Host Range, Symptomatology, and Electron Microscopy of a Persistent, Aphid-Transmitted Virus from Alfalfa in Michigan

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ABSTRACT

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A virus that was transmitted from alfalfa plants in Michigan resembles pea leaf roll virus because of its transmission in a persistent manner by the pea aphid, *Acyrthosiphon pisum*, nontransmissibility after sap-inoculations, and its host reactions on *Vicia faba, V. sativa, Pisum sativum, Trifolium incarnatum, T. subterraneum, Cicer arietinum, Lens esculenta, Medicago hispida*, and *Phaseolus vulgaris*. *Medicago sativa* infected with this virus usually was symptomless, but virus could be readily recovered from it. None of 20 nonleguminous species tested was susceptible to this virus. Electron microscopy of several infected host plants revealed spherical, viruslike particles (diameter 23 ± 1.5 nm) in some phloem cells. Virus concentration in cells varied from a few, widely scattered particles to dense masses; occasionally, crystalline arrays of these particles were observed. Identity of the virus in relation to other persistent, aphid-borne viruses which cause yellowing, leaf-rolling, and stunting in leguminous plants was reviewed.

Additional key words: pea aphid, pea leaf roll virus.

While testing field-collected pea aphids, *Acyrthosiphon pisum* (Harris), from alfalfa (*Medicago sativa* L.) at East Lansing, Michigan, we found that a few aphids carried a plant disease causal agent which was transmitted to broad bean (*Vicia faba* L.) and pea (*Pisum sativum* L.). The disease symptoms on the host plants were similar to those induced by pea leaf roll virus (PeLRV) which is known to occur in Europe (4, 5, 21, 25, 26, 34, 39, 40), Iran (15), and New Zealand (27, 42, 43). The host reactions and characteristics of aphid-transmission of this virus were investigated to determine its identity. Other than aphid-transmission characteristics, virtually nothing is known about the properties of PeLRV. This paper also reports the occurrence and distribution of spherical viruslike particles of the same size and morphology in leaf cells of several host plants infected with this virus.

MATERIALS AND METHODS

Maintenance of virus and aphids.—Broad bean (*Vicia faba* 'Board Improved Long Pod') was used for culturing the virus and, unless otherwise stated, as a virus source and test plant in aphid-transmissibility tests. The test seedlings were sprouted in vermiculite and after emergence were transplanted singly to pots and used in the experiments.

Unless otherwise stated, nonviruliferous first- and second-stage nymphs of an East Lansing Biotype (38) of the pea aphid, *A. pisum*, were used in aphid-transmission tests. *Myzus persicae* (Sulz.), *Aulacorthum solani* (Kaltenbach), *Aphis craccivora* Koch and *A. fabae* Scop. also were tested. All aphids except *A. craccivora* were maintained on healthy broad bean plants; *A. craccivora* was reared on *Chenopodium quinoa* Willd.

Transmission experiments.—In transmission studies, first- and second-stage nymphs of the pea aphid were fed on source plants for 72 hr and then were transferred to healthy test plants for 3 days in a controlled environmental chamber at 22 C. Usually aphids were tested for infectivity in groups of 5, 10, or 20 per test plant.

In host-range studies, at least 40 plants of each species or cultivar were inoculated by means of aphids. After 1-2 mo attempts were made to recover virus from a few randomly selected host plants with or without symptoms. The plants were reinfested with aphids for a 3-day acquisition-access period (AAP) and then assayed by transmission tests to broad beans, using 20 nymphs per seedling with 3-day inoculation-access period (IAP).

Electron microscopy.—In electron microscopic studies, young infected leaves from plants with distinct symptoms were cut into small pieces (about 0.1 cm × 0.3 cm) and fixed for 3-4 hr in 6% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). After this primary fixation, specimens were rinsed twice for 30 min with buffer and postfixed in 1% osmium tetroxide for 3-4 hr at room temperature. Specimens were dehydrated in a graded ethanol series with several changes of 100% ethanol and embedded in ERL epoxy resin (29). Ultrathin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate (41), and examined and electron-micrographed in a Philips Model 300 transmission electron microscope at 60 kV.

RESULTS

Transmission of the virus.—The virus originally was transmitted from naturally-infected alfalfa growing at Michigan State University. Pea aphids were collected at 50 locations in five alfalfa fields and were transferred (in groups of 20 from each location) to seedlings of broad bean, and pea (*P. sativum* 'Dart') for 1 day. They then were transferred to a second group of test plants for 1 day and then to a third group for 3 days. Ten percent of broad bean and pea plants developed yellowing of leaves, erect growth, and stunting of plants similar to those described for pea leaf roll virus infection (4, 5, 6, 15, 25, 34, 39, 40). Again in 1976, pea aphids were collected from different locations, and the tests showed that the virus could be transmitted from two more fields. In 1971 and 1973, we also observed in the experimental plots at East Lansing naturally-infected broad beans showing typical symptoms of pea leaf roll virus infection.

