Ilar-Like Characteristics of American Plum Line Pattern Virus and Its Serological Detection in *Prunus*

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ABSTRACT

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Purified American plum line pattern virus (AmPLV) retained infectivity in 0.02 M EDTA, pH 6.2, for 12–24 mo at 2 C. In other than ammonium phosphate, purified virus lost most of its infectivity at pH 7.0 or 8.0 in 2 hr at 24 C. AmPLV and a number of other viruses lost infectivity within a few minutes in potassium phosphate buffer at pH 8.0 if it had been prepared for more than 1 mo. In sucrose density gradients prepared with EDTA, AmPLV showed four components with sedimentation coefficients of about 95, 100, 114, and 126S. Particles of these components averaged 26, 28, 31, and 33 nm in diameter. All four components appeared to contribute to infectivity. In agar gel double diffusion serological tests, AmPLV was detectable in extracts of herbaceous, but not Prunus, hosts. Enzyme-linked immunosorbent assays gave positive tests on symptomless Prunus leaves. Buds from dormant material gave clear-cut test results.

Mechanical transmission of plum line pattern virus was described by Kirkpatrick et al (7). The virus was later characterized and its host range was described by Kirkpatrick et al (10) and Paulsen and Fulton (13,14). Paulsen and Fulton (14) produced an antiserum to the virus and demonstrated that it was serologically unrelated to the other mechanically transmitted viruses that infect stone or pome fruit trees. Two of these, apple mosaic virus and a strain of Prunus necrotic ringspot virus (PNRV), had been demonstrated to cause line pattern symptoms in plum (2,5,7). Thus, Seneveratne and Posnette (16) applied the designation "American plum line pattern virus" (AmPLV) to distinguish it from other viruses that cause similar symptoms in plum.

Detection of AmPLV in Prunus hosts has depended on graft inoculation of indicator species (9). This is necessarily slow and may be uncertain if PNRV or prune dwarf virus is also present. For this reason, the prevalence of the virus and its pathogenic effects are not clear. It has been reported, however, that it intensifies the symptoms of prune dwarf virus (9).

Properties of AmPLV suggest that it may be an Ilarvirus (17). Several Ilarviruses (4,17) are markedly stabilized in vitro by EDTA. An objective of the present research was to determine whether AmPLV was also stabilized by EDTA. This would facilitate investigation of its morphology and multicomponent nature. Particle stabilization might also facilitate production of an antiserum with a sufficiently high titer to permit detection of the virus in tissue of *Prunus* by enzyme-linked immunosorbent assay (ELISA).

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MATERIALS AND METHODS

Plants were grown in 10.2-cm (4-in.)-diameter pots containing a composted soil/sand/peat (4:1:1, v/v) mixture. This mixture was steamed for 20 min if seeds were to be planted in it. Healthy plants were grown in the greenhouse at about 24 C without supplemental illumination. Plants inoculated for producing virus for purification were illuminated artificially for 18 hr per day.

AmPLV caused distinct necrotic lesions on leaflets of *Lupinus albus* L., but *Vigna cylindrica* Skeels was used as an assay host because it was more susceptible. In preliminary work, this host was placed in the dark for 24 hr before inoculation. In later work, darkening seemed unnecessary if sufficiently young plants were used (7 days from seeding). Experiments were arranged so there would be four treatments to assay, and each was applied to a different half-leaf of each *V. cylindrica* plant. Usually there were four replications of each treatment.

The culture of AmPLV was the same one used by Paulsen and Fulton (14). It had been stored in chemically dehydrated petunia tissue at 2 C for more than 3 yr. Of several hosts tried as sources of virus for purification, inoculated leaves of *Nicotiana megalosiphon* Heurck & Muell. and *N. occidentalis* Wheeler yielded more virus than the others. *N. occidentalis* was used for most of the work because infected *N. megalosiphon* tended to become necrotic when days were long. Leaves were usually harvested 10 days after inoculation. The purification method was the same as had been used previously (14) except that antigen to be injected for antiserum production was taken from the virus zones in density gradient tubes. The average yield of virus from *N. occidentalis*, based on an assumed extinction coefficient of 5, was 2.26 mg per 100 g of fresh tissue. Hosts found unsatisfactory for virus production were *N. debneyi* Domin., *Cucumis sativus* L., and *Vigna cylindrica* (systemically infected leaves.)

