# Variation in the Aggregation Forms of Alfalfa Mosaic Virus Strains in Different Alfalfa Organs

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## ABSTRACT

The aggregation of two strains of alfalfa mosaic virus (AMV) was studied by electron microscopy in leaves, embryonic cotyledons, ovary walls, ovules, anthers, pollen, and bud receptacles of two alfalfa (*Medicago sativa*) clones. No differences were attributable to the clones. Aggregation forms varied with the virus strain and the alfalfa organ where it was located. Strain F1 formed aggregations that were different from those formed by U21 in leaves, bud receptacles, and embryonic cotyledons, but not in ovary walls, anthers, or pollen. Strain F1 was usually nonaggregated in leaf cells, and sometimes in bud-receptacle cells. It formed rafts of short particles in leaves, anthers, and pollen. In pollen, the rafts formed star-like aggregations. Large crystalline bodies formed in ovary walls, bud receptacles, and embryonic cotyledons. Strain U21 usually formed aggregations in leaves, bud receptacles, and pollen, but some nonaggregated particles also formed. Rafts of short particles formed in leaves, anthers, pollen, and embryonic cotyledons. Large crystalline bodies of long particles formed in ovary walls, bud receptacles, and embryonic cotyledons. The virus was seen only in cytoplasm of parenchyma cells, transfer cells, and vascular parenchyma cells; it was not associated with cell organelles. No AMV particles were observed in ovules.

*Additional key words:* virus morphology.

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Alfalfa mosaic virus (AMV) forms specific aggregations in alfalfa (11) and other crops (2, 7, 8, 9, 11). Hull and Plaskett (8) used this characteristic to study the behavior of AMV strains in mixed infections. This paper reports observations on aggregation of AMV in the male and female gametophytes of alfalfa in an attempt to learn more about transmission of the virus to seed through ovules and pollen (4).

While the aggregation forms of AMV are largely strain-specific, some variation has been reported: De Zoeten and Gaard (2) saw differences when two strains were studied in three plant species and we (11) noted a strain that formed two aggregation types in alfalfa leaves. This paper reports further evidence on variation in the aggregation forms of AMV in different cells and organs of alfalfa.

MATERIALS AND METHODS.—The alfalfa (*Medicago sativa* L.) organs studied were leaves, ovary walls, bud receptacles, pollen, anthers, ovules, and embryonic cotyledons of clones 1345 and 5, infected with AMV strains F1 and U21. Virus-free tissues were also studied. Methods of handling plants and virus strains have been given by Frosheiser (3), and the identifying characteristics of the strains have been described (10).

Strains U21 and F1 were studied because they were distinct in previous studies (10); strain F1 consistently formed one aggregation type, and strain U21 consistently formed two aggregation types. The alfalfa clones and AMV strains have been used for many studies on seed, pollen, and ovule transmission of AMV (4).

The youngest fully-expanded trifoliate leaves were collected from shoots about 20.3 cm (8 inches) tall; at least five leaves were studied from each of ten plants infected with each AMV strain. Leaves of only clone 5 were used. The alfalfa plants were grown in 13-cm diameter clay pots in the glasshouse at 20-25 C.

Ovaries were obtained from fully developed flowers before pollination. Twelve plants of each clone were studied: six were infected with strain U21 and six with strain F1. At least eight ovaries per plant were examined.

Bud receptacles were obtained from 2mm-long pieces of shoot apex collected from six plants of clone 1348 (three infected with each virus strain) and from four plants of clone 5 (two infected with each virus strain). Four buds were studied from each plant. The buds were sectioned at the point where all the bud tissues unite.

Shriveled and normal pollen was studied. The walls of the shriveled pollen had collapsed along the sutures. However, normal pollen was not shriveled and was observed before germination and just as pollen tube growth was initiated. Pollen was collected from six plants of each clone (three infected with each virus strain); 20 pollen grains from each plant were examined.

Unfertilized ovules in ovaries from fully developed flowers were studied. Five ovules were examined from each of 10 ovaries infected with either strain F1 or U21.

Cotyledons were removed from embryos dissected from seeds that had been soaked overnight in water. One cotyledon from each embryo was used for electron microscopy and the other was indexed on bean (4) to detect AMV infection. Six embryos of clone 1348 (three infected with strain U21 and three with strain F1) and 16 clone 5 (eight infected with each of the AMV strains) were examined.