Mechanical inoculation.—To determine whether the virus is sap transmissible, infected peas were ground in 0.1 M phosphate buffer (pH 7.0), and the resultant juice was rubbed on Carborundum-dusted seedlings of *P. sativum*, *V. faba*, *Chenopodium amaranticolor* Coste and Reyn., *Cucumis sativus* L., *Gomphrena globosa* L., and *Nicotiana glutinosa* L. These experiments were repeated four times using at least 20 seedlings of each species. The results of all attempts to transmit the virus by sap-inoculations were negative.

Host range and symptomatology.—Initially, three isolates of the virus were used for host-range and symptomatology tests, but since the symptoms caused by these isolates were identical only one isolate was studied in detail.

The first visible symptom on *V. faba* 'Broad Improved Long Pod' was an upward rolling and slight chlorosis of the younger leaves about 10-15 days after inoculation. The upward rolling was the most conspicuous symptom in *V. faba*. In the next 1-2 wk, infected plants showed an erect growth and yellowing; newly-developed leaves were small in size (Fig. 1). Infected broad beans also showed interveinal yellowing on the older leaves, especially near the leaf margin, and the leaves became thickened and leathery. In advanced stages (5-6 wk after inoculation) leaves became necrotic with marked chlorosis and a slight upward rolling (Fig. 2), the plants were stunted and defoliated, and flowering was usually sparse with negligible pod formation. Similar symptoms also were noticed in *V. faba* 'Broad Windsor' (Fig. 3). The sumptoms in *Vicia sativa* L. were slight stunting and yellowing of younger leaves.

The first symptom of virus infection in pea cultivars Dart, Wisconsin Perfection, Rocket, and Mars was faint yellowing on the youngest leaflets. As the leaflets matured they curled downward; this was accompanied by yellowing of the older leaves and dwarfing of the youngest (Fig. 4). Ultimately, the entire plant became markedly stunted with shortened internodes and petioles. Pea cultivars Midfreezer, Wando, Little Marvel, and Koroza did not develop symptoms following infestation with 10 aphids per seedling, and the virus could not be recovered by back-inoculation to broad beans. Pea cultivars Ranger, Sprite, and Signet showed moderate susceptibility.

Infected *Medicago sativa* L. 'Du Puits' showed no symptoms, but virus was recoverable. Occasionally, a mild transient yellowing of older leaves was noticed.

The most obvious symptoms in *Medicago hispida* Gaert. were severe stunting of the plant and yellowing and cupping of younger leaves.

Crimson clover (*Trifolium incarnatum* L.) plants infected with the virus were stunted, and mature leaves showed interveinal chlorosis together with a reddening of the leaf margin.

Symptoms of infection on *Trifolium subterraneum* L. 'Bacchus Marsh' commenced as a mild interveinal yellowing closely followed by a reddening of the margin of the older leaves. The younger leaves were yellow and cupped, and infected plants were smaller than healthy control plants. During the summer months the older leaves turned a deep red color, but at lower temperatures the plants showed only a brown to purple coloration of the leaf margin. The diseased plants often collapsed, especially during the summer months.

French beans, *Phaseolus vulgaris* L. 'Topcrop' and 'Bountiful' developed an interveinal chlorosis on leaves 3 wk after inoculation. The infected plants were smaller than the healthy control plants and the older leaves were twisted, rigid, thickened, yellow, and curled (Fig. 5).

Infected chickpea (*Cicer arietinum* L.) and lentil (*Lens esculenta* Moench) were characterized by chlorosis, stem necrosis, leathery downward-curled leaves and severely stunted plants. The symptoms were identical to those caused by pea leaf roll virus isolate from Iran (15, Fig. 5 and 7).

In our initial studies, it was observed that pea aphids did not feed well on nonleguminous plants which are nonhosts of that vector; however, they survived for about 2 days. Nault (23) used a "host alternating" technique and reported that the oligophagous grain aphids, *Macrosiphum avenae* (F.), *Rhopalosiphum padi* (L.), and *Schizaphis graminum* (Rondani) transmitted pea enation mosaic virus from pea to pea, a nonhost of the vector, if they first were placed on barley, a natural vector host, for a 48-hr incubation period following virus acquisition. On the assumption that viruliferous pea aphids can transmit the virus under study to nonleguminous plants, pea aphids which were reared on infected broad beans were transferred to plants of the following 20 species of nonleguminous plants belonging to nine families for a 48-hr IAP: *Amaranthus tricolor* L., *Beta vulgaris* L., *Brassica campestris* L., *B. napus* L., *B. nigra* Koch., *B. rapa* L., *Capsella bursa-pastoris* L., *Capsicum annuum* L., *Chenopodium quinoa* Willd., *Cucumis sativus* L., *Datura stramonium* L., *Gomphrena globosa* L., *Lycopersicon esculentum* Mill., *Malva parviflora* L., *Petunia hybrida* Vilm., *Physalis floridana* Rydb., *Spinacia oleracea* L., *Stellaria media* (L.) Cyrill., *Tetragonia expansa* Murr., and *Zinnia elegans* Jacq. The virus did not cause symptoms in any of these plants and could not be recovered by back-inoculation to broad bean.

