Biological, Serological, and Cytopathological Properties of Tomato Vein-Yellowing Virus, a Rhabdovirus Occurring in Tomato in Morocco

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We gratefully acknowledge the excellent technical assistance of Sabine Koerbler and Sabine Schuhmann.

Accepted for publication 12 July 1984.

ABSTRACT

El Maataoui, M., Lockhart, B. E. L., and Lesemann, D.-E. 1985. Biological, serological, and cytopathological properties of tomato vein-yellowing virus, a rhabdovirus occurring in tomato in Morocco. Phytopathology 75:109-115.

A previously undescribed disease of tomato (*Lycopersicon esculentum*) characterized by vein-yellowing, stunting, and plant deformation and occurring in the main tomato-growing areas of Morocco, is caused by a virus with bullet-shaped particles measuring 265 × 86 nm. Negatively stained particles showed variable dimensions depending on preparation methods. The virus also was found in the perennial weed *Solanum sodomaeum*. Its narrow host range consisted essentially of several solanaceous species. Symptoms produced were similar to those produced by potato yellow dwarf virus, but serological tests showed the two viruses to be unrelated. Virus particles were formed mainly by budding at the nuclear periphery and accumulated in the perinuclear space and adjacent endoplasmic reticulum (ER). Conspicuous aggregates of tubular material accumulated in cisternae of the ER. The name proposed for this apparently undescribed member of the plant rhabdovirus group is tomato vein-yellowing virus (TVYV).

Additional key words: tomato virus disease.

Tomato plants from commercial fields in several areas of the Atlantic coastal tomato-producing region of Morocco were observed to have symptoms (3) unlike those seen previously. Infected plants were stunted and had deformed leaves and pronounced vein yellowing (Fig. 1A). Infected plants either bore no fruit or abnormally small fruits with chlorotic spots (Fig. 1B). Other viruses occurring in tomato in Morocco such as tomato mosaic virus (ToMV) (15), tomato bushy stunt virus (TBSV) (6), potato virus Y (PVY) (15), alfalfa mosaic virus (AMV) (15), and cucumber mosaic virus (CMV) (15) were not observed by either electron microscopy or indicator plant reactions. The tomato virus was also isolated in both northern and southern areas of Morocco from *Solanum sodomaeum* Hort. ex. Dun., a perennial weed occurring ubiquitously throughout the country. Symptoms in this wild host consisted of severe mosaic.

MATERIALS AND METHODS

Virus source. Three isolates of the Moroccan tomato rhabdovirus were isolated from *Lycopersicon esculentum* Mill. 'Heinz 1350' collected at Mograne (TVYV-MOG) and 'Roma VF' collected at Larache (TVYV-TL4), and from *S. sodomaeum* collected at Oualidya (TVYV-SSO). The three isolates were cloned separately by serial single-lesion transfers in *Nicotiana rustica* L., and were then propagated in *N. glauca* Graham. For comparative testing the *constricta* strain of PYDV (CYDV, ATCC PV 233) and the *sanguinolenta* strain (SYDV ATCC PV 234) were obtained from the American Type Culture Collection and were propagated in *N. rustica*.

Mechanical inoculation and host range studies. Carborundum-dusted test plants were inoculated mechanically with extracts from locally or systemically infected leaf tissue of *N. rustica* in 0.05 M phosphate buffer, pH 7.2, containing 0.1% 2-mercaptoethanol.

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Electron microscopy. Particles were routinely detected in preparations obtained by briefly touching a carbon-pioloform coated grid to extracts produced by squashing infected leaf tissue in 2% ammonium molybdate, pH 6.5 (AM). To improve preservation, particles were fixed in crude extracts in 0.1 M phosphate buffer, pH 7.0, by floating grids with absorbed particles for 10 min on buffered 1% OsO_4 or 2.5% buffered glutaraldehyde (GA). Grids were then washed with water and negatively stained. Measurements were made in a Zeiss EM 10 C electron microscope by using a Morphomat 30 image analyzing system (Zeiss) calibrated with a carbon grating replica.

Pieces of systemically infected leaves of *N. clevelandii* and *N. glutinosa* were fixed, embedded in Epon, and cut as described previously (20).

