

# Identification of Barley Yellow Striate Mosaic Virus in Morocco and Its Field Detection by Enzyme Immune Assay

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## ABSTRACT

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Barley yellow striate mosaic virus (BYSMV), a member of the plant rhabdovirus group and previously reported only from Italy and France, was identified in all major cereal-growing areas of Morocco in surveys conducted during 1983-1985. BYSMV occurred on bread wheat, durum wheat, barley, and oats and was transmitted to sweet corn but not to field corn. Symptoms ranged from fine, broken chlorotic striations to complete chlorosis and plant death. Bacilliform virus particles were observed in leaf-dip preparations and in ultrathin sections ( $58 \times 330$  nm in situ) from infected plants and accumulated within the cisternae of the endoplasmic reticulum and occasionally in the perinuclear space between the inner and outer nuclear membranes. The virus was transmitted experimentally by the delphacid plant hoppers *Toya propinqua* and *Laodelphax striatellus*. No differences were found, in immunodiffusion tests, between antisera to Moroccan and Italian BYSMV isolates. Moroccan BYSMV (BYSMV-M) was also related serologically to maize sterile stunt and northern cereal mosaic viruses, two delphacid-transmitted plant rhabdoviruses occurring in Australia and Japan, respectively. Dot-EIA on nitrocellulose membranes was 30 times as sensitive as double-antibody sandwich microplate assay for detecting BYSMV-M in field samples.

During the 1982-1983 growing season, previously unrecorded chlorotic and striate chlorotic foliar symptoms were observed on bread wheat (*Triticum vulgare* Vill.) and durum wheat (*T. durum* Desf.) in several areas of northern Morocco. No fungal or bacterial pathogens were associated with the disease, which was also not transmissible mechanically. Bullet-shaped and occasional bacilliform rhabdoviruslike particles were seen in leaf-dip preparations of diseased but not of healthy leaves. Research was conducted during 1983-1985 to determine the identity of the causal agent, the method of transmission, and the distribution of the disease.

## MATERIALS AND METHODS

**Disease identification and field surveys.** The disease was identified initially by symptomatology and electron microscopy. Plants showing chlorotic streak symptoms were checked by electron microscopy for the presence of rhabdoviruslike particles. Subsequently, agar gel immunodiffusion tests and enzyme immune assay (EIA) were used in addition to symptomatology and electron microscopy. Surveys were conducted in the main cereal-growing areas of Morocco during the 1983-1984 and 1984-1985 growing seasons.

**Insect transmission.** Transmission tests were done using two cicadellid leafhopper species (*Exitianus capicola* Stall. and *Psammotettix* sp.) and two delphacid plant hopper species (*Toya propinqua* (Fieber) and *Laodelphax striatellus* (Fallén)) found frequently on cereals in Morocco. The insects were raised on virus-free bermudagrass (*Cynodon dactylon* L.) grown from seed. All transmission tests were done by mass transfer using insects from laboratory colonies that had been found in previous studies not to harbor known viruses. The individuals of a given insect species were allowed to feed for 7-10 days on virus-infected wheat and were then caged in groups of 20-25 on seedlings of healthy test plants of bread wheat, durum wheat, barley (*Hordeum vulgare* L. 'NK 38' and

'Capri'), oats (*Avena sativa* L. 'Clintland 64'), and *Zea mays* L. 'LG 55' (field corn) and 'Earliking' (sweet corn). Insects were allowed to feed for the duration of their lives on the test plants. No aphid transmission tests were done.

**Partial virus purification.** The Moroccan wheat rhabdovirus was purified from field-collected wheat leaf tissue showing typical symptoms. The virus was purified by a modification of the procedure described for tomato vein-yellowing virus (TVYV) (5), using Celite filtration (9,13) for clarification of the initial extract. Infected leaf tissue was finely minced and ground with washed sand in a mortar in cold 0.1 M glycine-HCl, pH 7.8, containing 0.2%  $\text{Na}_2\text{SO}_3$ . The homogenate was filtered through cheesecloth, centrifuged at 6,000 g for 10 min, and the supernatant clarified by gentle vacuum filtration through a Celite pad. The clarified extract was layered on a step gradient consisting of 5 ml each of 30 and 60% (w/v) sucrose in extraction buffer and centrifuged for 1 hr at 25,000 rpm in a Beckman SW 27 rotor. The light-scattering band at the 30/60% interface was collected, diluted in 0.01 M glycine-HCl, pH 7.5, and centrifuged for 25 min at 30,000 rpm in a Beckman Type 35 rotor. The resulting pellets were resuspended in 0.01 M glycine-HCl, pH 7.5, and clarified by a final low-speed centrifugation.