Attempts also were made to transmit this virus to the above-mentioned nonleguminous plants using *M. persicae* and *A. solani* that feed more readily on those plants. However, our tests were negative and the virus under study probably is limited to leguminous hosts.

Virus-vector relationship.—First- and second-stage

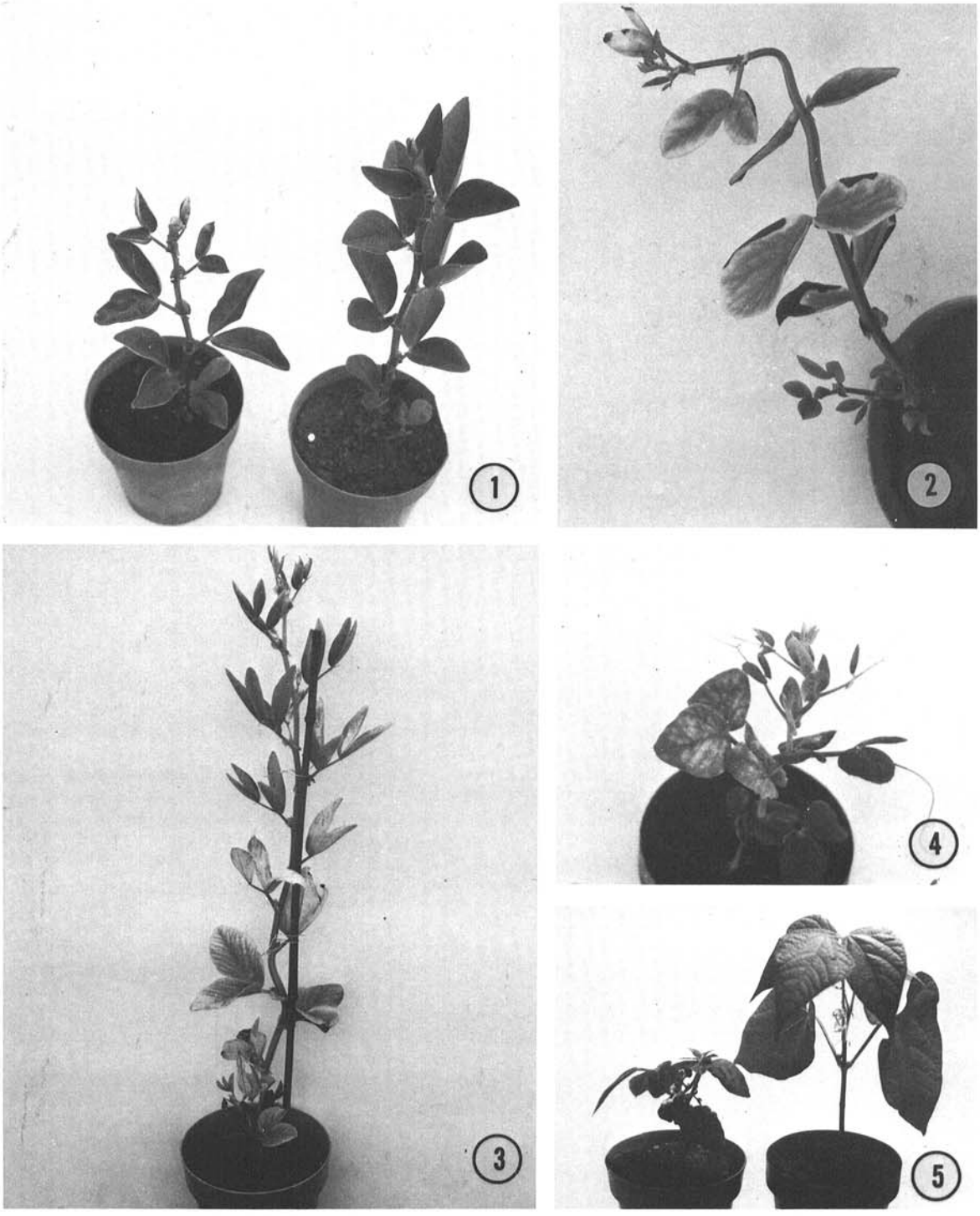


Fig. 1-5. Symptoms produced in various infected host plants by presumed pea leaf roll virus from alfalfa in Michigan. **1)** Left: infected, dwarfed *Vicia faba* 'Broad Improved Long Pod', 20 days postinoculation; and right, a healthy plant of the same age. **2)** Infected *V. faba* showing marked chlorosis, interveinal yellowing and necrosis, near the leaf margin, 5 wk postinoculation. **3)** *V. faba* 'Broad Windsor' plant showing erect growth, yellowing, and upward rolling of leaves with newly developed leaves small in size, 6 wk postinoculation. **4)** Typical symptoms of *Pisum sativum* 'Dart' with chlorosis and stunting, 21 days postinoculation. **5)** Symptoms of *Phaseolus vulgaris* 'Bountiful'. Left: an infected plant, 5 wk postinoculation and right, a healthy plant of the same age.

nymphs of *A. pisum*, *M. persicae*, *Aulacorthum solani*, *Aphis craccivora*, and *A. fabae* that had an AAP of 3 days on infected broad bean then were transferred to healthy broad bean seedlings for a 4-day IAP using 10 insects per test plant. *Acyrthosiphon pisum* transmitted the virus to 92% (23 infections out of 25 trials), *M. persicae* to 8% (2/25), *A. solani* to 80% (20/25), and *A. craccivora* to 32% (8/25) of the test plants. *Aphis fabae* failed to transmit the virus (0/75).

To assure that the low transmission efficiency by *M. persicae* was not due to our selection of an inefficient vector biotype of this aphid, transmission experiments were repeated using aphids which were collected from eight locations in Michigan. Two aphid isolates transmitted to 4%, five isolates to 8%, and one isolate to 12% of the test plants, respectively; these results confirmed that *M. persicae* is an inefficient vector of this virus.

