 °C. Plants for experiments were grown in sterilized soil, in glasshouses where the temperature ranged from 30 to 35 °C, and where the average humidity reached 90%. Young seedlings of *Chenopodium amaranticolor* were artificially illuminated for 16 h/day.

For mechanical transmission, frozen leaves of peanut or *C. amaranticolor* were ground in pH 7·1, 0·1 M potassium phosphate buffer (5 ml/g of tissue) containing

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0·02 M cysteine hydrochloride and 2·5 mg/ml bentonite. The sap obtained was rubbed manually on carborundum-dusted leaves, and in some experiments, on carborundum-dusted roots of young uprooted seedlings which were planted again after inoculation.

For studying *in vitro* properties, inoculum was prepared from *C. amaranticolor* as above, and *C. amaranticolor* plants used as indicators. The dilution end-point was determined by serial dilutions in 0·1 M phosphate buffer pH 7·1. The thermal inactivation point was tested by immersing 2 ml quantities of sap, contained in 5 ml tubes, in a water bath at various temperatures for 10 min and then placing the tubes in an ice bath. To test ageing *in vitro*, the infectivity of sap kept at 27 °C was checked every day, and resistance to air-drying was determined by testing daily the infectivity from *C. amaranticolor* leaves hung in the laboratory.

Density gradient columns (10–40% sucrose in 0·05 M sodium borate buffer, pH 8, containing 0·001 M disodium ethylene diamine tetra-cetate (EDTA)) were prepared in 3 × 1 in (7·6 × 2·5 cm) cellulose nitrate tubes. Gradients were centrifuged for 90 min at 51 000 *g* in a Beckman SW 25.1 rotor. They were fractionated from the top by displacing the tube contents with 50% sucrose solution. The optical density at 254 nm was recorded in the 3 mm flow cell of a LKB Uvicord absorptiometer.

Leaf dip preparations from healthy and diseased plants were made by the method of Hitchborn & Hills (1965) using carbon-coated grids. Virus preparations were stained with 1 % uranyl acetate + 0·5% EDTA at pH 7·0 and examined with a Philips EM300 electron microscope.

Antiserum against PCV was raised in a rabbit injected intramuscularly twice a week, for 5 wk, with 0·5–1 mg samples of freshly purified virus emulsified with Freund's incomplete adjuvant. The rabbit's left and right thigh muscles were injected alternately. Serum was collected 10 days after the last injection and stored at 4 °C, with an equal volume of glycerol. The serological tests were made using the microprecipitin technique (van Slogteren, 1955).

Aphid-transmission experiments were done in the laboratory with *Aphis gossypi* reared on eggplant (*Solanum melongena*) and *A. craccivora* reared on peanut (*Arachis hypogea*).

RESULTS

Host range and symptomatology

Peanut (*Arachis hypogea* cv. TE 3 and cv. 28206) is easily infected by mechanical inoculation with sap from diseased peanut, developing symptoms similar to those produced in the field (Plate, fig. 2).

C. amaranticolor also is very susceptible to infection by mechanical inoculation with sap from peanut, and the virus is easily transmitted from *C. amaranticolor* to *C. amaranticolor*. By contrast, the virus has not been transmitted from *C. amaranticolor* back to peanut, although the leaves of 210, and the roots of 180 plants were inoculated. This may be due to the potent inhibitors of infection known to occur in *C. amaranticolor* (Smookler, 1971). Despite this we were obliged to propagate the virus in *C. amaranticolor* for lack of seed of susceptible peanut cultivars. In studies on host range, plants were inoculated using sap from *C. amaranticolor*. Whether the plants showed symptoms or not the inoculated leaves and the upper uninoculated ones were

tested by back-inoculation to *C. amaranticolor*. Infection was obtained in the following species.

(1) *Leguminosae. Arachis hypogea* cv. TE 3 or 28206. Mottle and chlorotic ringspots appeared on new tip leaves, 7–10 days after inoculation. These symptoms rapidly faded, then disappeared when the leaves turned dark green. The plant then stopped growing and looked stunted (Plate, fig. 2).