Virus purification. The three isolates of TVYV (TVYV-MOG, TVYV-TL4, and TVYV-SSO), as well as CYDV and SYDV, were partially purified by a modification of the method described by Jackson and Christie (10) for sonchus yellow net virus (SYNV). Infected leaf tissue of *N. rustica* was blended (1:1.5, w/v) in 0.2 M sodium citrate, pH 7.8, containing 0.01 M $MgCl_2$ and 0.04 M Na_2SO_3. The homogenate was filtered through cheesecloth and centrifuged at 6,000 *g* for 10 min. The supernatant was gently vacuum filtered through a Celite pad and centrifuged through a step gradient of 5 ml each of 30 and 60% sucrose in extraction buffer for 1 hr at 25,000 rpm in a Beckman SW27 rotor. The material at the 30/60% interface was collected, diluted in extraction buffer, and centrifuged for 25 min at 30,000 rpm in a Beckman type 35 rotor. The resulting pellets were resuspended in 0.02 M sodium citrate, pH 7.8, containing 10% sucrose and clarified by a final low-speed centrifugation.

Antiserum production and serological tests. Antisera against TVYV-MOG, CYDV, and SYDV were prepared in rabbits by an intravenous injection of partially purified virus followed by four weekly intramuscular injections of partially purified virus emulsified in Freund's incomplete adjuvant. Blood was collected by sacrificing the animals 10 days after the last injection.

Double diffusion tests were done in 0.7% agarose containing 0.02% NaN_3, and prepared in distilled water. Fresh undiluted leaf sap from healthy and infected *N. rustica* either untreated, sonicated, or treated with 1% Triton X-100 was used as antigen.

Immunosorbent electron microscopy (ISEM) (2,19) was modified by using 0.1 M sodium citrate, pH 7.8, as the antigen extraction buffer and also for rinsing the grids after incubation.

Aphid transmission. *Aphis fabae* Scop., *A. gossypii* Glov., and *Myzus persicae* Sulz. were used in aphid transmission tests of TVYV. After a 1-hr preacquisition fast, adult apterae were allowed acquisition access periods of 10 min, 48 hr, or 72 hr, on systemically infected tomato plants before being transferred to healthy tomato and test plants of *N. rustica*. Fifteen aphids were used per test plant. After an inoculation access period of 7 days, the aphids were killed with insecticide.