The influence of acquisition access feeding period on transmission efficiency was tested by providing first- and second-stage nymphs with AAP's of 0.25, 0.5, 1, 2, 4, 8, 24, 48, and 96 hr on infected broad bean and transferring aphids in groups of 10 to individual healthy broad bean seedlings for an IAP of 7 days. The three shortest AAP's did not result in virus transmission and only 4% (1/25) of the test plants became infected when fed upon by aphids from the 2-hr AAP treatment (Table 1). Thus, the acquisition threshold lies between 1 and 2 hr. Transmission increased as a function of AAP, but 100% transmission was attained only by aphids from the 96-hr treatment, even though 10 aphids per plant were used.

To determine the inoculation threshold, first- and second-stage nymphs of *A. pisum* were fed on infected broad bean plants for 3 days and then transferred in groups of 10 for IAP's of 0.25, 0.5, 1, 2, 4, 8, 24, 48, 72, and 96 hr. The inoculation threshold was about 1 hr, as 4% (1/25) of the plants became infected following the 1-hr treatment, and even with a 24 hr IAP, only 60% (15/25) of the plants became infected (Table 2).

To estimate the latent period and the period the virus was retained in the vector, first- and second-stage nymphs of pea aphids were given an AAP of 12 hr and transferred in groups of 10 to test seedlings. Test aphids were transferred to new test plants, in series, at 4-hr intervals to three consecutive test plants, then daily for 12 days. The minimum latent period (from the beginning of the AAP) was 16-20 hr because 1/20 (5%) of the test plants became infected in the second group of test plants. Fifty percent of the test plants became infected on the fifth series of test plants, and by that time 48 to 72 hr had lapsed from the beginning of the AAP. The virus was retained for the 12 days of the test, although the transmission rate declined after the first week. Several of the aphid groups transmitted virus after they had molted to the adult stage; therefore, this virus is retained in the vector through the molting process.

TABLE 1. Influence of acquisition period on the transmission of presumed pea leaf virus by *Acyrthosiphon pisum*

Acquisition period (hr)	Transmission[a] (%)
0.25	0
0.5	0
1	0
2	4
4	12
8	28
24	80
48	92
96	100

[a]Twenty-five plants and 10 aphids per test plant were tested per acquisition access period. The aphids were left on the test plants for 7 days.

TABLE 2. Effect of inoculation period on transmission of presumed pea leaf roll virus by *Acyrthosiphon pisum* after an acquisition access period of 3 days on infected broad bean

Inoculation period (hr)	Transmission[a] (%)
0.25	0
0.5	0
1	4
2	12
4	16
8	28
24	60
48	84
72	96
96	96

[a]Twenty-five plants and 10 aphids per test plant were used per inoculation access period.

