AN ABSTRACT OF THE THESIS OF

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Title ISOLATION AND IDENTIFICATION OF DAHLIA VIRUSES

Abstract approved

(Major Professor)

Three viruses were isolated from dahlias using a modification of Yarwood's leaf-disc method of inoculation. Two of these isolates have been reported previously in dahlias. In addition, a fourth virus was isolated using a liquid-nitrogen transmission technique.

Dahlia virus isolate 1 possessed host range and thermal inactivation properties similar to those reported for dahlia mosaic virus. Attempts to partially purify and prepare an antiserum for dahlia virus 1 were not successful.

Dahlia virus isolates 2 and 3 showed host range and thermal inactivation properties similar to those obtained in tests with tomato spotted wilt and CMV virus type cultures. Dahlia virus 2 was partially purified using a procedure developed by Grogan and Kimble for CMV purification. An antiserum prepared to the virus did not react when tested with the CMV type culture. However, a positive reaction was obtained in gel diffusion tests when an antiserum prepared by Grogan to CMV (Imperial Strain 78) was tested with the dahlia isolate.

Dahlia virus 4 expressed symptoms on several hosts and possessed thermal inactivation properties similar to tomato ringspot virus. An antiserum prepared to this isolate reacted positively in a
microprecipitin test with tomato ringspot virus. Gel diffusion tests showed that extracts from tomato ringspot and dahlia virus 4 possessed antigens common to both viruses. However, tomato ringspot virus possessed a major antigenic component which was not present in extracts from plants infected with dahlia virus 4.

Attempts to eliminate viruses present in naturally infected dahlias using heat and chemical treatments were not successful.

Symptoms on Unwin's hybrid seedlings infected with dahlia virus isolates 3 and 4 differed from symptoms on the dahlia varieties from which the isolations were made. Seedlings infected with dahlia virus isolates 1 and 2 showed systemic symptoms which were similar to those on the original varieties.

Diagnosis of specific virus diseases in dahlia is difficult because of the variation in symptom expression resulting from infection with a single virus. Therefore, only a general diagnosis of a virus disease is possible based on the symptoms observed in naturally infected dahlias.
ISOLATION AND IDENTIFICATION OF DAHLIA VIRUSES

by

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ISOLATION AND IDENTIFICATION OF DAHLIA VIRUSES

INTRODUCTION

Virus diseases are the greatest limiting factor in commercial dahlia production in the United States and Europe (30). The three virus diseases most frequently described on dahlia are dahlia mosaic, cucumber mosaic, and tomato spotted wilt. Other virus diseases include dahlia ringspot, dahlia yellow ringspot, and dahlia oakleaf. Descriptions of these diseases have been based on symptom expression in the dahlia host. Recent evidence indicates that some variation in symptom expression may be attributed to strains of the same virus rather than to different viruses (10).

Viruses may be present in the dahlia host in a latent condition causing difficulty in detection and diagnosis of virus-diseased plants. The failure to detect and eliminate diseased plants has resulted in dissemination of infected dahlia varieties by vegetative propagation.

Virus symptoms are expressed most prominently on dahlias on the first growth in the spring. With warmer temperatures during the summer months (July and August) symptoms become less distinct. Nutrition also affects symptom expression. Application of fertilizers high in nitrogen can mask symptoms on plants which have previously shown distinct symptoms.

Symptom expression in infected dahlia hosts may be considered under three categories: 1) stunting and reduced vigor of the host; 2) mosaic, mottle, and ringspot symptoms on leaves of the affected host; and 3) color breaking in the flowers.

In addition to the direct injury of the dahlia host by these
viruses, indirect injury to other economically important hosts may also result. Both cucumber mosaic virus and tomato spotted wilt virus have wide host ranges (19, p. 31 and 136). Since both of these viruses are insect-transmitted, they may be transferred readily from dahlia to other hosts. Therefore, methods must be developed for early detection and diagnosis of the dahlia viruses under field conditions.

The objectives of this study were to isolate and identify the viruses principally responsible for virus diseases of dahlia in Oregon and to determine whether these diseases could be identified on the basis of symptom expression in the dahlia host.
REVIEW OF LITERATURE

Reports from the United States and Europe indicate that viruses cause the most important pathogenic diseases of dahlia (30). The literature concerning dahlia mosaic, tomato spotted wilt, cucumber mosaic, and tomato ringspot will be reviewed. Other viruses reported on dahlia include dahlia ringspot virus (7, p. 276), dahlia yellow ringspot virus (7, p. 280), dahlia oakleaf virus (7, p. 282), potato virus Y (27), mild vein mosaic virus (9), and sunflower virus (47).

