Component Ratio Differences in Strains of Alfalfa Mosaic Virus

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ABSTRACT

Viruses isolated from four woody ornamentals, *Hebe* (hybrid clone 'Co-ed'), *Ilex cornuta* 'Rotunda', *Viburnum opulus,* and *V. tinus,* were shown to be related to alfalfa mosaic virus (AMV) by serology and particle morphology. This is the first known report of AMV isolation from naturally infected *Hebe, I. cornuta,* and *V. tinus.* The isolates from the two *Viburnum* species are considered distinct strains; due to lack of measurable differences, the isolates from *Hebe* and *I. cornuta* are considered to be a single strain. This latter strain was distinguishable from each of the other two.

Each of the isolates was partially purified from tobacco by polyethylene glycol precipitation, then centrifuged through sucrose density gradients. Three prominent bands were visible in all cases. When gradients were removed through a continuously recording spectrophotometer measuring at 260 nm, three strong absorption peaks corresponding to the three visible bands appeared in the same place in the spectrum for each isolate, though the relative peak heights varied. The isolates from *Hebe* and *I. cornuta* produced similar patterns. Each of the strains tested, including one other known strain of AMV, was identifiable by its absorption pattern. This is suggested as a method of AMV identification and of strain differentiation. Phytopathology 61:1159-1163.

The basis for identifying a virus as alfalfa mosaic (AMV), and determining whether it is a new or previously described strain of AMV, varies with the report. Numerous criteria have been used, either singly, or in various combinations: thermal inactivation point (6), dilution end point (6), host range (11), symptoms on specific indicator hosts (6), serological relationships (1), particle morphology (4), and cross protection (2). As is the case with other viruses, the subjectivity of designating certain isolates of AMV as new strains or as additional sources of a known strain leaves much to be desired. Strain comparisons involving host reactions, which may be valid when restricted to differences between treatments within a given test, lose reliability when extended to tests run at different times or by other workers. This is largely due to differences in techniques between workers, plus differences in growing conditions at different times and places.

Four virus isolates displaying characteristics ascribed to alfalfa mosaic virus have been identified (13). The viruses had been isolated from four woody ornamentals, *Ilex cornuta* Lindl. 'Rotunda', *Viburnum opulus* L., *V. tinus* L., and *Hebe* 'Co-ed' (*Hebe* hybrid, horticultural clone, Pat. No. 2296, Select Nurseries, Inc., Brea, Calif.). This study was undertaken to study the strain relationships of these isolates of AMV.

MATERIALS AND METHODS.—The isolate of AMV used as a standard was obtained from P. R. Desjardins, Department of Plant Pathology, University of California, Riverside, and designated AMV-R. Each isolate was subcultured twice from single local lesions on cowpea (*Vigna sinensis* Savi). All isolates used gave necrotic local lesions on inoculated leaves of tobacco (*Nicotiana tabacum* L. '*Xanthi nc*'), and then systemically invaded the plant. The isolates were propagated in tobacco; local lesion assays for all strains were carried out on cowpea. The indicator plants tested, along with symptoms observed, are listed in RESULTS. Only those hosts contributing to strain differentiation are listed.

The method of virus purification employed was a variation of the polyethylene glycol precipitation method of Gooding & Hebert (5). Leaf tissue was frozen for 4-24 hr, then ground in a chilled mortar with an equal weight of 1% K_2HPO_4 buffer containing a reducing agent, 0.1% K_2SO_3, to prevent oxidation. This amount of K_2SO_3 was slightly more than the minimum required to prevent visual darkening of the homogenate. All subsequent steps were kept cold. The homogenate was filtered through muslin, then centrifuged at 22,000 *g* for 20 min. Polyethylene glycol 6000 (PEG) was added to the supernatant, at the rate of 4.0 g/100 ml of supernatant, and the mixture stirred for 15 min. This was centrifuged 15 min at 5,000 *g*. The supernatant was discarded and the pellet resuspended in buffer equal in volume to 1/10-1/5 the volume of the original supernatant, using either 0.01 M K_2HPO_4 or 0.0003 M Beckman borate buffer, pH 9.2. Resuspension took place overnight, and the preparation was centrifuged at 10,000 *g* (10,000 rpm) for 20 min. The final step of Gooding & Hebert's method of further virus purification (using NaCl) was not used, as it resulted in a severe loss in infectivity.