RESULTS

Virus stability. During purification, AmPLV infectivity was maintained best by 2-mercaptoethanol (2-ME). Polyvinyl-pyrrolidone-10 at 1% and sodium diethyldithiocarbamate at 0.02 M were included in some homogenization mixtures. Inclusion of sodium diethyldithiocarbamate usually resulted in a marked decrease in infectivity after several hours and it was not used routinely.

When purified virus was resuspended in 0.02 M EDTA, pH 6.2, infectivity was maintained for 12–24 mo at about 2 C. Thus, AmPLV was similar to a number of other Ilarviruses in being stabilized by EDTA.

A number of trials were done to test the effect of different buffers at 0.03 M, pH 8.0, on the infectivity of purified AmPLV at an A_{260} of 0.03. Ammonium phosphate resulted in greater infectivity than any of the other buffers (Table 1) and maintained infectivity at room temperature much better than sodium phosphate buffer. Infectivity in potassium phosphate was very low at pH 8.0, but satisfactory at pH 7.0.

Because the effect of potassium phosphate was unexpected, additional trials were made attempting to clarify its role. In freshly prepared potassium phosphate at pH 8.0, AmPLV was nearly as infective as in sodium phosphate buffer. After 3–4 wk, however, the potassium phosphate buffer at pH 8.0 rapidly inactivated AmPLV. The pH of the buffer had not changed detectably. Type of storage container (soft glass, Pyrex, cellulose nitrate, polyethylene, or polycarbonate) did not alter the development of inhibitory properties. Also, boiling aged potassium phosphate buffer or filtering it through activated charcoal had no effect. Buffer made from three lots of crystalline potassium phosphate and allowed to stand for 1 mo inactivated AmPLV. Buffer (pH 8.0) with 0.02 M sodium phosphate and 0.01 M aged potassium phosphate was nearly as detrimental as 0.03 M potassium phosphate.

TABLE 1. The effect of buffer composition and pH and time following dilution on the infectivity of purified American plum line pattern virus[a]

Buffer[b]	pH	Avg. lesions after 15 min	Avg. lesions after 2 hr
Sodium phosphate	8.0	146	5
Potassium phosphate	8.0	13	0
	7.0	143	0
Ammonium phosphate	8.0	172	223
Tris buffer	7.0	40	0
Sodium borate	8.0	88	4
Sodium citrate	8.0	1	...

[a]Virus concentration 0.01–0.02 mg/ml.
[b]Buffer concentration, 0.03 M.

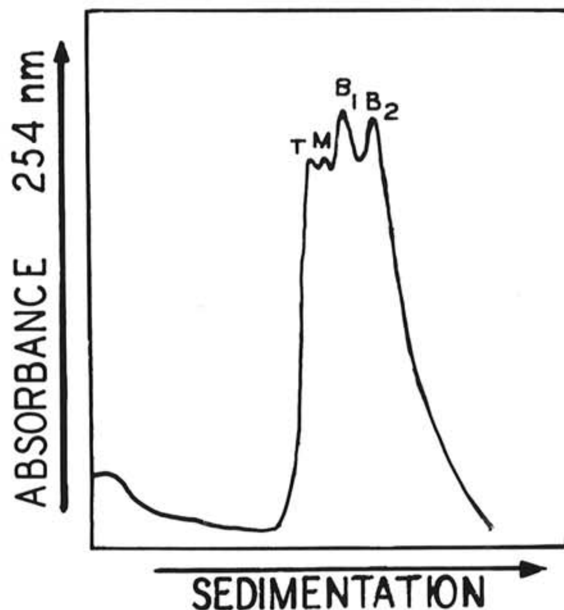

Fig. 1. Ultraviolet scan of a sucrose density gradient tube containing American plum line pattern virus. The sucrose was made up in 0.03 M EDTA, pH 6.2, and centrifuged 4 hr at 25,000 rpm in an SW 25.1 rotor.