The plant tissues were fixed with 4% glutaraldehyde (0.01 M phosphate buffer pH 6.8) for 2 hours at 23 C, postfixed with 2% osmium tetroxide in the same buffer for 1 hour at 23 C, dehydrated with a graded acetone series, and embedded in Epon 812. Sections were cut with an LKB Ultratome II ultramicrotome using glass and diamond knives, mounted on 400 × 100-$\mu$m copper mesh grids, stained with saturated uranyl acetate for 25 minutes, and stained with Venable's lead citrate for 5 minutes (5). Finally, the sections were examined with a Philips EM-300 electron microscope.

RESULTS.—*Location of AMV in alfalfa cells.*—Particles of both strains of AMV were observed only in the cytoplasm of the infected cells, except in leaves where they were also seen in vacuoles and intercellular spaces. The virus did not appear to be associated with cell organelles. AMV was seen only in parenchyma cells of leaves and bud receptacles; in epidermis, parenchyma, and vascular parenchyma cells of the ovary walls; in parenchyma and transfer cells of anthers; in pollen; and in parenchyma cells of embryonic cotyledons (Table 1). No AMV particles were observed in the 50 ovules studied.

Even though many virus particles were seen in the cytoplasm, the ultrastructure of the infected cells appeared not to be affected by the virus. In leaf cells, however, virus strain U21 occasionally caused the tonoplast to detach from the cytoplasm (11).

*Types of aggregations observed.*—AMV particles were aggregated in four distinct forms in the cells of alfalfa tissues (Table 1). Nonaggregated particles were sometimes seen. Although the individual particles usually were difficult to distinguish from ribosomes, they were recognized easily when they became numerous enough to darken the cytoplasm in the electron micrographs (Fig. 1). This darkening is called "opaque cytoplasm". Some aggregations were short or long rafts consisting of virus particles 18-60 nm long (Fig. 3, 4, and 5) aligned together in parallel array. In cross-section, the particles were packed together hexagonally (Fig. 3 and 5). Short rafts contained between four and ten virus particles in parallel array (Fig. 5), and long rafts contained 15-75 particles (Fig. 3 and 4). Usually, the rafts were arranged randomly in the cytoplasm, but in pollen they were often oriented about a central point as part of a larger star-like aggregation (Fig. 6). Some aggregations were large crystalline bodies (Fig. 2) composed of what appeared to be many very long virus particles (more than 790 nm long). These particles were arranged beside each other in parallel array, and were packed hexagonally in cross-section. The crystalline bodies, which were rectangular or crescent-shaped, varied in size and shape. Some large bodies extended halfway across the cell, but others were less than the diameter of a mitochondrion in size.

*Variation in aggregations in alfalfa organs.*—The AMV aggregation types in different alfalfa organs are summarized in Table 1.

*In leaves.*—The particles of strains F1 and U21 were found in mesophyll cells; U21 was found also in vascular parenchyma.

Particles of strain F1 were usually nonaggregated and made the cytoplasm electron-opaque, as in Fig. 1. Occasionally, a short raft containing six or eight short particles was seen. Sometimes a few particles of F1 were grouped together between the cell wall and a membrane,