Effect of antibiotic treatment on the disease.—Since infected plants showed the stunting and yellowing characteristic of many other yellowing diseases which have been shown in recent years to be caused by *Mycoplasma* (20) and aphids have been reported to transmit mycoplasmal disease agents (10, 22), we attempted to effect disease remission by treating infected broad bean with oxytetracycline-HCl (Terramycin) for 24 hr as a root application at 200, 100, and 50 μg/ml dilutions in 0.001 M phosphate buffer (pH 7.0). Repeated attempts showed that antibiotic treatment did not suppress symptoms and no difference in transmission rate was detected when treated and untreated plants were used as sources for aphid-transmission tests. In parallel experiments, in which aster yellows-infected aster plants were treated with antibiotics, remission of symptoms in most plants was noticed in 2-3 wk. This suggests that the disease is not caused by a mycoplasmalike organism (MLO). Recently, Kaiser and Schalk (16) reported from Iran that tetracycline-HCl treatment of foliage or roots of pea leaf roll-infected broad bean had no apparent effect on the disease. Our tests also showed that penicillin G and chloramphenicol did not suppress the symptoms in broad bean or delay the appearance of symptoms when they were applied before infective aphids were placed on them.

Appearance and distribution of viruslike particles in infected plant tissues.—Since the antibiotic treatment was negative and because no mycoplasmalike organisms (MLO) were observed in infected plants, attempts were made to detect virus particles in tissues from infected plants by electron microscopic examination. Abnormalities not detected in healthy broad bean plants

were noticed in some cells of the phloem in infected plants. These cells contained viruslike particles (VLP) which were electron-dense and roughly spherical in shape. We detected only a few VLP in infected broad bean plants; however, abundant VLP were observed in phloem of infected crimson clover, *T. incarnatum*. Cross sections of a vascular bundle showed only one or two cells in the phloem bundle containing VLP (Fig. 6). The VLP appeared densely stained and polyhedral in shape (Fig. 6, inset). No such particles were seen in healthy leaves.

The particles were scattered singly or in masses throughout the lumen of affected cells and were interspersed with remnants of cell organelles (Fig. 7 and 8). At higher magnification, the VLP were uniform in shape when viewed in cross section (Fig. 9) and measured 23 ± 1.5 nm in diameter. There also was some crystallization of VLP (Fig. 10). Because the internal structure of the cells was disrupted radically, it was not always possible to discern the type of phloem cells that contained the masses of VLP. Most affected cells appeared, however, to be necrotic sieve tube cells.

Similar particles also were seen in virus-infected *M. hispida* (Fig. 11), *T. subterraneum* 'Bacchus Marsh' and *P. sativum* 'Dart'.

DISCUSSION

The virus reported here has the same host range and symptoms, especially on broad bean, pea, lentil, chickpea, french bean, and crimson clover, as that reported for pea leaf roll virus (PeLRV) from Germany (25, 34), the Netherlands (5), England (4, 39), several other European countries (21, 26), and Iran (15). Like PeLRV, this virus is a persistent aphid-transmitted virus and is not transmitted by sap-inoculation. Our results showed that *A. pisum* was a more efficient vector than *M. persicae* and that *A. fabae* failed to transmit the virus under test conditions. These results are in agreement with the transmission data reported for PeLRV (3, 25, 35, 36, 37). All of the pea cultivars known to be resistant to PeLRV (7) that we tested proved to be nonhosts of our virus. This is strong evidence that the virus reported here is closely related to PeLRV and may be the same virus. Since PeLRV has not been purified, serological identification is not possible. Although host range, symptomatology, and transmission data are all that is available for diagnosis in this case, it is believed to be particularly reliable because of the senior author's prior experience with PeLRV in Germany (34, 35, 36, 37).

The name pea leaf roll virus is preferred because this was the name originally given by Quantz and Völk (25) and since it has been used by many other researchers (12, 15, 39, 42). Other synonyms used in the literature are bean leaf roll virus (1, 3, 4, 25), tip yellows virus of pea (40), pea top yellows virus (1, 5), pea yellows virus (26), pea virus 8 (26), and *Viciavirus chlorogenum* (25). Because of the similarity in names with potato leaf roll virus (PLRV), we prefer the abbreviation PeLRV (43) for pea leaf roll virus.

It is noteworthy that several viruses described in Australia, Japan, and New Zealand closely resemble PeLRV in symptoms induced in various legumes. Among these are subterranean clover stunt, soybean dwarf, yellow dwarf of pea, milk-vetch dwarf, and subterranean clover red leaf viruses.

The symptoms caused by our isolate on *P. vulgaris* are similar to those caused by subterranean clover stunt virus (11, 28) and pea leaf roll virus from Iran (15) and England (4).