**Antiserum production and serological tests.** Antiserum against the Moroccan wheat rhabdovirus was produced in rabbits given a sequence of five injections of partially purified virus emulsified in Freund's incomplete adjuvant. The initial injection was administered subcutaneously and was followed by three intramuscular injections 1 wk apart. Ten days after the final injection, blood was collected by sacrificing the animals.

Immunodiffusion tests were done in 0.8% agarose in distilled water containing 0.02%  $\text{NaN}_3$ . Undiluted leaf sap from infected and healthy wheat, barley, oats, and maize were used as antigens without prior detergent or sonication treatment. Antiserum to an Italian isolate of BYSMV was supplied by O. Lovisolo, Istituto di Fitovirologia Applicata,

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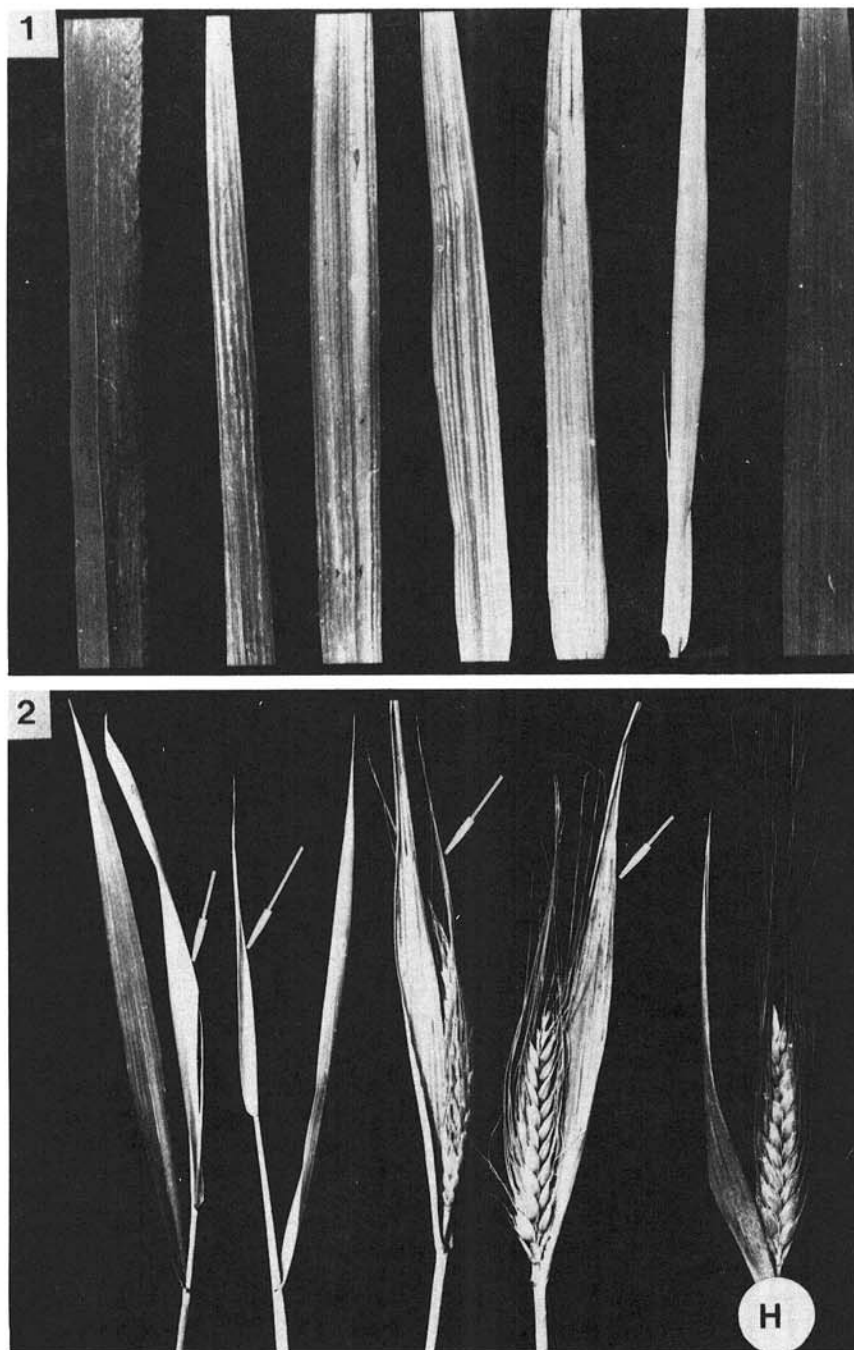
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Torino, Italy. Immunodiffusion tests with antisera to maize sterile stunt (MSSV) (6) and northern cereal mosaic (NCMV) (14) viruses were done by R. S. Greber, Queensland Department of Primary Industries, Queensland, Australia. Antiserum to a Hawaiian isolate of maize mosaic virus (MMV) was supplied by R. E. Gingery, Ohio Agricultural Research and Development Center, Wooster.