Seed transmission. Seeds from systemically infected *N.*

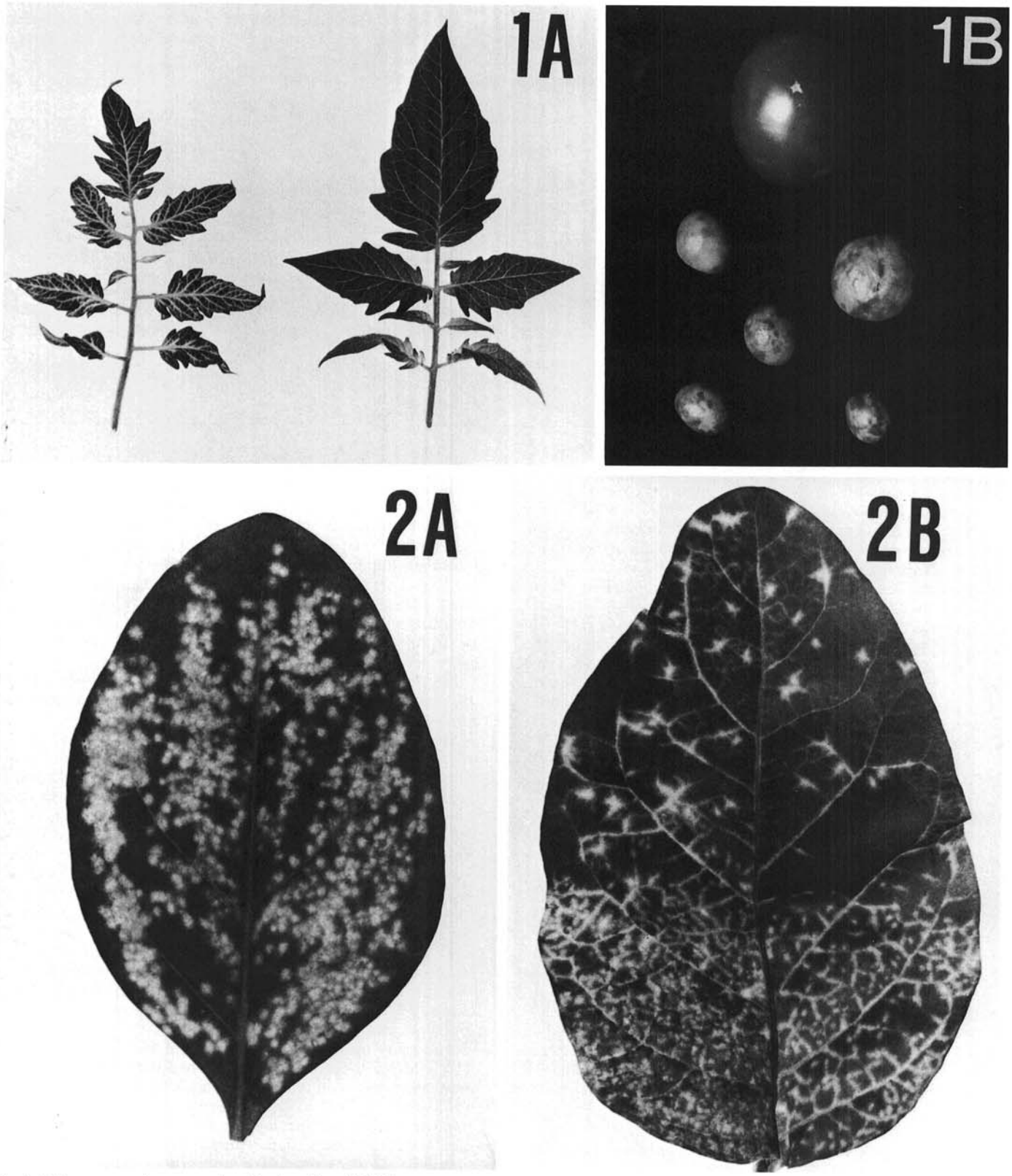

Fig. 1–2. Symptoms of tomato vein-yellowing virus (TVYV) on tomato and *Nicotiana rustica*. **1A,** Typical vein-yellowing in Heinz 1350 tomato at left, healthy leaf at right. **1B,** Symptoms of TVYV infection on tomato fruits, healthy fruit at top. **2A,** Local lesions on inoculated leaves of *N. rustica* 25 days after inoculation. **2B,** Systemic vein-yellowing and chlorotic spotting in *N. rustica*.