(2) *Chenopodiaceae. Chenopodium amaranticolor*, *C. quinoa* and *C. album.* At 35 °C local yellow spots appeared 2 days after inoculation; later they became ringspots and line patterns which extended along the veins (Plate, fig 3). Inoculated leaves wilted, then fell between the sixth and eighth days. There was no systemic infection. At 24 °C no symptoms developed. Virus could be recovered from the leaves of plants whose roots were inoculated, but no shoot symptoms were produced. *C. ackenii*: inoculated leaves showed slight vein-clearing after 4 days and turned yellow on about the sixth day; no systemic infection. *C. ambrosioides*: no symptoms developed, but virus could be recovered from inoculated leaves; there was no systemic infection. *C. botrys*: inoculated leaves showed yellow blotches, followed by necrosis; there was no systemic infection. *C. hybridum*: inoculated leaves showed necrotic stippling after 7 days; symptomless systemic infection. *C. murale*: between the fifth and eighth days following inoculation, broad green ringspots could be seen in the inoculated leaves, together with oak-leaf patterns. the leaves then turned yellow and were shed on about the eleventh day; the infection was not systemic. *C. opuliforum:* two days after inoculation, numerous yellow spots developed and sometimes became ringspots; leaves fell on about the fifth day; there was no systemic infection. *C. polyspermum*: systemically infected and developed a slight mottle; no symptoms in inoculated leaves. *C. rubrum*: inoculated leaves developed vein-clearing on the third day after inoculation, then turned yellow; broad green ringspots and line patterns appeared on the eighth day; no systemic symptoms.

(3) *Amaranthaceae. Celosia argentea* cv. plumosa; when plants were inoculated very young, systemic mottle and slight distortion developed after 1 month.

Non-host plants

The following species failed to become infected in our tests. The number of plants tested of each species is given in parentheses.

Aizoaceae: *Tetragonia expansa* (26). Amaranthaceae: *Amaranthus caudatus* (35). *A. retroflexus* (10), *A. spinosus* (10), *Gomphrena globosa* (26). Apocynaceae: *Amsonia tabernaemontana* (18), *Vinca rosea* (12). Bombacaceae: *Ceiba pentandra* (16). Caricaceae: *Carica papaya* (18). Chenopodiaceae: *Beta vulgaris* (26). Compositae: *Zinnia elegans* (10). Convolvulaceae: *Ipomea coccinea* (20), *I. oleracea* (12). Cruciferae: *Brassica oleracea* (36), *Capsella bursa-pastoris* (26). *Cucurbitaceae: Cucumis melo* (28), *C. savitus* (10). Euphorbiaceae: *Euphorbia biumbellata* (16), *Manihot utilissima* (16). Gramineae: *Chloris vulgare* (40), *Oryza sativa* (26), *Poa annua* (25), *Sorghum vulgare* (40). Leguminosae: *Cajanus* sp. (21), *Canavalia ensiformis* (14), *Cassia occidentalis* (21), *Crotalaria retusa* (5), *Desmodium intertum* (3), *Glycine max* (19), *Indigofera frutescens* (3), *Lupinus annuum* (35), *Melilotus alba* (26), *Pisum sativum* (23), *Stylosanthes gracilis* (29), *Tephrosia vogeli* (5), *Trifolium repens* (20), *Vicia faba* (27), *Vigna cylindrica* (20),

V. sinensis (44), *Voandzia subterranea* (9). Malvaceae: *Abutilon indicum* (18), *Hibiscus asper* (13), *H. esculentus* (32). Passifloraceae: *Passiflora suberosa* (20). Phytolaccaceae: *Phytolacca americana* (15). Rosaceae: *Potentilla* sp. (24). Solanaceae: *Capsicum annuum* (28), *Datura innoxia* (4), *D. metel* (14), *D. stramonium* (4), *Lycopersicon esculentum* (28), *Nicotiana clevelandii* (12), *N. glutinosa* (15), *N. tabacum* Samsun (12), *N. tabacum* Xanthi (10), *Physalis alkekengii* (21), *P. floridana* (5), *Solanum nigrum* (17). Tropaeolaceae: *Tropaeolum majus* (9). Umbelliferae: *Daucus carota* (8).