Soybean dwarf virus (SDV) reported from Japan (33) is persistent in the aphid vector *Aulacorthum solani* (30), and the symptoms on broad bean and crimson clover (31) are similar to symptoms reported for PeLRV. However, SDV is not transmitted by *A. pisum*, *M. persicae*, or *A. craccivora* (32), whereas the virus under study as well as PeLRV are transmitted by these aphids (3, 15, 16, 34, 35, 36).

According to Inouye et al. (13), yellow dwarf of pea is caused by milk-vetch dwarf virus and is transmitted by *Aphis craccivora*. Again, the symptoms on peas and broad beans are yellowing, leaf-rolling, and dwarfing. However, unlike our virus, it was not transmitted by pea or green peach aphids.

Our virus also caused reddening of the older leaves of *T. subterraneum* similar to the symptoms described for subterranean clover red leaf virus (SCRLV) from Australia (2, 17) and New Zealand (43). Cockbain and Gibbs (4) also reported from England that their isolate of PeLRV caused reddening (brown to purple) of the older leaves and petioles of *T. subterraneum*. However, according to Kellock (17), SCRLV in Australia does not produce obvious symptoms on broad beans and peas, whereas Wilson and Close (43) were able to isolate SCRLV from field samples of these crops in New Zealand. The virus reported here is transmitted by *M. persicae* and *A. craccivora*, whereas these aphids failed to transmit SCRLV (43).

All of the above viruses seem to be similar, but differ slightly in host range, symptomatology, and vector specificity. At present, symptomatology and vector relationship data are the only criteria for identifying them. Possibly, some are strains of the same virus, but this cannot be determined until they have been purified and antisera produced for the testing of serological relations. So far only SDV (19, 32) and milk-vetch dwarf virus (24) have been purified.

According to the criteria enumerated by Jensen (14) to differentiate barley yellow dwarf virus, the viruslike particles we observed in infected tissues were distinguishable from other host cell components such as ribosomes, and the crystallization of the particles (Fig. 10), and absence of VLP in healthy plants is strong presumptive evidence of their viral nature. Localization and distribution of virus particles in plant cells is similar to those of potato leaf roll virus (18) and barley yellow dwarf virus (14). The present virus is phloem-restricted in all hosts examined, and the particles appear to be limited to a very few cells in the phloem region. Owing to the very low virus titer in the leaves, virus purification may be difficult.

In the absence of a method of purification and antiserum for this virus or the pea leaf roll virus, the present virus cannot be positively identified as pea leaf roll virus. On the basis of host reactions, aphid-transmission characteristics, and phloem-specific host cell localization of the virus, it appears to be a member of the luteovirus group (9) and similar to pea leaf roll virus. The luteovirus group is characterized by small polyhedral particles, persistent aphid-transmission and phloem-