Dahlia Mosaic Virus

Dahlia mosaic virus is synonymous with dahlia—yellows virus, dahlia stunt virus, dahlia dwarf virus, dahlia runting virus, dahlia leaf-curl virus, dahlia rosette virus, and dahlia virus 1 (19, p. 85).

The geographical origin of dahlia mosaic virus is unknown, but it has been observed in Western Europe (25, 26, 34, 43), Guatemala (33), and New South Wales (50). In the United States it was first reported in Maryland in 1909 by Norton (35) and in Massachusetts in 1911 by Stone (44). In 1922, Howe (22) suggested that "stunt" or "dwarf" in dahlia was probably not different from what was known as the mosaic disease. Brierley (7, p. 240) found the mosaic disease widespread in Connecticut, New Jersey, and New York.

The first report of transmission of a virus disease of dahlia was made in 1928 by Brandenburg (6, p. 57) who transmitted dahlia mosaic virus from dahlia to dahlia by means of grafting. Brierley (7, p. 254-264) succeeded in transmitting dahlia mosaic virus by placing healthy
and mosaic-diseased dahlias together in cages with the green peach aphid, *Myzus persicae* (Sulz.). Only members of the genus *Dahlia* were infected by the virus in this manner.

Additional hosts for the virus in the family Compositae, including *Sanvitalia procumbens* Lam., *Verbesina encelioides* (Cav.) B. & H., *Zinnia elegans* Jacq. (Lilliput types), and *Coreopsis douglasii* (Gray) Hall were later described by Brierley (8). He recovered dahlia mosaic virus on sub-transfer of *Myzus persicae* (Sulz.) from these hosts. Aphids starved 2-4 hours acquired the virus after an infection feeding period of 1-5 minutes, and transmission occurred after a test period of 5-15 minutes. In *Verbesina* and *Zinnia*, a 1-minute feeding followed by a 5-minute test period resulted in infection. Infective aphids lost their ability to transmit the virus after the second hour, indicating the non-persistent nature of the virus.

Several other methods of virus transmission have been attempted with varying success. No transmission was achieved when sap from leaves showing mosaic symptoms was injected into stems, petioles, and midveins of dahlia (6, p. 57). Brierley (7, p. 250), in 1933, described five methods used to transmit other plant viruses which were not successful in transmitting dahlia mosaic virus from dahlia to dahlia. These included 1) rubbing expressed sap of mosaic leaves over the leaf surface of healthy plants with a cheesecloth, 2) applying expressed sap to leaves using a glass rod of the type described by Samuel (39, p. 499), 3) pricking expressed sap into leaf axils by means of insect pins, 4) superimposing diseased leaves on healthy leaves and pushing insect pins through the chlorotic areas of the diseased leaves into the
healthy leaves, and 5) inserting a piece of diseased tissue with an associated bud into the slit stem of a healthy plant. Brierley (8) later reported poor transmission of dahlia mosaic virus from dahlia to dahlia using the leaf-rubbing technique. Four trials resulted in infection of 3/60 plants. An attempt to transfer the virus from dahlia to Verbesina produced infection in 1/10 plants. Further tests made by Brierley (9) indicated that Verbesina could be used in indexing for dahlia mosaic virus when inoculations were made early in the season.

Smith (43) observed that the most prominent symptom produced by dahlia mosaic virus was conspicuous vein banding on the new growth, which became less distinct as the plant matured. Older leaves appeared mottled. Infected plants were often short and bushy, exhibiting a condition known as stunt. Brandenburg (6, p. 47) described a color break in the flowers of the variety Paradiesvogel caused by dahlia mosaic, but Brierley (7, p. 245) observed that the flowers of dahlia mosaic diseased plants were usually normal. Symptom expression varies widely among varieties, and certain varieties carry the virus in a latent condition (30).

Brierley (8) reported thermal inactivation of dahlia mosaic virus at temperatures ranging from 85°-90°C. To determine the inactivation temperature, leaves of Verbesina encelioides were ground in a mortar with a minimum of water (about 2 parts of water to 1 part of leaves by weight). Sap extracts treated at 80°C for 10 minutes produced infections in 10/10 plants of Verbesina; at 85°C, 1/10 infections; and at 90°C, 0/10 infections.

Partial purification and development of an antiserum for dahlia
mosaic virus has been reported by Cornuet et al. (11). Dahlia was used as the source of the virus for purification. To prevent precipitation and denaturation of the virus, sap extractions were made in the presence of a nicotine sulfate solution, containing potassium cyanide and sodium bisulfite. The virus was purified by ammonium sulfate precipitation.

Morel and Martin (32) reported the elimination of dahlia mosaic virus from the dahlia variety Rose Reve by growing apical meristems in agar culture. The resulting leafy shoots were grafted on young virus-free seedlings. Growth from the shoot grafts developed normally and symptoms did not appear.