To determine whether sensitivity to aged pH 8.0 potassium phosphate buffer was a property peculiar to AmPLV, the infectivities of a number of other viruses, purified or in fresh sap, in this buffer were compared with infectivities in aged, pH 8.0, sodium phosphate buffer. All test plants were inoculated within 15 min of virus dilution.

The following viruses were less than 8% as infectious at pH 8.0 in aged potassium phosphate as in sodium phosphate: alfalfa mosaic, tobacco streak, prune dwarf, Prunus necrotic ringspot, Tulare apple mosaic, cucumber mosaic, tomato ringspot, tobacco rattle, and tomato bushy stunt. In contrast, the following viruses were more than 85% as infectious at pH 8.0 in aged potassium phosphate as in sodium phosphate: tobacco mosaic, Southern bean mosaic, turnip mosaic, tobacco necrosis, potato yellow dwarf, and potato virus X. There was no correlation of the effect of potassium phosphate with the assay host species. Sensitivity to aged potassium phosphate buffer was thus not a property peculiar to AmPLV.

Multicomponent nature of AmPLV. The stabilizing effect of EDTA on AmPLV suggested that virus morphology might be preserved. In density gradient centrifugation, therefore, sucrose solutions were made up in 0.03 M EDTA, pH 6.2. When purified virus was centrifuged in an SW 25.1 rotor for 4 hr in linear gradients ranging 7–25% sucrose, four peaks were resolved in ultraviolet scans of the tubes (Fig. 1).

Virus component peaks were collected separately and components from three or six gradient tubes were pooled. The virus was pelleted by centrifugation for 4 hr in a Spinco 40 rotor and, when resuspended in 0.03 M EDTA, again centrifuged in density gradient tubes. Infectivity tests were made after four cycles of density gradient centrifugation, but because the four peaks

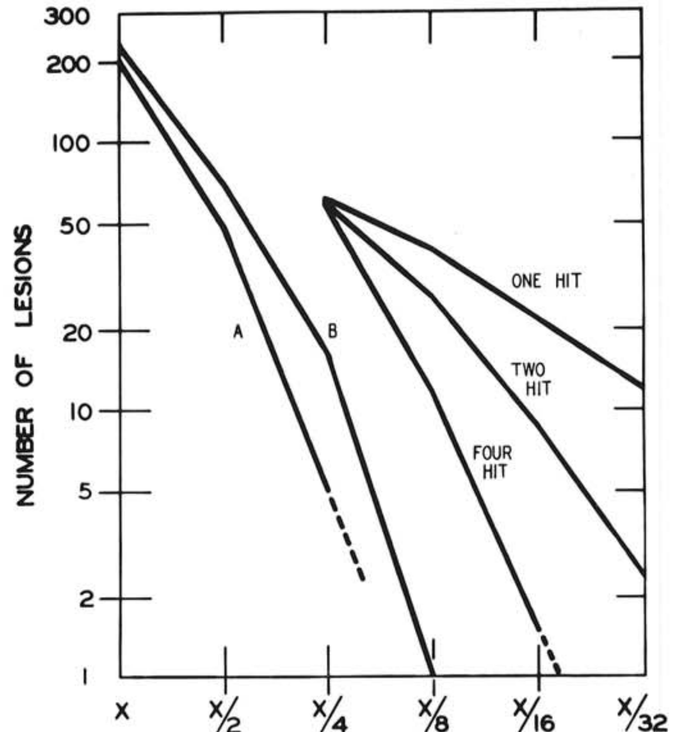

Fig. 2. Infectivity dilution curves (from assay on *Vigna cylindrica*) of American plum line pattern virus **A,** in tissue extract, or **B,** purified virus. Theoretical one-, two-, and four-hit curves are positioned to the right. Relative dilutions are expressed as fractions (x, x/2, x/4, ...).

sedimented close to each other, separation of the components may have been incomplete. Furthermore, the specific infectivity of different preparations of virus varied, so that comparisons between preparations were unreliable.