Inoculations to the roots of *Gomphrena globosa*, *Abutilon indicum*, *Beta vulgaris*, *Solanum nigrum* and *Vigna cylindrica* did not cause infection.

Properties in vitro

Dilution end-point. Infectivity in *C. amaranticolor* sap decreased greatly between dilutions 10^{-2} and 10^{-3}. Symptoms were produced after inoculation with sap diluted to 10^{-4} but not at 10^{-5}.

Thermal inactivation point. The infectivity of sap freshly expressed from inoculated *C. amaranticolor* leaves was much decreased after 10 min at 60 °C, and abolished after 10 min at 64 °C.

Resistance to air-drying. Infected *C. amaranticolor* leaves lost infectivity after drying for 37 days.

Longevity in vitro. The infectivity of *C. amaranticolor* sap was lost after 22–27 days at about 27 °C.

Effect of freezing. Infectivity decreased little during 15 months in leaves of *C. amaranticolor* stored at –20 °C. It also persisted for a year in frozen sap. Infectivity was retained in leaves alternately frozen and thawed every day for 50 days.

Purification

PCV was purified from *C. amaranticolor* leaves frozen for at least 3 wk. Many procedures, including those used for the purification of other viruses having similar morphology, failed because of the tendency of PCV to aggregate during the first steps of purification. Borate buffer, pH 8, was found best for the initial extraction and for storing the purified virus.

Among the different methods of clarification tested, including freezing, treatment with chloroform, butanol, carbon tetrachloride, diethyl ether or Triton X-100, the best was emulsification with a mixture of chloroform and butanol.

Frozen leaves were ground (1 g/2·5 ml) with 0·5 M sodium borate buffer (pH 8) containing 1 M urea, 0·02 M sodium bisulphite, 0·01 M cysteine hydrochloride and 0·001 M EDTA, for 2 min in a Waring Blender. A 9:1 mixture of chloroform and butanol (1 ml of the mixture/1 g leaf) was added and the mixture blended again for 1 min. The extract was centrifuged for at least 5 min at 3000 *g* and the pellets resuspended and blended once more with the same volume of extracting buffer, then recentrifuged. The pellets were discarded, the two supernatant fluids were pooled and polyethylene glycol (mol. wt 20000) added, to a concentration of 5 %. The mixture was left overnight at 4 °C, centrifuged for 20 min at 15000 *g* and the supernatant fluid discarded. The pellets were resuspended in 0·05 M borate buffer (pH 8) containing 1 M urea, 0·001 M EDTA and 0·1 % 2-mercapto-ethanol (1/5 of the initial volume).

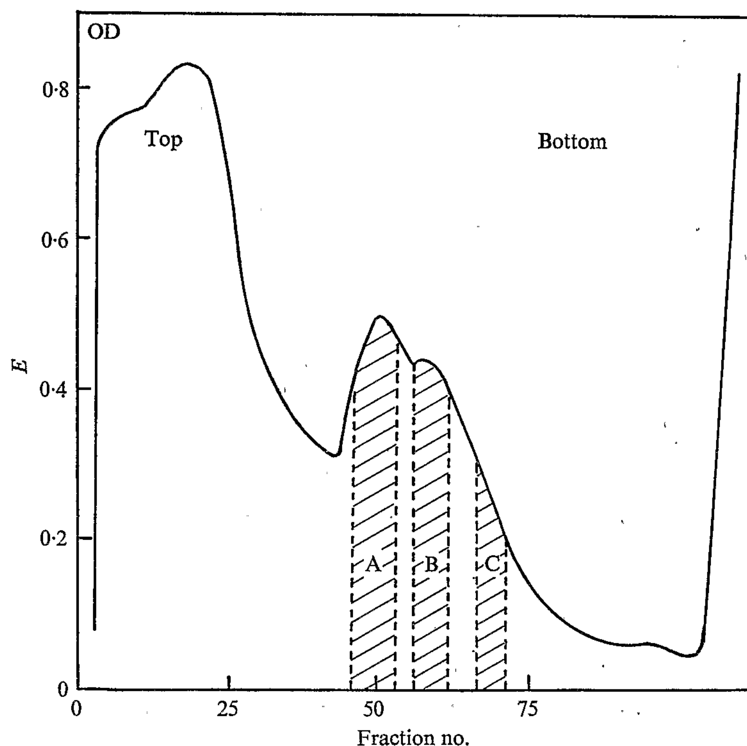
After removal of the insoluble material by centrifugation for 15 min at 12000 *g*, the virus was sedimented by centrifugation at 78000 *g* for 1 h. It was then resuspended in borate buffer for 2 h (1 % of the initial volume) and further purified by centrifugation for 15 min at 12000 *g*, then 30 min at 150000 *g*. After this high-speed centrifugation, a thin layer of brownish material came away from the top of the pellet and was discarded.