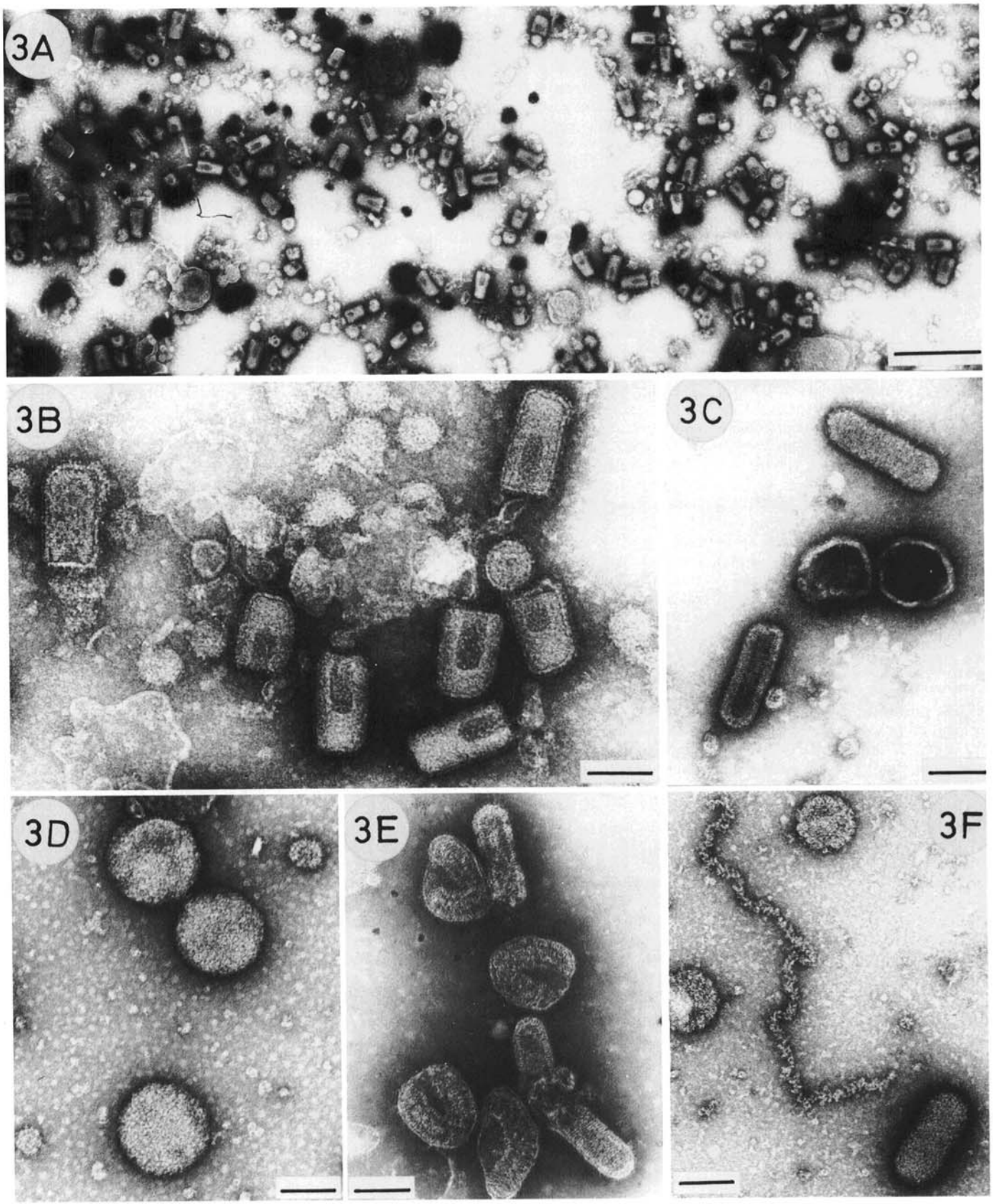

Fig. 3. Negatively stained particles of TVYV in crude extracts of leaves of *Nicotiana rustica.* **A,** Preparation extracted in ammonium molybdate (bar = 500 nm). **B,** Higher magnification of same preparation. **C,** Particles fixed in OsO_4 and stained in uranyl acetate, showing apparently complete structure. **D,** Particles unfixed and stained in uranyl acetate. **E,** Particles fixed in OsO_4 and stained in ammonium molybdate. **F,** Particles fixed with glutaraldehyde and stained in ammonium molybdate. (Bars in B–F = 100 nm).

glutinosa, N. rustica, and *S. sodomaeum*, were collected and germinated. Fifty seedlings were selected at random from each seed lot, transplanted, and observed for symptom development. Plants showing any deviation from normal appearance were examined by electron microscopy for the presence of virus particles.

RESULTS

Host range and symptoms. All three TVYV isolates produced identical symptoms on test plants. Apart from *Gomphrena globosa* L., which reacted with reddish chlorotic local lesions, the host range of TVYV was restricted to the Solanaceae. The most sensitive test plant was *N. rustica* which reacted at 15–20 days after inoculation with chlorotic local lesions (Fig. 2A) followed by systemic vein-yellowing and mosaic (Fig. 2B). Similar symptoms were produced on *N. benthamiana* Domin., *N. clevelandii* Gray, *N. edwardsonii* (Christie and Hall), *N. debneyi* Domin., *N. glauca* Grah., *N. glutinosa* L., *N. megalosiphon* Heurk & Muell., *N. paniculata* L., *N. sylvestris* Speg. & Comes, and *N. tabacum* L. 'Xanthi,' 'Samsun,' and 'White Burley.' Except for *Physalis floridana* Rybd., which was very sensitive, the other solanaceous species tested had low percentages of infection and long incubation periods prior to symptom appearance. These plants included *Capsicum annuum* L., *Datura stramonium* L., *D. meteloides* DC., *Nicandra physaloides* (L.) Gaertn., *Petunia hybrida* Vilm., *Physalis ixocarpa* Brot., *Lycopersicon esculentum*, *L. pimpinellifolium* Mill., *solanum melongena* L., *S. nigrum* L., and *S. sodomaeum* L.