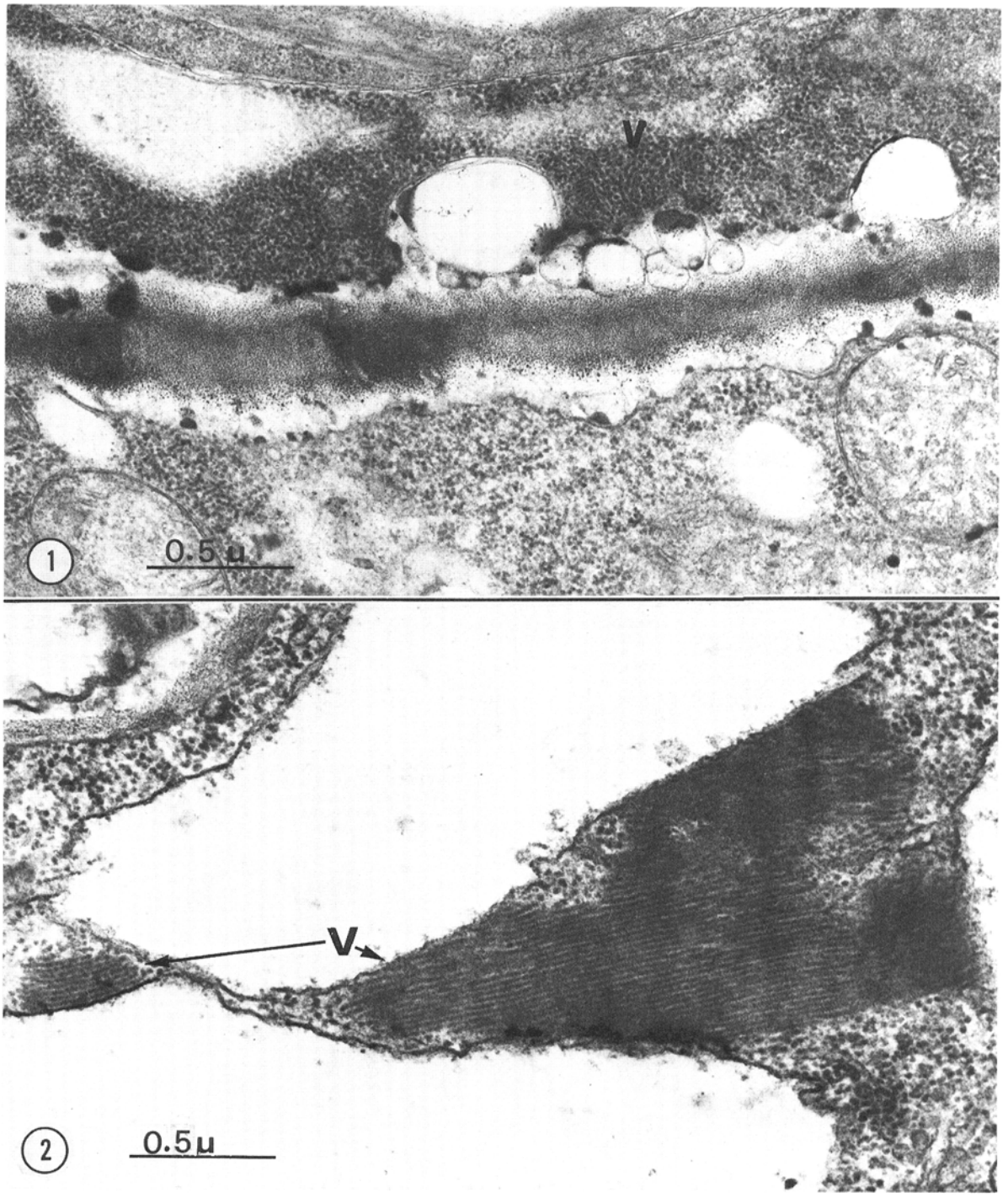


**Fig. 1-2.** Alfalfa mosaic virus in alfalfa parenchyma. **1)** Parenchyma cells of alfalfa bud receptacle. The cell in the lower half of the figure was apparently virus free, and the cell in the upper half was infected with alfalfa mosaic virus (cytoplasm electron-dense because of the presence of many nonaggregated virus particles). **2)** Large crystalline body made up of long alfalfa mosaic virus particles in a parenchyma cell of the ovary wall of alfalfa.

but they were not seen in vacuoles or intercellular spaces (10).

Particles of strain U21 were sometimes nonaggregated and made the cytoplasm electron-opaque, but more often they were aggregated as either short or long rafts, as in leaves than those of F1. Particles of U21 were seen in vacuoles and intercellular spaces (11).

*In ovary walls.*—The particles of both strains were

aggregated into large crystalline bodies (Fig. 2) in epidermal, parenchymal, and vascular parenchymal cells. The virus was found only in cells along the suture of the ovary.