Antisera were produced against all isolates, and used in Ouchterlony agar-diffusion serology (10).

Electron microscopy was done, using an RCA EMU-3F. The preparations were fixed in 2% Formalin for 10 min, then either shadowed with uranium or negatively stained with 1% phosphotungstic acid (PTA), pH 7.2, and in both cases coated lightly with carbon.

Preliminary separations of AMV components by density-gradient centrifugation indicated that this might be a method of strain identification. Tests were made using samples purified by the PEG precipitation method. The gradient consisted of 6.0-ml layers each

of 5, 10, 15, 20, and 25% (w/v) sucrose in 0.01 M K_2HPO_4. The gradients were allowed to equilibrate without mixing for 24-30 hr at 4 C, after which the virus sample was layered on top. Sample volume consisted of 0.2-0.5 ml, depending upon concentration of the virus in the sample. Centrifugation was done in a swinging-bucket rotor at 50,000 *g* for 5 hr. After centrifugation, the tubes were viewed against a black background with a narrow-beam overhead light. The samples were usually removed via a glass capillary tube (1-mm inside diameter) supported at the top of the tube by a cork, and extending to the bottom of the tube. This capillary tube was connected to plastic capillary tubing (1-mm inside dimension), which in turn was connected to a Beckman Flowthru cuvette inside a Beckman DG-G spectrophotometer, measuring at a wavelength of 260 nm. The outlet of the cuvette was in turn attached to another plastic capillary tube of the same diameter which fed through a peristaltic pump and then into collection bottles. The samples were removed at the rate of 4 ml/min.

To test the effect of the buffer used on density-gradient profiles, various buffers were used both in resuspending the PEG pellets and in making up the sucrose gradients. Two buffers were used to resuspend the pellets: either 0.0003 M sodium borate, pH 9.0, or 0.01 M potassium phosphate, pH 8.5. The following buffers were used to make the gradient: 0.0003 M sodium borate, pH 7.0, pH 8.0, pH 9.0, or 0.01 M sodium phosphate, pH 9.0. The isolate from *V. opulus* was used for all these tests.

Virus in tobacco plants was extracted by the PEG precipitation method at 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, 13, and 28 days after the plants were inoculated with the *V. opulus* isolate. Density-gradient profiles were made of the preparations to determine if the profiles, and thus the component ratios, varied with time after inoculation.

RESULTS.—*Serology.*—Antiserum produced against each of the isolates reacted positively with its own antigen, and with each of the other four isolates in agar-diffusion serology. In addition, AMV antiserum obtained from K. A. Kimble (Davis, Calif.) reacted positively with each of the five isolates tested.

Particle morphology.—Partially purified preparations of each of the isolates had particles corresponding to those described for AMV (2). Uranium-shadowed or PTA negatively stained preparations had spheres and bacilliform rods of various lengths.

Host reactions.—The *Hebe* and *I. cornuta* isolates were easily distinguishable from any of the other three isolates if inoculated at the same time to comparable tobacco plants, though they were indistinguishable from each other. The symptoms were much more severe than with any of the others; stunting up to about 60% was common, and some leaves were so distorted as to be hardly recognizable as tobacco. Leaves had a mottling of yellow mixed with areas of darker-than-normal green.

The two *Viburnum* isolates are considered distinct strains based on reactions on *Petunia hybrida* Vilm., variety Happy Talk, Rose Pink (the *V. opulus* isolate produced local lesions; the *V. tinus* isolate did not); on *Cucumis sativus* L. (the *V. opulus* isolate produced a mottle of extremely dark green mixed with very light green, as well as severe stunting; the *V. tinus* isolate produced only a very mild mottle); and by the reaction on *Datura stramonium* (the *V. opulus* isolate produced a marked systemic glazed-leaf appearance; the *V. tinus* isolate produced little or no glaze).