No symptoms were observed on any of 32 other test plants belonging to other families. No virus was recovered from these plants by back-inoculation to *N. rustica*, and no viruslike particles were seen in leaf dip preparations.

Symptomatological comparison between TVYV, CYDV, and SYDV. A restricted range of test plants was selected to compare symptoms produced by TVYV, CYDV, and SYDV (Table 1). The solanaceous species, except for potato, reacted to TVYV infection with severe systemic symptoms including lethal necrosis, whereas CYDV and SYDV produced only mild systemic symptoms. The two PYDV strains, however, produced typical yellowing and dwarfing symptoms on potato, which was symptomlessly infected by TVYV.

Virus purification. Using Jackson and Christie's (10) SYNV purification procedure, which has been successfully employed for PYDV (4,5,12), no typical TVYV particles were found in the final suspension. Similar results were obtained with the PEG method of Hsu and Black (9) for PYDV, and with the LNYV purification procedure described by McLean and Francki (8,18). By electron microscopic examination it was found that soon after the extraction step in the buffers used in these and other purification procedures (14) the TVYV particles were disrupted. In phosphate and tris-HCl buffers no identifiable structures were visible. In glycine, particles were broken and shorter than normal. In 0.2 M sodium citrate, however, the particles were undamaged, and the addition of 0.01 $MgCl_2$ or 10% sucrose to the citrate buffer further stabilized the particles. The modified SYNV purification procedure using 0.2 M sodium citrate was adopted, and gave good results with TVYV and with both strains of PYDV. Partially purified preparations were relatively free of contaminating host material and were infectious when inoculated to healthy *N. rustica*.

Electron microscopy and particle morphology. Crude extracts (Fig. 3A) and partially purified preparations contained bullet-shaped particles. Particle dimension and structure depended on extraction, fixation, and staining procedures. Unfixed particles in AM extracts (Fig. 3A and B) were predominantly bullet-shaped, and measured about 150 × 75 nm but such preparations contained many obviously broken particles. After fixation with glutaraldehyde, particles up to 192 nm long were observed and after fixation with OsO_4 (Fig. 3C) particles were up to 234 nm long. Unfixed particles stained with uranyl acetate were often spherical (Fig. 3D). They probably represent swollen bullet-shaped or bacilliform particles. Preparations fixed with OsO_4 and stained with AM showed distorted nucleoprotein particles enclosed in inflated membranes (Fig. 3E). In many preparations, especially after fixation with glutaraldehyde, unraveled nucleoprotein threads were seen (Fig. 3F). Particles were totally disrupted in PTA.

Most well-preserved fixed particles were bacilliform (Fig. 3F), but when they were penetrated by the stain it could be recognized that the nucleoprotein did not fill one of the rounded ends which was formed by only the particle membrane. Correspondingly, viral particles in ultrathin sections consisted mainly of a bullet-shaped nucleoprotein core with a more or less regular membrane sac at the flat end of the nucleocapsid (Fig. 4B). Pairs of bullet-shaped particles with their flat ends towards each other sometimes formed bacilliform particles (Fig. 4B). Single bullet-shaped particles measured 265 × 86 nm and double particles, 535 × 86 nm. Cross sections of particles often showed a central canal with ringlike or irregular internal elements (Fig. 4B).

Cytopathology. Yellow areas of infected leaves contained conspicuous alterations of cell structures (Fig. 4A). Nuclei were enlarged by accumulations of virus particles bounded by the inner membrane of the nuclear envelope and protruding into the interior of the nuclei. The virus accumulations in the perinuclear space also protruded into the cytoplasm and also filled adjacent elements of the endoplasmic reticulum (ER). The virus particles originated mostly from the inner membrane of the nuclear envelope. Conspicuous masses of tubular material occurred within inflated ER cisternae in infected cells (Fig. 4A, C, and D). The origin of this material is not known. The tubules had approximately the diameter of virus particles but were up to 20 μm long. This material occurred in the same arrangement in *N. glutinosa* and *N. clevelandii*, the two host plants studied.