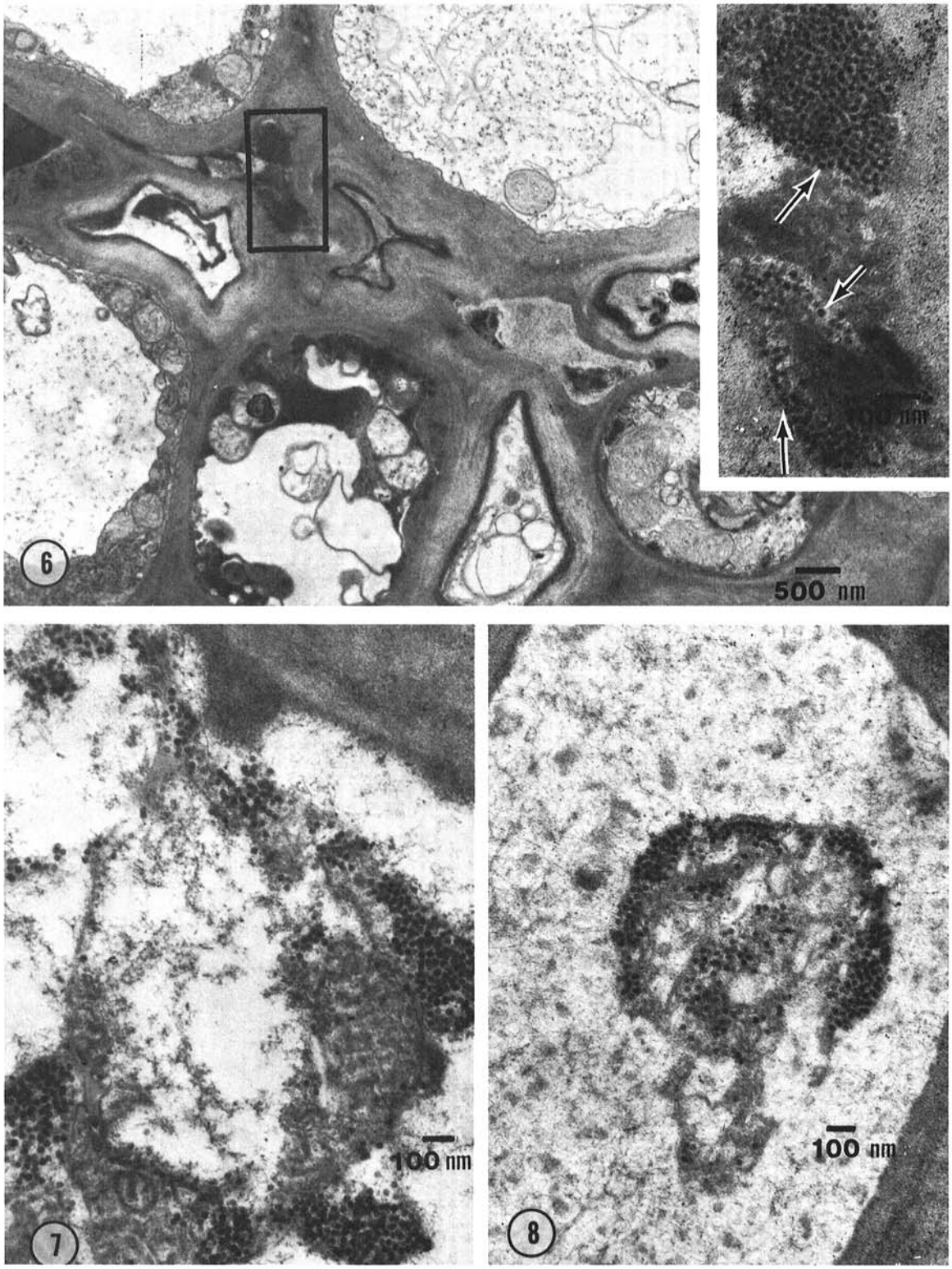
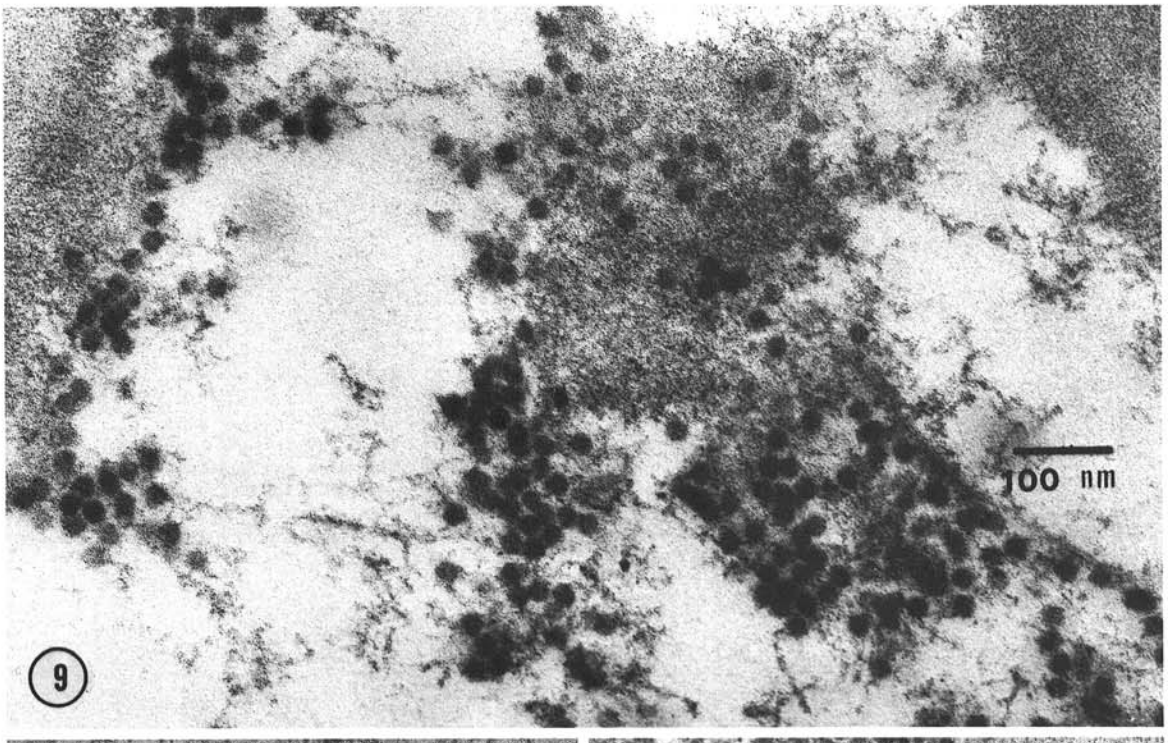
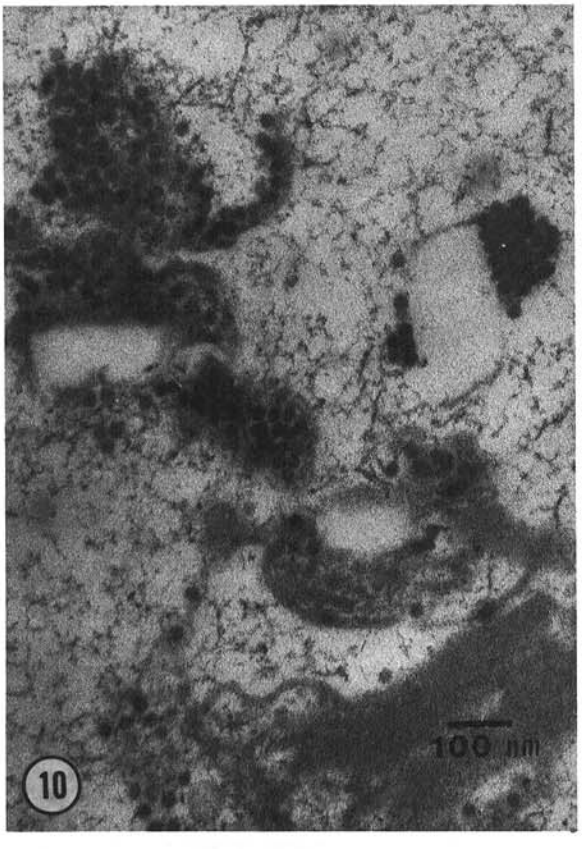