AMV-R infection on cowpea produced small (less than 1-mm diam) reddish-brown local lesions; the *V. tinus* isolate produced larger (2- to 3-mm diam) gray-brown local lesions. Except for this difference, and the generally more severe symptoms of the *V. tinus* isolate in tobacco, the two were not easily distinguishable.

Sucrose density-gradient centrifugation.—The density gradients in the tubes were viewed with an overhead light following the 5-hr separation run. Three distinct, light-scattering bands were visible with all isolates tested, and are designated b_1, b_2, and b_3, respectively, from the top. Above these three bands were one or two other faint bands which had poorly delimited boundaries. The absorption peaks corresponding to the visible bands b_1, b_2, and b_3 occurred in almost exactly the same place with each isolate, although the relative peak heights varied. The various strains could be distinguished on the basis of the absorption pattern of these three bands. The patterns produced by *I. cornuta* and *Hebe* 'Co-ed' isolates were indistinguishable from one another; all other isolates gave distinct patterns. Typical patterns of these three bands for the isolates are shown in Fig. 1-A, B, C, D. The absorption pattern produced by healthy tobacco run through the same purification scheme had no peaks in the area occupied by b_1-b_3, but did have 1-3 absorption peaks in the gradient above this area, as shown in Fig. 1-F. The extracts from virus-infected plants produced 3-5 peaks in the upper area (above b_1); however, for reasons not understood, these varied considerably from one separation to the next for any given isolate, and hence are omitted from Fig. 1-A, B, C, D. Figure 1-E illustrates an entire spectrum for the *V. opulus* isolate. It is included only to indicate relative positioning of the various peaks, and is not to be considered typical with regard to peak heights above b_1.

Effect of buffer.—Using potassium phosphate (pH 8.5) as the liquid in a sucrose density gradient, the *V. opulus* isolate produced a peak pattern as in Fig. 1-D. Gradients made up in sodium borate buffer (pH 9.0) induced 2 major changes: (i) a small extra peak