Single components were essentially noninfectious on *V. cylindrica*, producing only one or two lesions on four half-leaves when inoculated at concentrations ranging from 2 to 10 μg/ml. Pairs of components inoculated at the same total concentration gave averages of one to 10 lesions on four half-leaves. Lesion counts (four half-leaves) for three- and four-component mixtures were $T+M+B_1$, 25; $T+M+B_2$, 13; $T+B_1+B_2$, 54; $T+M+B_1+B_2$, 117. Each of the four component preparations seemed to contribute to infectivity. The distribution of nucleoprotein particle types of AmPLV resembled that of prune dwarf virus (4), but differed in that the four types seemed to occur in approximately equal amounts.

Dilution-infectivity trials with AmPLV were run with twofold dilution steps, in 0.03 M pH 8 phosphate buffer with purified virus and with that buffer containing 0.02 M 2-ME for crude extracts. The slopes of the resulting curves were very steep, both with purified virus and with sap extracts (Fig. 2). Both curves are consistent with the evidence that multiple particle types are necessary for infection.

Particle morphology. Virus from the four zones of the density gradient tubes was prepared for electron microscopy by fixation for 1 hr in 0.5% glutaraldehyde before negative staining with 1% neutral sodium phosphotungstate. Grids were examined in a JEM 7 electron microscope.

Particles from the various zones did not differ appreciably in morphology (Fig. 3). Measurements of 100 particles of each type were made in two directions at right angles. The average diameters of particles were top, 26 nm; middle, 28 nm; bottom-1, 31 nm; bottom-2, 33 nm. A few particles were asymmetric, but when an "asymmetry index" was calculated for each particle type (greatest average diameter divided by the smallest average diameter), the index for top was 1.08; middle, 1.09; bottom-1, 1.3; bottom-2, 1.23. Thus, the degree of morphological difference among particle types was not as great as that found with prune dwarf (4) or Prunus necrotic ringspot viruses (11).

Sedimentation rates of the particle types of AmPLV were estimated by comparing them with those of tobacco streak virus by centrifuging both viruses simultaneously in sucrose density gradients. S values for the four components of AmPLV were estimated as top, 95S; middle, 100S; bottom-1, 114S; and bottom-2, 126S. Unfractionated virus from sucrose density gradients had an $A_{260/280}$ nm absorbance ratio of 1.67.

Serological detection of AmPLV. Antigen for rabbit injection consisted of virus from sucrose density gradients concentrated by centrifugation. Injections were made intramuscularly twice a week with 1.8–2.0 mg of virus emulsified in 1 ml of Freund's incomplete adjuvant. Bleedings were made 25, 32, 39, and 47 days after the initial injection. Serum of these bleedings had titers of 1:640, 1:1,280, 1:1,280, and 1:2,560 as determined by microprecipitin tests.

In developing antiserum, the schedule of two injections a week was used because it had been found efficient with several unstable Ilarviruses (3). In comparing the antigenicity of AmPLV with a number of these and other viruses for which antisera had been produced similarly, it was apparent that antiserum titers induced were approximately the same for similar amounts of antigen injected of Prunus necrotic ringspot, apple mosaic, prune dwarf, tobacco streak, tomato aspermy, and tobacco mosaic viruses.

AmPLV was readily detectable by agar gel double diffusion tests with sap from systemically infected *V. cylindrica* or *N. occidentalis*. A single sharply defined zone of precipitation formed between antigen and antibody wells. No reaction was obtained, however, with sap or crushed tissue of infected *Prunus mahaleb* L. or *P. persicae* (L.) Batsch.

Enzyme-linked immunosorbent assay (ELISA) was done with the 1:2,560-titer antiserum and the double antibody sandwich method of Clark and Adams (1). The alkaline phosphatase-*p*-nitrophenyl phosphate system was used. Reactions were compared by measuring absorbance at 405 nm of 1:10 dilutions of well contents. In most trials, infected tissue of *Prunus* was ground in sufficient extraction buffer to give a 1:10 dilution (w/v). In some trials, a small amount of coarse Al_2O_3 was added to tissue as an abrasive during grinding. This did not affect the results.