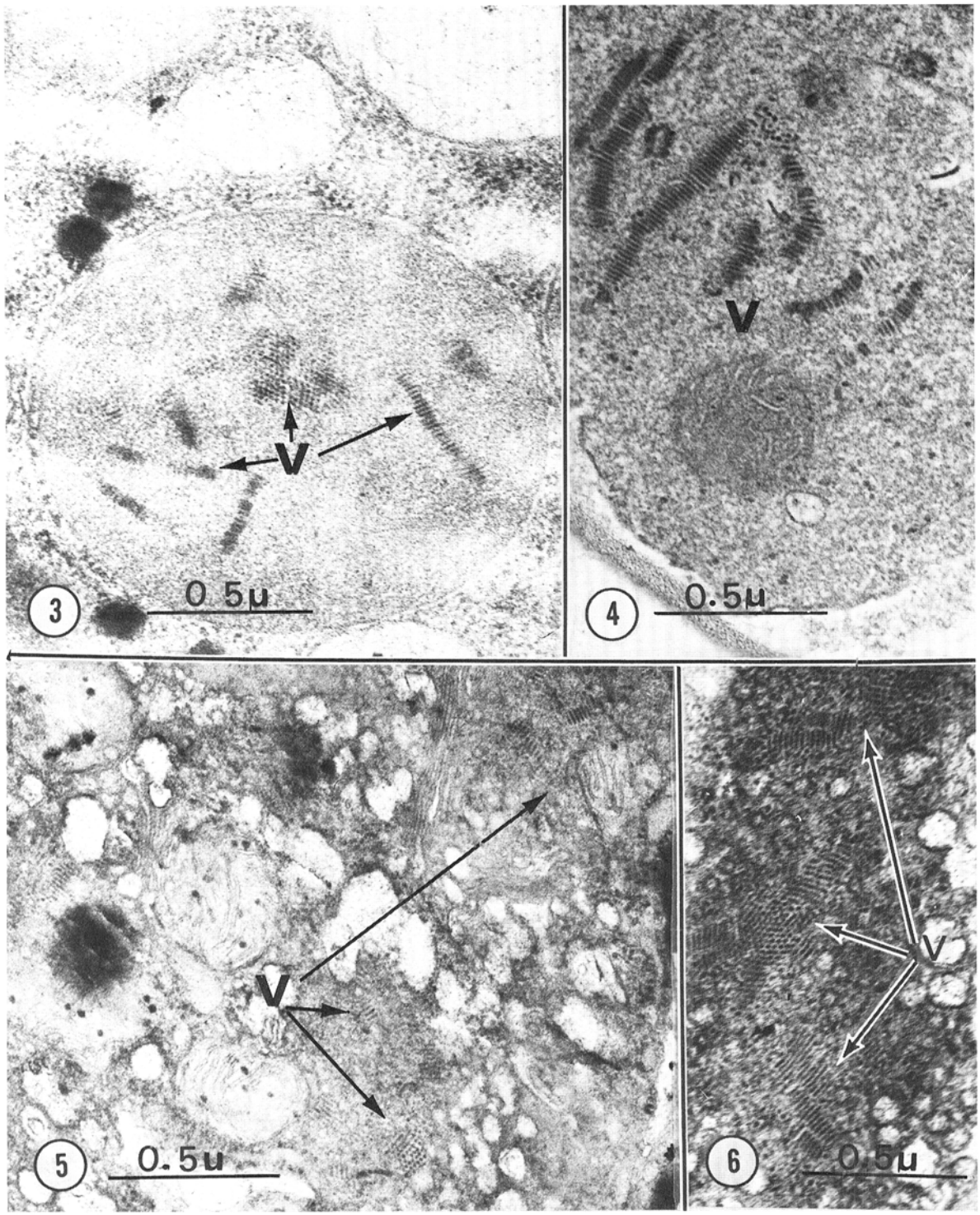


**Fig. 3-6.** Rafts and aggregates of alfalfa mosaic virus in alfalfa flower parts. **3)** Rafts of alfalfa mosaic virus particles in a membrane-bound body in a parenchyma cell of embryonic cotyledon of alfalfa. **4)** Long rafts of alfalfa mosaic virus particles in a parenchyma cell of alfalfa anther. **5)** Short rafts of alfalfa-mosaic-virus particles in alfalfa pollen. **6)** Star-like aggregation of alfalfa-mosaic virus particles in alfalfa pollen.

*In bud receptacles.*—The particles of both strains were in the bud receptacles. The aggregations of both strains were large crystalline bodies, as in ovary walls. Also, cytoplasm containing strain F1 was sometimes electron-opaque, as in Fig. 1.

*In anthers.*—Both strains were easily found in transfer and parenchyma cells located in the tapetum, in the form of short particles aggregated into long rafts (Fig. 4).