A small translucent pellet of purified virus remained, which was resuspended in 0·05 M borate buffer (pH 8) containing 0·001 M EDTA. In some experiments, the virus was centrifuged through a sucrose density gradient column after the first cycle of ultracentrifugation.

Taking $E_{260}^{0\cdot1,\ 1\,\mathrm{cm}}$ 3·1, as for soil-borne wheat mosaic virus (Brakke, 1971) virus yields were estimated to be 10–15 mg/kg leaf.

Density gradient centrifugation

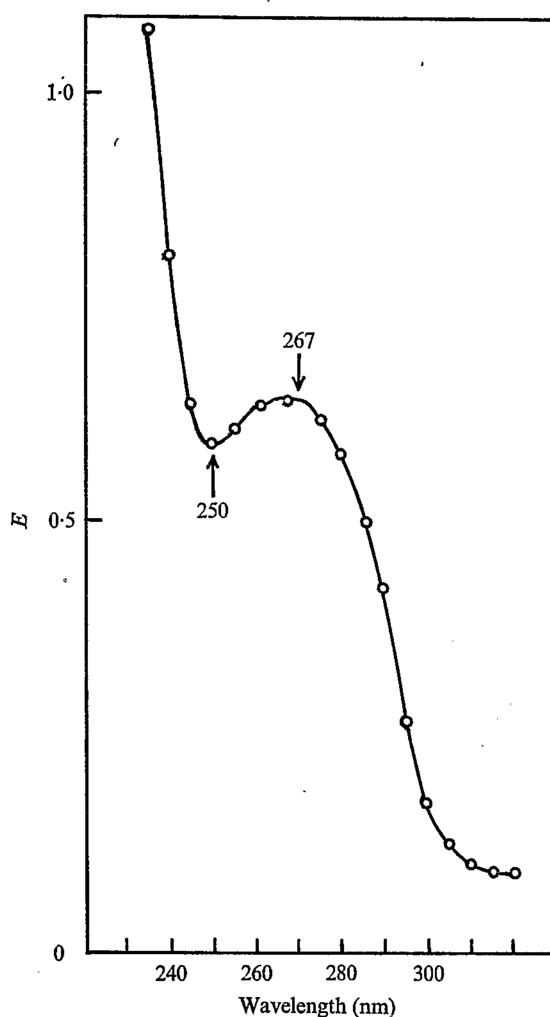
At the end of the run, a zone of light-scattering material could be seen 25–30 mm below the meniscus. This zone contained two peaks, the upper representing unaggregated virus particles and the lower representing aggregated particles (Text-fig. 1).



Text-fig. 1. The ultraviolet absorbance profile at 254 nm of a sucrose density gradient. Purified virus preparation (2 ml) was layered on a gradient of 10–40 % sucrose in 0·05 M borate buffer (pH 8) containing 0·001 M EDTA. Centrifugation was for 90 min at 51000 *g* in a SW 25.1 rotor. Fractions representing the hatched zones A, B and C were pooled and used for electron microscopy (see Text-fig. 3).

Ultraviolet absorption spectrum

The absorption spectrum of purified virus had a maximum at 267 and a minimum at 250 nm and was typical of a nucleoprotein (Text-fig. 2). The maximum/minimum ratio was 1·08 ± 0·01 (with no correction for light-scattering), and the E_{280}/E_{260} ratio of 1·09 ± 0·01 suggests a nucleic acid content of 4·5 % (Layne, 1954).



Text-fig. 2. Ultraviolet absorption spectrum of purified PCV.