Serology. In preliminary experiments, the results obtained with untreated plant sap were superior to those obtained with sonicated or detergent-treated sap, and undiluted sap of *N. rustica* was used thereafter in all immunodiffusion tests.

Antisera prepared against TVYV, CYDV, and SYDV all had homologous titers of 1/32 in double-diffusion tests and did not react with sap of healthy *N. rustica*. In gels prepared in distilled water, all three viruses produced a single precipitin line, but in gels

TABLE 1. Symptomatological differentiation between tomato vein-yellowing virus (TVYV) and the CYDV and SYDV strains of potato yellow dwarf virus (PYDV)

	Virus/test plant reaction[a]					
	CYDV		SYDV		TVYV	
Test plant	Local	Systemic	Local	Systemic	Local	Systemic
Datura meteloides	cLL	0	cLL	0	cLL	M
Gomphrena globosa	scLL	0	scLL	0	lcLL	0
Nicotiana glutinosa	scLL	mM	scLL	mM	lcLL	svMD
Physalis floridana	scLL	mM	scLL	mM	lcLL	smVND
Solanum sodomaeum	*(scLL	VY)	scLL	0	lcLL	svM
Solanum tuberosum	0	St	0	St	0	0

[a] Symbols: s = small; l = large, m = mild; sv = severe; C = chlorotic; LL = local lesions; M = mosaic; D = death of plant; VN = vein-necrosis; VY = vein-yellowing; St = stunting; 0 = no symptoms; and *() = rarely infected.