AmPLV was readily detectable in infected, symptomless leaves of greenhouse-grown *P. mahaleb* and *P. persicae*. Absorbance readings were 6–7 times those of healthy leaves. Purified virus at 4 ng/ml (the lowest concentration tested) gave readings 4.2 times those of controls. For detection by inoculation to *V. cylindrica*, about 1,000 times this amount of virus was required.

Tests with senescent leaves of plum, peach, and *P. mahaleb* gave erratic results. Some infected field material of plum, collected in September, gave absorbance readings about twice those of healthy leaves. Other senescent leaf material did not give readings significantly different from healthy controls. Preparations of such leaves at 1:5 in extraction buffer, too viscous to be pipetted, resulted in no higher absorbance readings than 1:10 preparations. The leaf disk method as used by Romaine et al (15) was not effective.

A somewhat better source of test material than senescent leaves of *Prunus* was the green inner bark of twigs, which usually gave readings 2–3 times those of healthy bark. Entire buds of dormant infected twigs appeared to be reliable test tissue and resulted in readings 4–6 times those of uninfected buds.

Much of the healthy Prunus material gave rather high absorbance readings. In an effort to minimize this effect, a method suggested by Hill et al (6) was tried. This involved incubating wells with ovalbumin (Sigma A-5253) at 1 mg/ml after coating with antiviral gamma globulin and washing. The absorbance readings with healthy tissue were not significantly lowered by this treatment, but readings with infected tissue were about double those on plates not treated with ovalbumin. Thus, the secondary coating with ovalbumin was used in subsequent tests.

Some of the Prunus material tested was also infected with Prunus necrotic ringspot or prune dwarf viruses. These did not interfere with detection of AmPLV. It is apparent that ELISA is a rapid and reliable method for detecting AmPLV in either growing or dormant tissue of *Prunus* and in the presence of other viruses.

DISCUSSION

The instability in sap and the multicomponent nature of AmPLV are typical characteristics of Ilarviruses. The stabilization of infectivity by EDTA also is a property shared by many (although

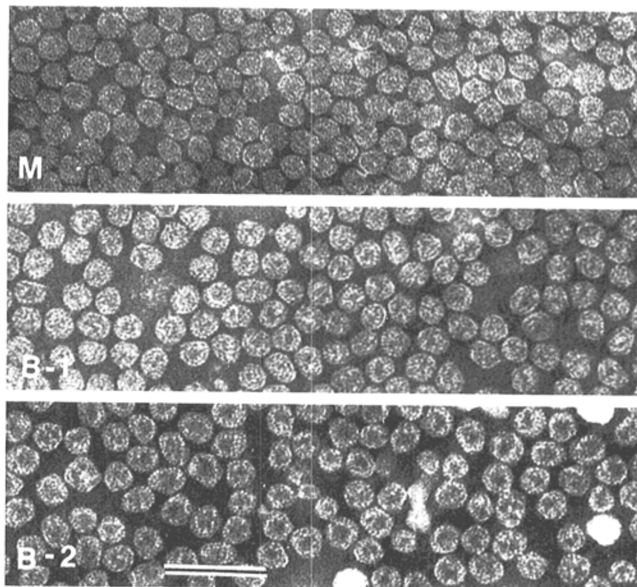

Fig. 3. Purified American plum line pattern virus negatively stained with neutral potassium tungstate. M, middle zone; B-1, first bottom zone; B-2, second bottom zone from sucrose density gradients. Bar represents 100 nm.

not all) Ilarviruses (17). Morphologically, AmPLV is more similar to Prunus necrotic ringspot and tobacco streak viruses (12) than to prune dwarf virus (4) in that nucleocapsids differ in size rather than morphology. These characteristics suggest that AmPLV should be grouped with Ilarviruses.