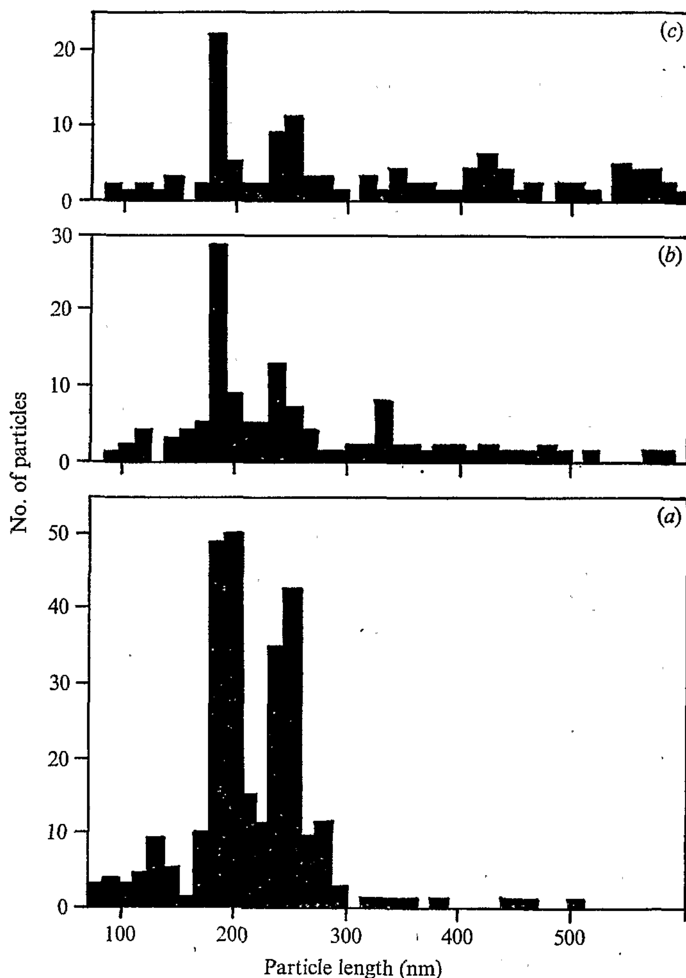
Electron microscopy

Leaf-dip preparations made from diseased peanut and *C. amaranticolor* leaves always showed rod-shaped particles. On the contrary, preparations made from healthy leaves never showed particles. Micrographs made from purified preparations

confirmed the existence of two types of particles (Plate, fig. 4) as previously observed in dip-preparations (Thouvenel *et al.* 1974). Length measurements of 346 particles gave average values of 245 ± 15 nm for the large particles and 190 ± 10 nm for the small ones (Text-fig. 3). The width of both types was 21 ± 3 nm.

Serology

Homologous antiserum reacted with purified PCV when diluted to 1/2048 but not further. Purified virus reacted with antiserum at OD_{260} = 0·05 but not at 0·01. Crude chloroform-clarified extract of diseased peanut leaves reacted strongly with the antiserum; by contrast, crude chloroform-clarified extract of diseased *C. amaranticolor* leaves reacted very faintly, presumably because the concentration of virus is small.



Text-fig 3. Length distribution of PCV particles in samples from a sucrose density gradient. (*a*), (*b*) and (*c*) indicate particles from zones A, B and C of Text-fig. 1 respectively.

There was no reaction between purified PCV and antisera to the following viruses (homologous titres in parentheses); tobacco rattle virus strains CAM (1/512), PRN (1/256), Lisse (1/1024) and a common English strain (1/512); pea early-browning virus strains SP 5 (1/64) and E116 (1/128), soil-borne wheat mosaic virus isolate J-B (1/1 600) or to a virus causing a disease of beet resembling 'rhizomania' (1/100) (Putz & Vuittenez, 1974).

Means of spread

Aphids (*Aphis gossypii* and *A. craccivora*) were starved for 3 h before being allowed to feed for 1 day on diseased peanut. They were then transferred to feed for 1 day on healthy peanut seedlings. Using ten aphids per seedling, no transmission of virus was obtained to any of twenty seedlings.