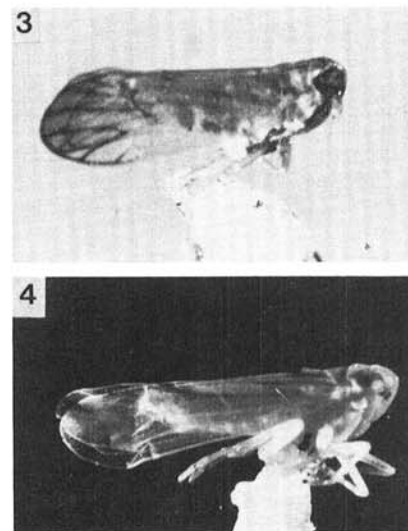
Double-antibody sandwich enzyme immune assays (DAS-EIA) using alkaline phosphatase conjugate were done according to standard procedure (2).

Polystyrene plates were coated with 1  $\mu\text{g/ml}$  purified IgG. Leaf samples were extracted and diluted in phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween 20 and 2% polyvinyl pyrrolidone (PVP, mol wt 40,000). Enzyme conjugate was used at a 1/500 dilution. Absorbances were determined spectrophotometrically at 405 nm using a Microelisa Minireader (Dynatech Corp., Chantilly, VA). EIAs on nitrocellulose membranes (dot-EIA) were done essentially by the procedure described for potato viruses S, X, and Y (1) except for slight

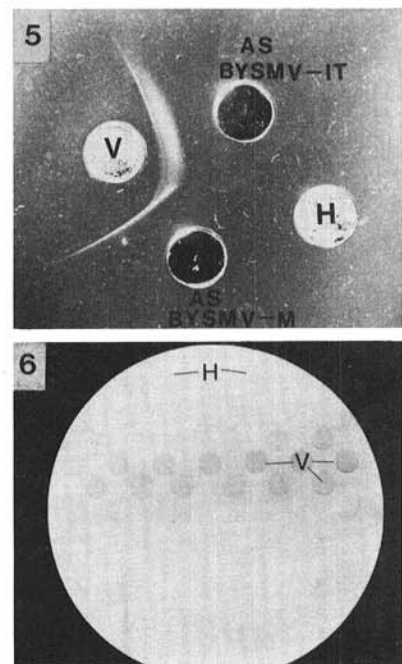
modifications. Nitrocellulose membranes (type HA 0.45- $\mu\text{m}$  pore size, Millipore Corp., Bedford, MA) were coated by immersion for 2 hr at room temperature in 1  $\mu\text{g/ml}$  purified IgG. Bovine serum albumin (BSA) was used as a blocking



**Figs. 1 and 2.** Symptoms of barley yellow striate mosaic virus, Moroccan isolate (BYSMV-M), in naturally infected durum wheat (*Triticum durum*). (1) Healthy leaves at extreme left and right. Infected leaves in center show progressive symptom development from broken chlorotic streaks to complete chlorosis. (2) Healthy normal green flag leaf and awns at extreme right. Arrows indicate characteristic chlorotic terminal laminae and awns associated with BYSMV-M field infection.



**Figs. 3 and 4.** Delphacid plant hopper vectors of Moroccan barley yellow striate mosaic virus (BYSMV-M): (3) *Toya propinqua*, adult male ( $\times 16$ ), and (4) *Laodelphax striatellus*, adult female ( $\times 16$ ).