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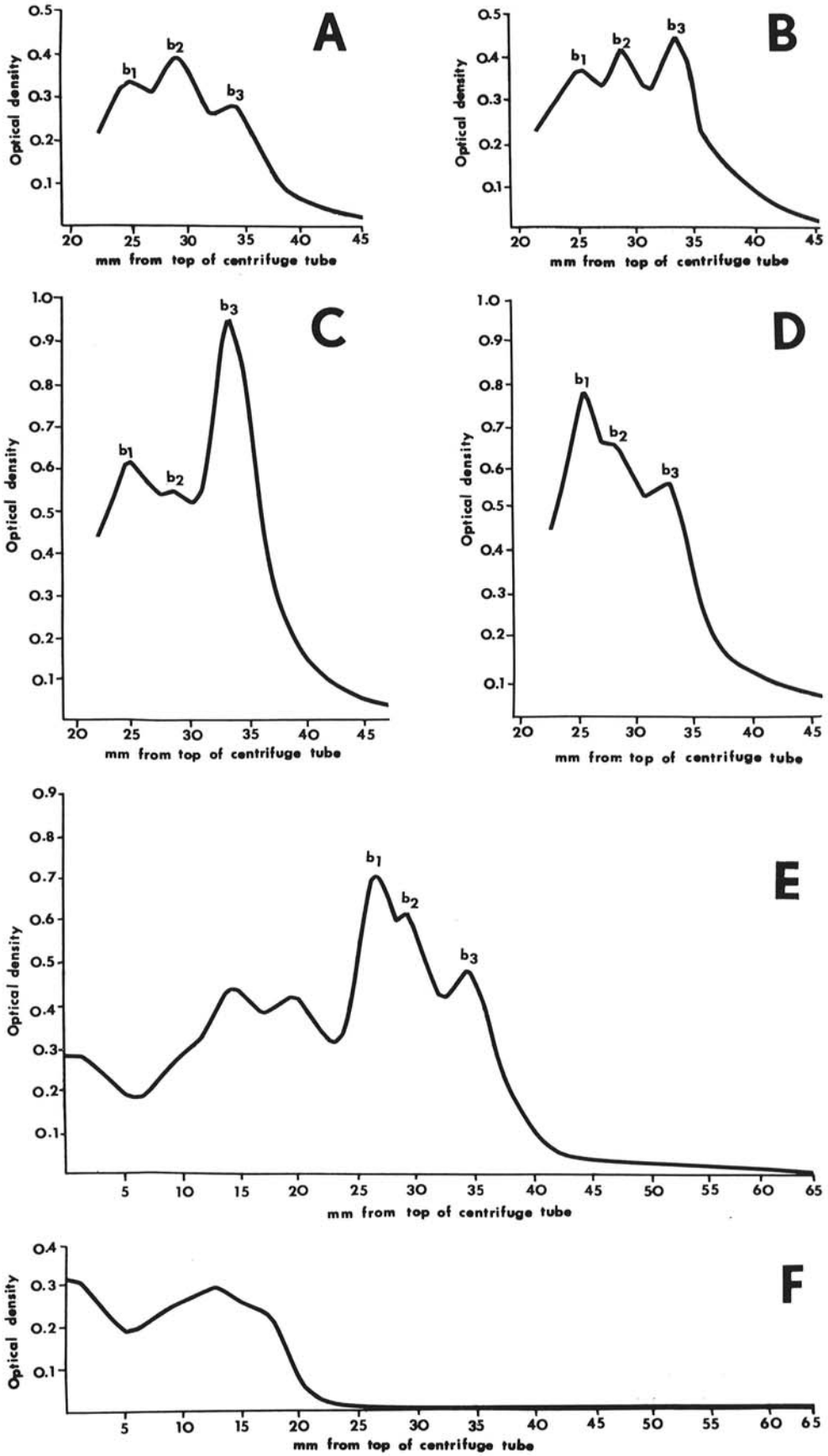
Fig. 1. A, B, C, D) Absorption patterns of b_1, b_2, and b_3 by isolates of AMV. **A)** AMV-R. **B)** Isolate from *Viburnum tinus*. **C)** Isolates from *Hebe* 'Co-ed and *Ilex cornuta*. **D)** Isolate from *V. opulus*. **E)** Entire absorption pattern produced by isolate from *V. opulus*. **F)** Entire absorption pattern produced by comparable extract from healthy tobacco.


A
B
C
D
E
F
Optical density
mm from top of centrifuge tube
b1
b2
b3

appeared below b_3; and (ii) the height of peak b_2 was increased. Healthy tobacco extract run on the same type of gradient using borate buffer had an absorption peak in the area occupied by b_2, and the increase in b_2 is assumed to be an additive effect of host and virus components. The extra peak, appearing below the b_3 area with the extract from virus-infected tobacco, did not appear with healthy tobacco extract. No explanation can be offered for this peak. On the assumption that the higher pH effected these changes, 0.0003 M sodium borate was used to make up gradients, at pH 9.0, 8.0, and 7.0. No differences were found among these three.

The possibility that the sodium ion had an effect was tested by making up gradients in 0.01 M sodium phosphate, pH 9.0; 0.01 M sodium citrate, pH 8.6; or 0.01 M potassium phosphate, pH 8.5. The two phosphate buffers gave the same effects. The citrate buffer gradient had only a small effect: the components (host and virus alike) moved 8-10% slower in the citrate than in the phosphate.

Resuspending the PEG pellets in 0.01 M potassium phosphate (pH 8.5) versus 0.0003 M sodium borate (pH 9.0) made no obvious difference when the components were separated on 0.01 M potassium phosphate sucrose gradients.

Effect of time after inoculation.—No evidence of absorption peaks b_1, b_2, or b_3 was found for the first 3 days after inoculation. On the 4th day, peak b_1 and peak b_2 were detected. By the 6th day after inoculation, all three peaks were present, and by the 7th day they were present in a pattern similar to Fig. 1-D. This pattern held true at 4 weeks. No extractions were made beyond 4 weeks after inoculation.