Fig. 6-8. Sections of infected crimson clover showing viruslike particles (VLP). **6)** A cross section of leaf showing portion of phloem. Only the areas indicated by the box contained identifiable, electron-dense VLP. Inset: An enlargement of the cell indicated in Fig. 6. Note the occurrence of clusters of electron-dense spherical particles (arrows) scattered throughout the necrotic sieve tube. **7)** Portion of a phloem cell with electron-dense spherical particles. **8)** A cross section of leaf phloem showing electron-dense masses of VLP.





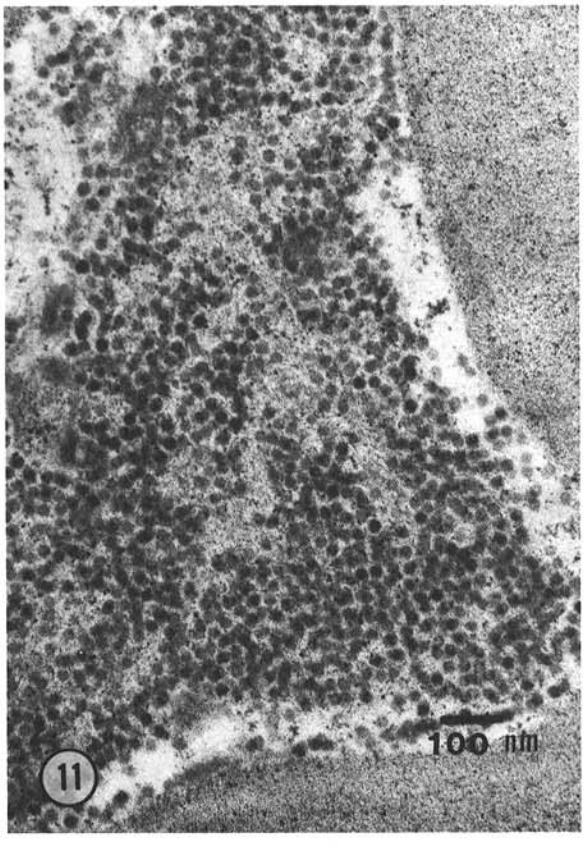


Fig. 9-11. Viruslike particles (VLP) in phloem of infected plants. **9)** VLP at a higher magnification. **10)** Small crystalline aggregates of VLP in infected crimson clover. **11)** VLP in a phloem cell of infected *Medicago hispida*. They occupied nearly all available space.

specific tissue reaction.

Duffus and Russell (8) suggested the possibility that beet western yellows virus (BWYV) probably is involved in several of the yellowing diseases. Since the virus under study failed to infect nonleguminous plants and is apparently restricted to legumes, it is unlikely that it is BWYV.

Since broad bean was used as the test plant in our tests and it is apparently insensitive to this virus, it was necessary to use 10 aphids per test plant to achieve experimentally satisfactory levels of transmission. In order to establish clearly the characteristics of the virus-vector relationship, it will be necessary to find a test plant that will develop clearly-defined symptoms following inoculation by single viruliferous aphids.

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