**Figs. 5 and 6.** Serological reactions of Moroccan barley yellow striate mosaic virus (BYSMV-M). (5) Immunodiffusion reaction of BYSMV-M in infected oat sap (V) with antisera to Moroccan (AS-BYSMV-M) and Italian (AS-BYSMV-It.) BYSMV isolates. H = healthy oat sap. (6) Detection of BYSMV-M in field samples of durum wheat (*Triticum durum*) by dot-EIA on nitrocellulose membranes. V = location of positively reacting infected samples. Positively reacting samples are red-pink when observed directly. H = healthy durum wheat controls.

agent at 3% (w/v), and samples were applied in plastic templates for 1 hr at 4 C. After rinsing, membranes were incubated in alkaline phosphate conjugate (1/500) for 2 hr at 30 C. Tris-HCl (0.01 M, pH 7.4) was used in all steps. The substrate and color development system used was Fast Red TR salt and AS-MX phosphate (Sigma Chemical Company, St. Louis, MO). Color development on membranes was evaluated visually.

**Electron microscopy and cytopathology.** Leaf-dip preparations for electron microscopic examination were made using 1.5% sodium phosphotungstate, pH 7.0 (PTA), 2% ammonium molybdate, pH 6.8, or uranyl acetate. For cytopathological studies, young infected durum wheat leaf tissue was fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 6.8, or 2–3% glutaraldehyde in 0.1 M potassium-sodium phosphate buffer (PSPB), pH 7.2. After rinsing in PSPB, the tissue was fixed in 2% osmium tetroxide in PSPB, rinsed again in PSPB, dehydrated in a graded series of ethanol followed by propylene oxide, and infiltrated and embedded in low-viscosity embedding medium. Ultrathin sections

of embedded tissue were stained in uranyl acetate and lead citrate, then examined in the electron microscope.

## RESULTS

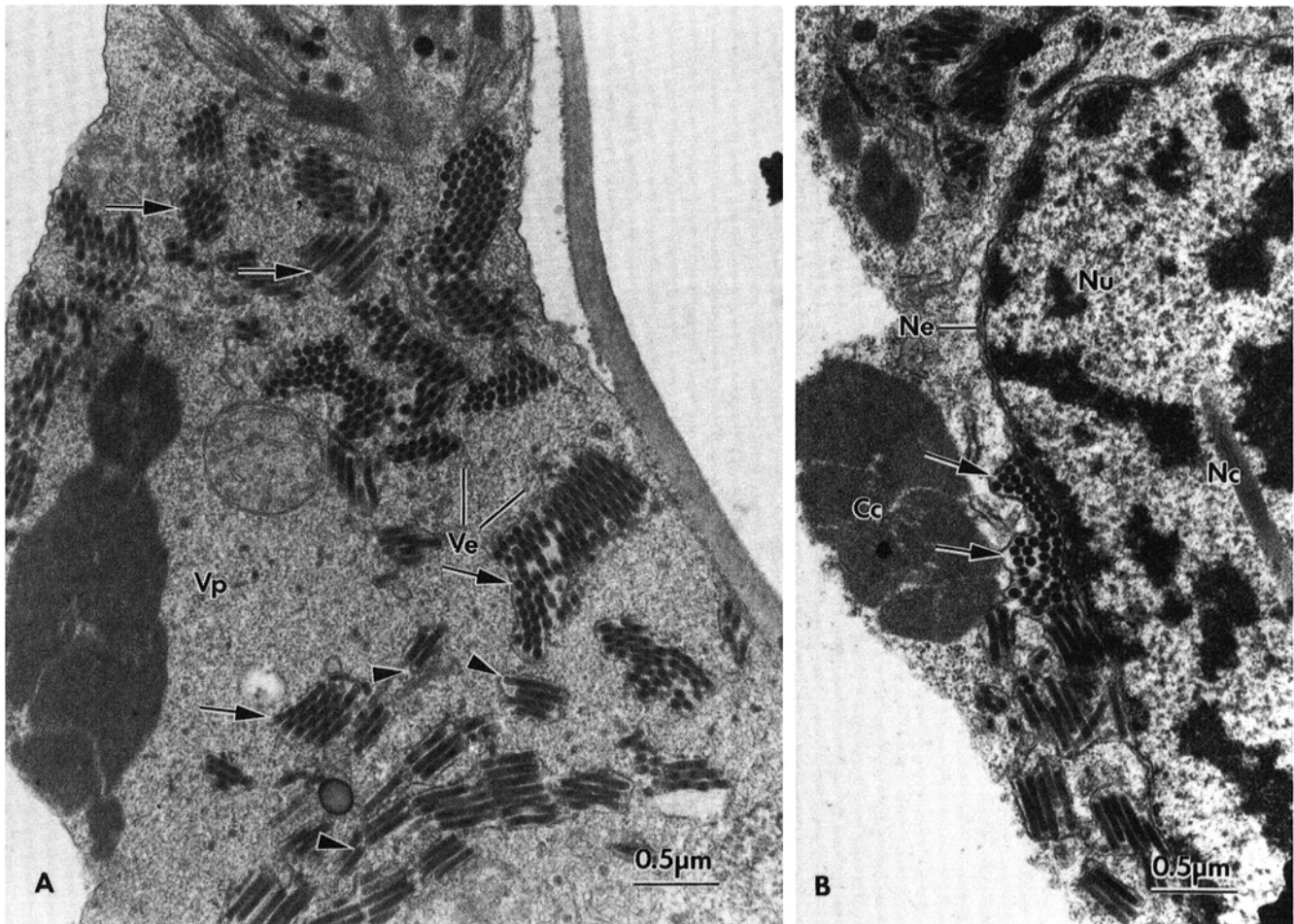
**Symptoms, disease identification, and distribution.** Symptoms of rhabdovirus infection were similar on bread wheat, durum wheat, barley, and oats. Initial symptoms consisted of broken, fine, chlorotic lines, then progressed to chlorotic striations and eventually to complete leaf chlorosis (Fig. 1). A characteristic feature of the disease in the field was the prominent chlorosis of terminal and flag leaves and awns (Fig. 2). Basal tillers arising from infected plants were often totally chlorotic and eventually died.