Previous studies (Germani & Dhery, 1973) suggested that PCV is soil-borne. To confirm this, peanut seeds were sown in soil collected from a disease outbreak area. Symptoms of peanut clump disease were visible in 132 out of 250 seedlings and PCV was recovered from them. No symptoms occurred in forty control seedlings grown in sterilized soil.

Soil from fields producing infected crops contained numerous stubby root nematodes (*Trichodorus* sp.) and much of the fungus *Pythium* sp., possible vectors of PCV. However, the mode of transmission of PCV is still an open question, and is under active investigation.

DISCUSSION

In the host-range experiments inoculations were made with sap from *C. amaranticolor* because we lacked a source of clumped peanut plants. It seems possible that failure of the virus to infect species outside the Chenopodicaeae and Amaranthaceae may have been caused by the same inhibitors in the sap of *C. amaranticolor* that prevented infection of peanut. Despite the fact that transmission from *C. amaranticolor* back to peanut has not yet been achieved, there is strong evidence that PCV is the agent causing peanut clump disease. The virus has always been recovered by manual inoculation from diseased peanuts but never from healthy plants. Similarly, electron microscope examination of leaf-dip preparations detected the virus-like particles in samples from diseased plants only. Moreover, PCV antiserum prepared using virus extracted from *C. amaranticolor* reacted specifically with crude sap of diseased peanut.

The existence of particles of two lengths (190 and 245 nm), as well as transmission in soil, are points of similarity between PCV, tobraviruses (tobacco rattle and pea early-browning viruses) and several yet unclassified viruses including barley stripe mosaic, beet necrotic yellow vein, broad bean necrosis, hart's tongue fern, lychnis ringspot, poa semi-latent, potato mop-top, soil borne wheat mosaic and the virus described by Putz & Vuittenez (1974) associated with a rhizomania-like disease of beet.

The symptoms produced by PCV on *C. amaranticolor* are similar to those of broad bean necrosis virus (Inouye & Asanti, 1968) and even more so to those of potato mop-top virus (Harrison, 1974). Both PCV and potato mop-top virus give only a local infection; a single lesion may spread over half a leaf, but never to the whole plant.

However, PCV differs in particle dimensions from these and the other viruses listed above, moreover, broad bean necrosis and potato mop-top viruses have optimal temperatures for multiplication of 15 °C whereas that for PCV is 35 °C. Finally, we found no serological relationship between PCV and tobacco rattle, pea early-browning or soil-borne wheat mosaic viruses or a virus associated with a rhizomania-like disease of beet. With tobacco rattle (Harrison, 1970), pea early browning (Harrison, 1973) and soil-borne wheat mosaic viruses (Gumpf, 1971), infectivity is associated with the long particles, though with the two tobraviruses both components are necessary for production of nucleoprotein particles. For the time being it seems impossible to test the infectivity of each PCV component because of the very small quantity of virus found, in *C. amaranticolor*, and because the virus aggregates during purification. However identification of the vector should allow PCV to be classified with more confidence than at present.

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EXPLANATION OF PLATE

Fig. 1. Symptoms of the clump disease of peanut in the field (healthy plant on left).

Fig. 2. Peanut infected by inoculation with peanut sap (left), and healthy peanut (right).

Fig. 3. Symptoms of PCV in an inoculated leaf of *C. amaranticolor*.

Fig. 4. Electron micrograph of purified PCV, stained with uranyl acetate.