*In pollen.*—Nonaggregated particles made the cytoplasm electron-opaque, especially in shriveled pollen. In normal pollen, the virus particles were aggregated into short rafts or into very long rafts (Fig. 5). Often, the long rafts were associated in a larger star-like aggregation (Fig. 6).

*In embryonic cotyledons.*—Two different aggregations formed in embryonic cotyledons. Strain U21 formed short to medium-length rafts containing short particles in membrane-bound bodies (Fig. 3) or free in the cytoplasm. In addition, both strain U21 and F1 formed aggregations of long particles grouped together in crescent-shaped crystalline bodies with ragged ends located in the cytoplasm (Fig. 2).

DISCUSSION.—In this study we confirmed the report of Frosheiser (4) that pollen from AMV-infected alfalfa plants contains aggregated and nonaggregated virus particles.

We did not find AMV in ovules even though Frosheiser (4) found it was transmitted to embryos through 3-10% of the ovules. Perhaps we did not sample enough ovules even though we examined 50 taken from ovaries that contained the virus. It appeared there was some physiological barrier to the movement of AMV along the suture from the ovary into the ovules. It was present in cells of the ovary wall along the suture from the epidermis to the vascular bundles but not between the vascular bundles and the transfer cells bordering the locule. This

TABLE 1. Summary of information about aggregation of two strains of alfalfa mosaic virus (AMV) in different alfalfa organs

| Alfalfa organ and AMV strain | Cell type containing AMV | Type of AMV aggregation |
|---|---|---|
| Leaves | | |
| F1 | Parenchymna | Particles usually nonaggregated (opaque cytoplasm) occasional short rafts, as in Fig. 1. |
| U21 | Parenchyma | Particles sometimes nonaggregated (opaque cytoplasm); usually short or long rafts made of short particles, as in Fig. 4 and 5. |
| Ovary walls | | |
| F1 and U21 | Parenchyma, vascular parenchyma, and epidermis. | Large crystalline bodies made up of long particles (Fig. 2). |
| Bud receptacle | | |
| F1 | Parenchyma | Same as ovary wall, plus opaque cytoplasm (Fig. 1 and 2). |
| U21 | Parenchyma | Same as ovary wall (Fig. 2). |
| Anthers | | |
| F1 and U21 | Parenchyma and transfer cells | Short particles in long rafts (Fig. 4). |
| Pollen | | |
| F1 and U21 | Pollen | Opaque cytoplasm in shriveled pollen. Short particles in short and long rafts (Fig. 5); long rafts frequently associated in star-like aggregations (Fig. 6). |
| Embryonic cotyledon | | |
| F1 | Parenchyma | Long particles in crescent-shaped crystalline bodies in cytoplasm, as in Fig. 2. |
| U21 | Parenchyma | Short particles in short to medium long rafts in cytoplasm or membrane bound bodies (Fig. 3); long particles in crescent-shaped crystalline bodies in cytoplasm, as in Fig. 2. |
| Ovules | | |
| F1 and U21 | Not seen | Not seen |

area was the only one in the ovary in which the virus was seen. There was no apparent structural barrier to prevent the virus from moving through these cells into the ovule because plasmodesmata were common.

It is possible that a few nonaggregated AMV particles were present in some of the ovules. If this is true, it would be difficult to see them because many AMV particles are about the same size as ribosomes, and therefore difficult to distinguish from ribosomes. In other tissues, nonaggregated AMV could be recognized only when the particles were so numerous that the cytoplasm was made electron-dense (See Fig. 1, for example).

The strains of AMV were selected for this study because they were readily transmitted through pollen and ovules (4), and because they formed different aggregations in alfalfa leaves (11). In the present study, they were easily recognized by their distinctive appearance in leaves, as expected, however, their aggregation forms varied in other organs. It is now clear that if particle aggregations are to be used to help characterize strains of AMV, it will be necessary to make the studies on standard plant organs. In addition to having many aggregation types, the physical and biological properties of AMV strains are quite diverse (1, 3, 4, 6, 10).

As reported previously (11), we saw no relationship between cell organelles and AMV aggregations; the virus particles were seen only in cytoplasm and appeared to be randomly placed among the cell organelles. We also saw no relationship between the formation of aggregations and visible structural damage to the cells. Often we saw aggregations in cells that were without any apparent injuries.

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