The disease was identified in all major cereal-growing areas of Morocco, on all four cereals mentioned, and occurred during all growing seasons between 1982–1985. The percentage of plants showing advanced symptoms was relatively low. Assays were not done to determine the percentage of field plants infected by the virus.

**Insect transmission.** The wheat rhabdo-

virus was transmitted by the delphacid plant hoppers *T. propinqua* (Fig. 3) and *L. striatellus* (Fig. 4) from infected wheat to healthy wheat, barley, and oats. Symptoms on infected test plants were similar to those occurring in the field. The cicadellid leafhoppers *E. capicola* and *Psammotettix* sp. did not transmit the virus. The delphacids also transmitted the virus from infected wheat and oats to sweet corn cultivar Earliking but not to the field corn cultivar LG 55. Symptoms on sweet corn consisted of fine broken or continuous chlorotic lines, but no severe chlorosis developed as it did in the cereals. Both positive and negative transmission results were verified in all instances by electron microscopy and EIA microplate assay.

**Partial virus purification.** Partially purified wheat rhabdovirus preparations from infected wheat contained bullet-shaped and broken particles and were contaminated with numerous membrane fragments. No other viruslike particles were present, and no further purification was attempted because partially purified suspensions were judged satisfactory for antiserum production.



**Fig. 7.** Leaf cells of durum wheat infected with the Moroccan isolate of barley yellow striate mosaic virus. (A) Bacilliform virus particles (arrows) are present within cisternae of the endoplasmic reticulum. Some particles (arrowheads) reveal electron-transparent ends. A granular inclusion or viroplasm (Vp) containing many small vesicles (Ve) is associated with the endoplasmic reticulum. Scale bar = 0.5  $\mu$ m. (B) Virus particles in the perinuclear space (arrows). The outer membrane has become widely separated from the inner membrane of the nuclear envelope (Ne). Also shown are the nucleus (Nu), a nuclear crystal (Nc), and a cytoplasmic crystal (Cc). Scale bar = 0.5  $\mu$ m.

**Serology.** In immunodiffusion tests, antiserum to the Moroccan wheat rhabdovirus had a homologous titer of 1/16, using undiluted infected oat leaf sap as antigen. Positive reactions in immunodiffusion were also obtained with infected wheat, barley, and sweet corn. The virus reacted in immunodiffusion tests with antiserum to an Italian isolate of BYSMV (BYSMV-It) but not with antiserum to maize mosaic virus (MMV) or Cynodon chlorotic streak virus (CCSV) (7). No spur formation was observed when the wheat rhabdovirus antigen was tested against homologous and BYSMV-It antisera (Fig. 5), indicating a close serological relationship between the Moroccan wheat rhabdovirus and BYSMV-It. The Moroccan wheat rhabdovirus was therefore designated BYSMV-M. No

BYSMV-It antigen was available for comparative tests. In separate immunodiffusion tests, BYSMV-M reacted with antisera to MSSV and NCMV (R. S. Greber, *personal communication*).