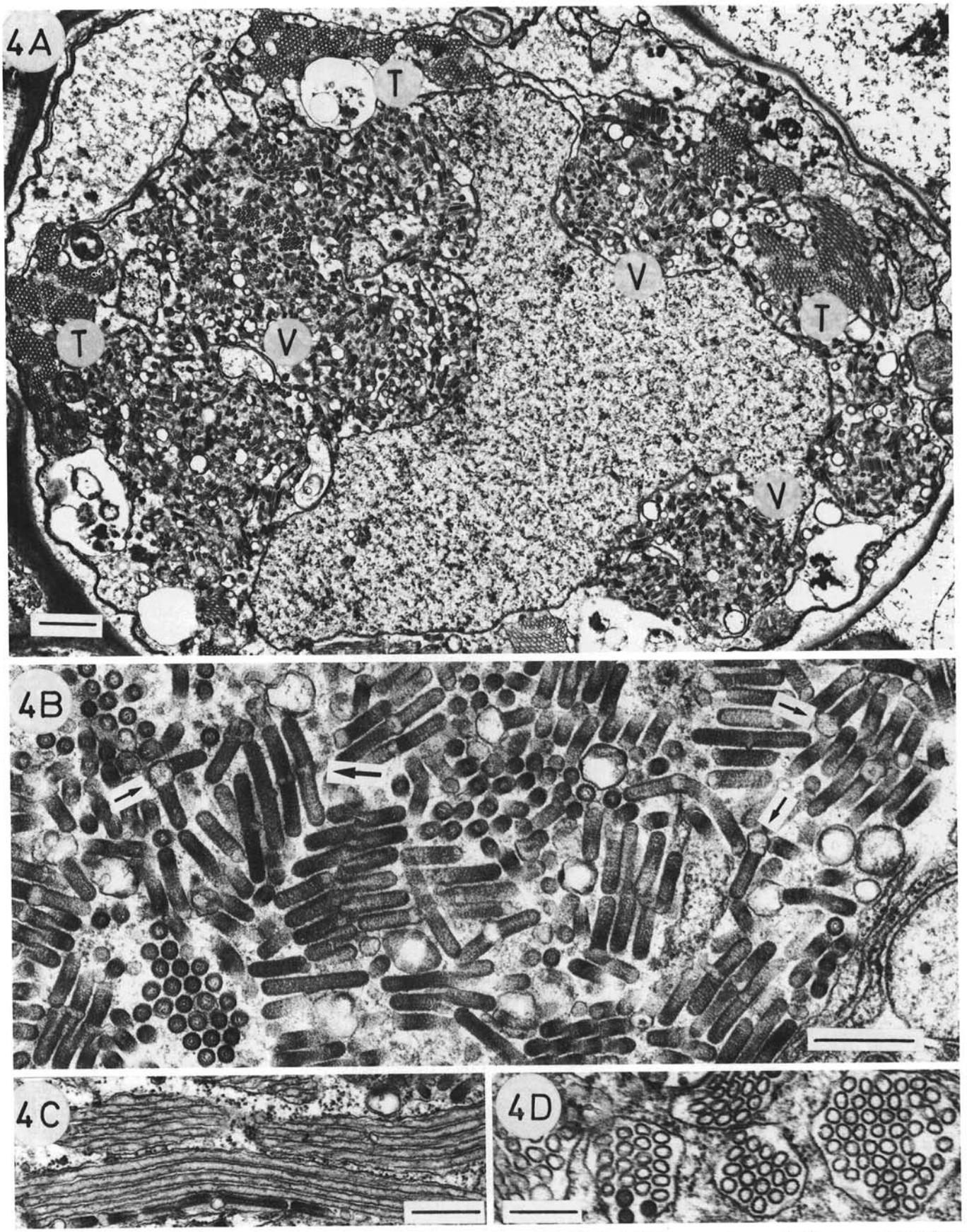

Fig. 4. Ultrathin sections from a leaf of *Nicotiana clevelandii* systemically infected with TVYV. **A,** Xylem parenchyma cell with accumulations of virus particles (V) protruding into the nucleus and the cytoplasm, aggregates of tubules in cross section (T) in the cytoplasm (bar = 1,000 nm). **B,** Detail of virus particle accumulation with bullet-shaped particles showing membrane sacs at one end (small arrows), bacilliform double particles (large arrow). Bar represents 500 nm. **C and D,** Longitudinal cross sections of aggregates of cytoplasmic tubules within cisternae of endoplasmic reticulum. Bar in **C** = 500 nm and in **D** = 300 nm.

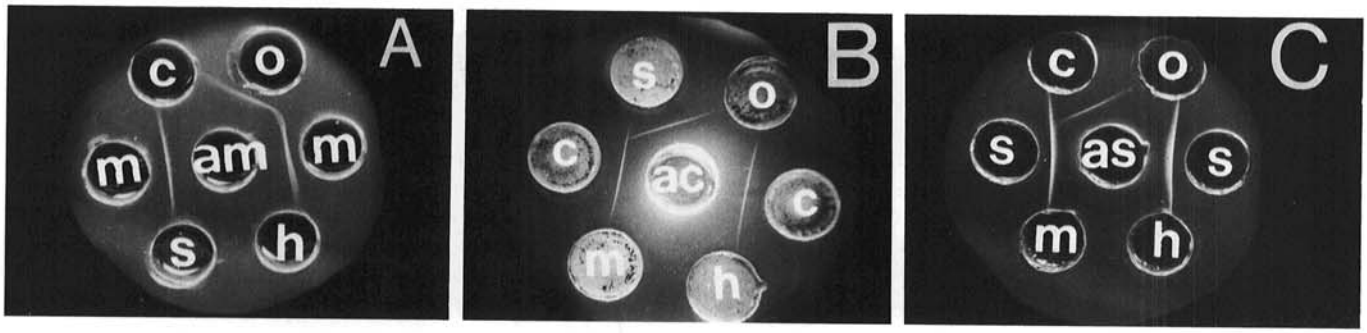

Fig. 5. Immunodiffusion tests of tomato vein-yellowing virus (TVYV) and the *constricta* (CYDV) and *sanguinolenta* (SYDV) strains of potato yellow dwarf virus (PYDV). Reaction of the three virus antigens and healthy plant sap against: **A,** TVYV-MOG antiserum, **B,** CYDV antiserum, and **C,** SYDV antiserum. Symbols: m = TVYV-MOG (Mograne tomato isolate), o = TVYV-SSO (isolate from *Solanum sodomaeum*), c = CYDV (ATCC PV 233), s = SYDV (ATCC PV 234), h = healthy *Nicotiana rustica* sap, am = antiserum to TVYV-MOG, ac = antiserum to CYDV, and as = antiserum to SYDV.