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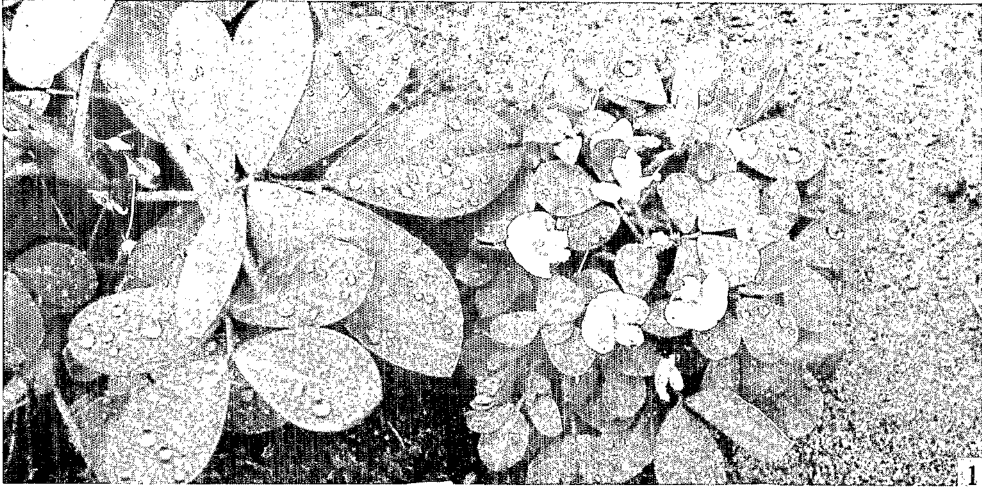

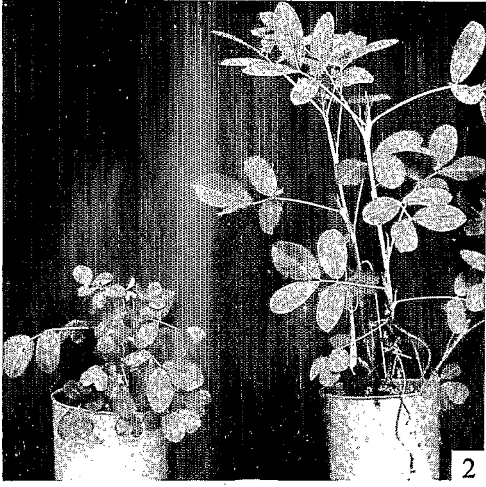

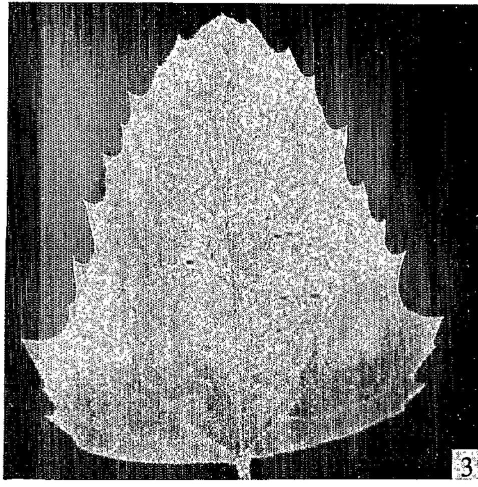

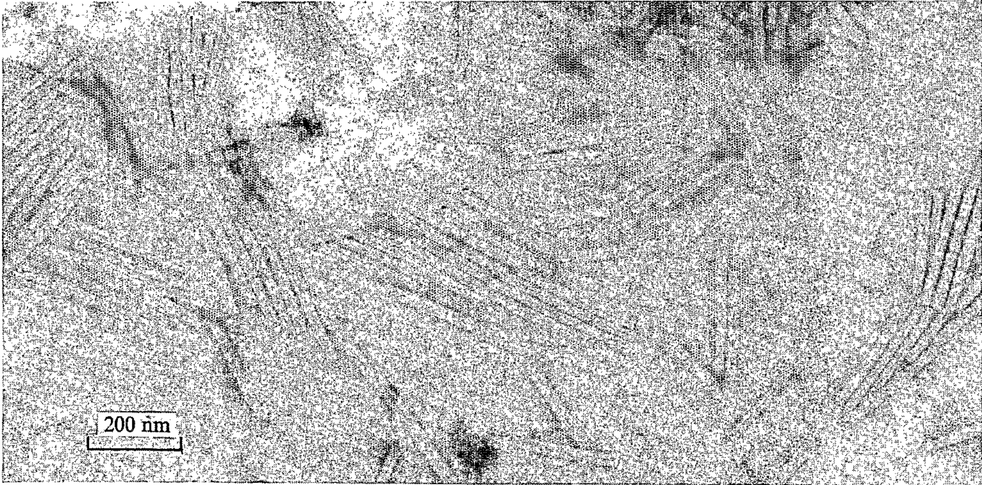

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