In DAS-EIA microplate assays, antigen samples were routinely applied at a 1/10 dilution and yielded absorbance values ( $A_{405nm}$ ) ranging from 0.23 to 1.10 compared with 0.01 to 0.05 for healthy controls. Positive results were also obtained using dot-EIA on nitrocellulose membranes (Fig. 6). The limit of reliable detection of BYSMV-M in wheat leaf samples was at a 1/800 tissue dilution in DAS-EIA ( $A_{405nm} = 0.11$  compared with 0.00 for healthy controls). The limit of reliable detection of BYSMV-M by dot-EIA was at a leaf tissue dilution of 1/25,600 as assessed visually.

Microplate DAS-EIA was used to

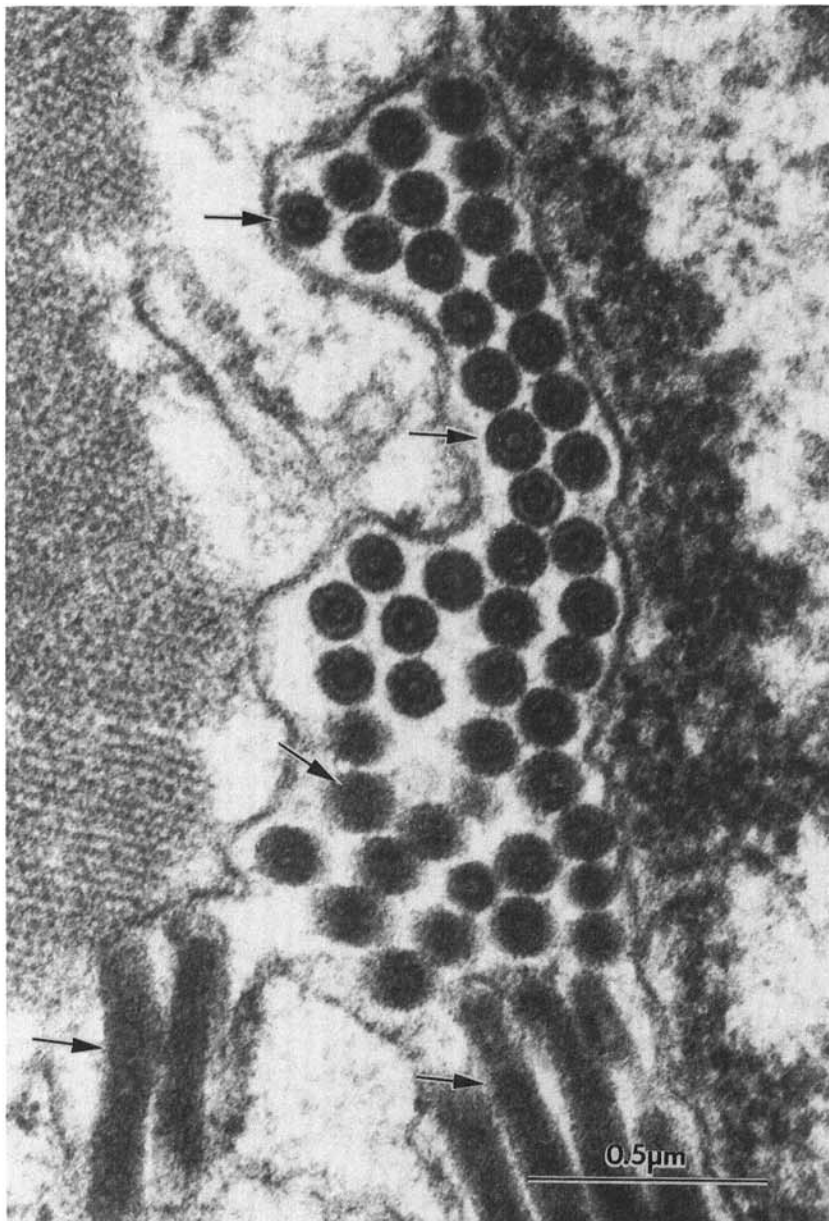
detect BYSMV-M in field samples collected at several locations throughout Morocco. The virus was detected by this method in samples that showed very faint or inconspicuous symptoms and in which virus particles could not readily be detected by electron microscopy.