prepared in phosphate or tris-HCl buffers, two or sometimes three precipitin lines were produced. All comparative serological tests were done in agarose gels prepared in distilled water and containing 0.02% NaN_3. In repeated tests, TVYV-MOG antiserum failed to react with CYDV and SYDV antigens, while TVYV-MOG and TVYV-SSO from *S. sodomaeum* gave confluent precipitin lines with this antiserum (Fig. 5A). Antisera prepared against CYDV and SYDV reacted strongly against the respective homologous antigen, and produced spurs in heterologous combinations (Fig. 5B and C), as described previously (4,12). Using the CYDV and SYDV antisera prepared by us, as well as those obtained from the American Type Culture Collection (ATCC PVAS 233, and ATCC PVAS 234), no reaction was obtained with any of the three Moroccan tomato rhabdovirus isolates TVYV-MOG, TVYV-TL4, or TVYV-SSO.

In ISEM tests, grids coated with TVYV antiserum trapped large numbers of particles from TVYV-infected leaf extracts, but not from CYDV- or SYDV-infected leaf extracts. Conversely, grids coated with CYDV and SYDV antisera trapped large numbers of virus particles in both homologous and heterologous combinations, but did not trap TVYV particles.

Aphid and seed transmission. *Aphis fabae, A. gossypii,* and *M. persicae* failed to transmit TVYV from tomato to tomato or to *N. rustica* after any of the three acquisition access periods used. In seed-transmission tests no viruslike symptoms were observed in plants produced from seed collected from infected *N. glutinosa, N. rustica*, and *S. sodomaeum* after such plants were kept in the greenhouse for 2 mo after transplanting.

DISCUSSION

Based on bacilliform and bullet-shaped particle morphology in situ and in extracts, TVYV falls into the plant rhabdovirus group (7,11,17,21). In its biological properties (host range and symptoms) and particle dimensions TVYV resembles the two other mechanically transmissible plant rhabdoviruses infecting solanaceous species, PYDV and eggplant mottled dwarf virus (EMDV) (1,16, and D. E. Lesemann, *unpublished*). However, EMDV infects *C. amaranticolor* and *C. quinoa* (16) which were not infected by TVYV. Eggplant, a natural host of EMDV, was infected only with difficulty by TVYV.

Comparative host range tests with TVYV, CYDV, and SYDV showed that all three viruses produced similar symptoms on test plants. Differences in symptoms produced by TVYV and by the two PYDV strains, noted in Table 1, were reproducible and reliable, but do not appear to constitute sufficient basis to establish a separate identity for the Moroccan tomato rhabdovirus. In this respect, the results of the comparative serological tests were more conclusive. Complete absence of heterologous reaction between TVYV and the two PYDV strains is taken as evidence that TVYV is a distinct and apparently previously undescribed member of the plant rhabdovirus group. This conclusion is sustained by the cytological comparison. TVYV induces the accumulation of tubular material which is not found in association with PYDV, EMDV, or any other described plant rhabdovirus. However, a rhabdovirus isolated from tomato in Portugal (D. Louro, and D.-E. Lesemann, *unpublished*) resembles TVYV in biological and morphological properties. The significance of observed differences in cytological effects and in serological properties have yet to be determined. The relationship of TVYV to a virus reported to produce a yellow-vein disease of tomato in Nigeria (13) is unknown, since no morphological properties of the latter virus were described. The Nigerian virus was reported to produce no symptoms on *N. debneyi*, which is readily infected by TVYV.

The natural occurrence of TVYV in *S. sodomaeum* in widely separated areas of Morocco suggests that this plant may be an important perennial reservoir from which the virus may spread to susceptible crops. In Morocco, *S. sodomaeum* has also been found to be a natural reservoir of ToMV (15) and of an unidentified potyvirus infecting tomato (Lockhart and El Maataoui, *unpublished*).

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