Difficulty in separating the nucleoprotein components of AmPLV by density gradient centrifugation prevented a clear demonstration of the particle types required for infectivity. Some preparations showed shoulders on leading or trailing peaks in sucrose density gradients, suggesting that more than four components were present. Thus, it is possible that preparations thought to contain mostly single components actually contained additional undetected components. On the other hand, the proportion of virus sedimenting at any one rate varied somewhat with different preparations, and it was not clear whether partial degradation of nucleoprotein particles was occurring when more than four components were detected.

Serological evidence indicated that the amount of virus in infected tissue of *Prunus* was very low. This is reflected in the difficulty in transmitting the virus by mechanical inoculation, which is usually possible only with young leaves showing symptoms, or by concentrating the virus in extracts of *Prunus* by centrifugation (8). It is possible that certain parts of trees may at times contain either no virus, or so little that ELISA would not detect it. Thus, in interpreting results of ELISA, particularly with dormant material, less emphasis should be placed on negative than on positive tests. This qualification, however, might also apply to transmission assays for the virus if certain parts of trees are virus-free, or become so at certain seasons.

LITERATURE CITED

1. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay (ELISA) for the detection of plant viruses. J. Gen. Virol. 34:475-483.
2. Fulton, R. W. 1965. A comparison of two viruses associated with plum line pattern and apple mosaic. Zast. Bilj. 85-88:427-430.
3. Fulton, R. W. 1968. Serology of viruses causing cherry necrotic ringspot, plum line pattern, rose mosaic, and apple mosaic. Phytopathology 58:635-638.
4. Halk, E. L., and Fulton, R. W. 1978. Stabilization and particle morphology of prune dwarf virus. Virology 91:434-443.
5. Gilmer, R. M. 1956. Probable coidentity of Shiro line pattern virus and apple mosaic virus. Phytopathology 46:127-128.
6. Hill, J. H., Bryant, G. R., and Durand, D. D. 1981. Detection of plant viruses by using IgG in ELISA. J. Virol. Meth. 3:27-35.
7. Kirkpatrick, H. C. 1955. Infection of peach with apple mosaic virus. Phytopathology 45:292-293.
8. Kirkpatrick, H. C., Cheney, P. W., and Lindner, R. C. 1964. Mechanical transmission of plum line pattern virus. Plant Dis. Rep. 48:616-618.
9. Kirkpatrick, H. C., and Fulton, R. W. 1974. Plum line pattern. Pages 166–175 in: Virus Diseases and Noninfectious Disorders of Stone Fruits in North America. U.S. Dep. Agric. Handb. 437. Washington, DC.
10. Kirkpatrick, H. C., Lindner, R. C., and Cheney, P. W. 1967. A selective host range for certain mechanically transmitted stone fruit viruses. Plant Dis. Rep. 51:786-790.
11. Loesch, L. S., and Fulton, R. W. 1975. Prunus necrotic ringspot virus as a multicomponent system. Virology 68:71-78.
12. Loesch-Fries, L. S., Halk, E. L., and Fulton, R. W. 1977. Ilarvirus (necrotic ringspot) group, subgroup B. Pages 371–375 in: The Atlas of Plant and Insect Viruses. K. Maramorosch, ed. Academic Press, New York.
13. Paulsen, A. Q., and Fulton, R. W. 1968. Hosts and properties of a plum line pattern virus. Phytopathology 58:766-772.
14. Paulsen, A. Q., and Fulton, R. W. 1969. Purification, serological relationships and some characteristics of plum line-pattern virus. Ann. Appl. Biol. 63:233-240.
15. Romaine, C. P., Newhart, S. R., and Anzola, D. 1981. Enzyme-linked immunosorbent assay for plant viruses in intact leaf tissue disks. Phytopathology 71:308-312.
16. Seneveratne, S. N. deS., and Posnette, A. F. 1970. The identification of viruses isolated from plum trees affected by decline, line pattern and ringspot diseases. Ann. Appl. Biol. 65:115-125.
17. Van Vloten-Doting, L., Francki, R. I. B., Fulton, R. W., Kaper, J. M., and Lane, L. C. 1981. Tricornaviridae—a proposed family of plant viruses with tripartite, single-stranded RNA genomes. Intervirology 15:198-203.