**Electron microscopy and cytopathology.** BYSMV-M particles were stable in unfixed preparations stained with PTA, ammonium molybdate, and uranyl acetate. In this respect, BYSMV-M was unlike TVYV (5), which is disrupted rapidly by PTA. For routine examination of leaf-dip preparations of field samples, PTA was therefore used as negative stain. Particles of BYSMV-M were observed readily in plants showing pronounced symptoms but less readily in plants showing faint or doubtful symptoms. No viruslike particles were ever observed in symptomless plants.

**Cytopathology.** When the durum wheat leaves with chlorotic streak symptoms from Morocco were examined in the electron microscope, bacilliform virus particles typical of a rhabdovirus were observed in nearly all tissues. Whereas most occurred in parenchyma cells of the phloem, others were present in sieve elements, parenchyma cells of the xylem, mesophyll cells, and epidermal cells. The Moroccan virus particles were usually found within cisternae of the endoplasmic reticulum (Fig. 7A). Frequently, these cisternae were associated with a granular inclusion (viroplasm) in the cytoplasm that contained groups of small vesicles (Fig. 7A). Some of the bacilliform virus particles had electron-transparent ends (Fig. 7A). Occasionally, virus particles were seen within an expanded region of the perinuclear space (Fig. 7B). The bacilliform virus particles of BYSMV-M measured 54 nm in diameter and 340 nm long (average of 100 particle measurements) (Fig. 8) compared with intracellular TMV particles that measured 11 nm wide and 290 nm long in tobacco leaf tissue. Particles of the virus were also found in thin sections of leaves of barley and sweet corn infected with BYSMV-M.

## DISCUSSION

The virus we have described, occurring naturally in cereals in Morocco, is clearly BYSMV, which to date has only been recorded in Italy (3) and France (11,12). This is therefore the first documented report of BYSMV in northern Africa and also the first report of natural infection of oats by BYSMV. Chlorotic streak symptoms were observed on durum wheat and barley in Algeria, Tunisia, Cyprus, and Syria, and rhabdovirus particles similar to those of BYSMV-M were seen in thin sections of durum wheat leaf samples from Algeria and Cyprus. However, the association of BYSMV infection with field symptoms observed in countries other than Morocco remains to be determined.



**Fig. 8.** Enlargement of Figure 7B showing some ultrastructural details of virus particles (arrows) sectioned transversely and longitudinally. Scale bar = 0.25  $\mu$ m.

The cytopathology of the Moroccan virus was identical to that which appeared earlier in the literature (4) for BYSMV. In addition, some cytopathological aspects, such as the accumulation of virus particles in the cytoplasm and the presence of granular inclusions or viroplasm, were similar to those described for northern cereal mosaic virus (14). Recent reports of serological relationships between BYSMV-M, BYSMV-It., MSSV, and NCMV (R. S. Greber, *personal communication*), and between NCMV and wheat rosette stunt virus (WRSV) (8), suggest that these may constitute a related group of dephacid-transmitted plant rhabdoviruses of Gramineae.

Although BYSMV-M was found infecting cereals throughout Morocco, no natural reservoirs of the virus have yet been identified, and this aspect of the epidemiology of the virus remains an important question to be resolved. In France, *Agropyron repens* L. was reported to be the principal reservoir of BYSMV (11,12), but this plant was not found in or around the cereal fields sampled in Morocco. Both *T. propinqua* and *L. striatellus*, the experimental vectors of BYSMV-M, occur in high numbers in cereals in Morocco. Apart from low numbers of *Peregrinus maidis* Ashmead on maize (B. E. L. Lockhart, *unpublished*), no other delphacid plant hoppers have been found on Gramineae

in Morocco, and it is therefore likely that *T. propinqua* and *L. striatellus* are also natural vectors of BYSMV in Morocco. *Laodelphax* has been reported to be a vector of BYSMV in Italy (3) and in France (10,12). *T. propinqua* has not previously been reported as a vector of BYSMV.

Both DAS-EIA and dot-EIA were found to be reliable and sensitive methods for detecting BYSMV in field samples, and it is likely that other variations of EIA can also be used in BYSMV detection elsewhere in the Mediterranean cereal-producing area.

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