Component infectivity.—Several infectivity assays were made of the components of the strains from *V. opulus, V. tinus,* and *I. cornuta.* While no attempt was made to assure complete component separation, the tests did indicate (i) that component b_1 is only weakly, if at all, infectious; and (ii) that component b_3 is more infectious than b_2, using equal volumes of each component as they were separated on density gradients. Components b_1, b_2, and b_3 of the strain from *V. opulus* contributed 1, 47, and 52%, respectively, of the total local lesions produced by that strain; the components of the strain from *V. tinus* contributed 0, 20, and 80%, respectively, of the total local lesions produced by that strain; and the components of the strain from *I. cornuta* produced 0, 34, and 65%, respectively, of the total local lesions produced by that strain.

DISCUSSION.—The isolates from *Hebe, I. cornuta, V. opulus,* and *V. tinus* are serologically related to the known strains of AMV used. Based on this, and on the similarity of particle morphology, the four new isolates are all considered to be AMV. For reasons listed in the introduction, it is not possible to tell if any of the new isolates worked with here are previously unreported strains, or whether they correspond to strains already published.

The isolates from *Hebe* and *I. cornuta* are considered to be a single strain, based on a lack of any measurable differences between the two. The severity of symptom expression on tobacco infected with this strain readily distinguished it from the other strains tested. Symptom expression as a basis for strain differentiation is not always a good criterion; however, since these tests were done using equivalent plants, grown under identical conditions, and inoculated at about the same time and in the same manner, and because of the wide differences in disease severity, it is used here as an aid in strain differentiation. In the same manner, the *V. opulus* isolate is distinguishable from the *V. tinus* isolate by the more severe symptoms of cucumber infected with the former. Based on this and other differences listed in RESULTS, the two *Viburnum* isolates are considered to be separate strains.

Schmelzer (12) isolated AMV from *V. opulus.* No report has been found of AMV isolation from naturally infected *V. tinus, I. cornuta,* or *Hebe*; therefore, these last three named are considered to be new listings of natural hosts of AMV.

The breakdown into strains is reinforced by the absorption patterns from density-gradient separations. It is felt that the consistency of absorption patterns found for each strain make the differences in particle density a more objective and reliable means of strain separation than symptom differences. The particle separations done with the *V. opulus* strain were performed over a 6-month period with wide differences in environmental conditions. The same basic peak pattern held true for b_1, b_2, and b_3 throughout the period, whereas symptom expression in inoculated tobacco varied considerably during the same period. Thus, these differences are reproducible and probably a characteristic of the strain.

The possibilities for extending strain identification based on absorption peak patterns is limited by the number of peak-height sequences which are possible. Assuming height changes between each two peaks, only six combinations of relative peak heights are possible for three peaks; viz., 1-2-3, 1-3-2, 2-1-3, 2-3-1, 3-1-2, and 3-2-1. If one includes the possibility of equal peaks, the number is increased to 13. Since there are reported to be as many as 44 strains (9), this approach is clearly inadequate to identify them all. It would be of interest, however, to examine them all in this manner and then to try groupings based on similarities in sedimentation profiles. These groupings could then be compared for host range and symptoms.

The different fractions (b_1, b_2, and b_3) appear to have exactly the same sedimentation rate for each strain, suggesting that sedimentation profiles may be useful in identifying a new virus as AMV. Since AMV often occurs in combination with other viruses (7), it may be possible to show AMV present and perhaps even separate it out. On the other hand, if two or more AMV strains occur together, the method would be of little help in either determining that or in separating the two, unless the method is combined with other techniques, such as single-lesion subculture and/or propagation in strain-selective hosts.

Virus extraction by PEG precipitation, first reported

with AMV by Clark in 1958 (3), is considered to be a good method for AMV, with certain limitations. In purification from tobacco, some host material is left, as indicated by serological tests. The method was selected for its simplicity. Although purification by precipitation may be selective for certain components, as shown for Mg^{++} precipitation (8), this was not considered to be detrimental for the purposes here. Several attempts to purify the virus by differential centrifugation resulted in virus yields (judged by infectivity assay) much lower than that produced by the PEG precipitation method.

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