**Tomato Spotted Wilt Virus**

Tomato spotted wilt virus (TSWV), which occurs throughout the world, has received considerable attention since its discovery in Australia in 1915 (43). Smith (41) reported the virus on Solanum capsicastrum in Britain in 1931. Thrips tabaci Lind. transferred a virus from dahlia which produced symptoms on Datura and tobacco similar to the Solanum isolate. In a later report (42, p. 319-320) he proved that dahlia was a host for spotted wilt by cross inoculation tests in which spotted wilt was transferred from dahlia to tomato and from tomato to dahlia by Thrips tabaci. He observed that the virus was systemic in dahlia and the roots from infected plants usually gave rise to diseased plants.

McWhorter (29) reported the occurrence of TSWV in Oregon in 1941. He stated that dahlias seemed to be the chief factor in distribution of
spotted wilt viruses in the Northwest and that the tip blight disease of tomato, a form of TSWV occurring in Oregon, could be transferred to dahlias. The introduction of this virus into several areas of Oregon by dahlias was noted.

Roland (37) described tomato spotted wilt virus on dahlia in Belgium in 1948. Mildner (31) isolated a virus from dahlia which possessed properties similar to TSWV.

Dahlia ringspot, yellow ringspot, and dahlia oakleaf symptoms in Britain were caused by TSWV, according to Brunt (10). However, dahlia ringspot, yellow ringspot, and dahlia oakleaf viruses were described previously by Brierley (7, p. 276-283) as separate dahlia viruses.

Smith (43) observed that the severity of disease symptoms induced by TSWV on dahlia varied according to the variety and stage of development. Symptoms were most evident on new growth early in the season and consisted of single or concentric rings with line patterns. Summer growth was symptomless or showed only slight mottling. Bonnemaison (4) reported that dahlia infected with spotted wilt were often stunted. The young leaves were chlorotic and flowers were smaller.

Best (2, p. 1) reported that suspensions of TSWV in buffer solutions at pH 7.0 became noninfectious at room temperature after a few hours. When infective juice was added to a solution buffered at pH 7.0 through which pure hydrogen was bubbled, virus activity remained constant for four hours. The control suspension was inactivated after the same period of time. Sodium bisulfite preserved virus activity for six days, but activity was reduced to 20% of its original value. The virus
retained activity in sealed tubes for 35 days in a suspension of phthalate-phosphate-borate buffer at pH 7.5 containing 0.01 M cysteine. No activity remained after 42 days. Other reducing agents which helped to preserve activity included glutathione, thioglycollic acid and ascorbic acid; however, none of these substances preserved activity for more than a few hours.

TSWV was inactivated when exposed to a temperature of 42°C for 10 minutes (19, p. 136).

TSWV was partially purified using sodium bisulfite as a preservative (3). A 50% reduction in virus activity resulted using this procedure.

Holmes (21) attempted to inactivate TSWV in dormant roots with a hot-water treatment but found that roots were injured by heating. No data were presented. He eliminated TSWV from the dahlia variety Rhythm without heat treatment by propagation of small tip cuttings taken from infected plants (20, 21).

Cucumber Mosaic Virus

Cucumber mosaic virus (CMV) is prevalent in dahlias in Europe but has not been widely reported in the United States (8). The virus may be transmitted mechanically and is carried in a non-persistent manner by more than 60 species of aphids (24, p. 20-21).

In 1951, Brierley (9) reported the isolation of CMV from 3 of 18 dahlia varieties. However, he found no evidence that American strains of CMV can infect Unwin's and Coltness' hybrids. When a dahlia seedling clone from Unwin's hybrids was inoculated with two strains of CMV
isolated from dahlia, no symptoms appeared and CMV could not be reisolated from the inoculated seedlings. He concluded that the Unwin's hybrid clone is immune to or highly resistant to CMV. Another experiment was conducted in which seedling dahlias of the Coltness hybrids were inoculated with five strains of CMV. Four of these strains were originally from vegetables and included Doolittle's type, Price's type, and Wellman's celery strain. The other strain was from Easter lily. None of the inoculated seedlings developed symptoms nor could the virus be reisolated from them.

Smith (43) described CMV symptoms on dahlias in Europe as mottling of light and dark green patches on the leaves without blistering. On some varieties there appeared light green whorls or ring and line patterns which differed from the concentric rings of spotted wilt of dahlia. Brierley (9) noted that naturally infected dahlia varieties expressed a mild diffuse mottling without distortion of the leaves or vein mosaic. He observed a mild flower color break in the variety Ogden Reed. Although some dahlia varieties infected with CMV did not show definite symptoms of the virus, they could serve as a source of the virus for infection of other hosts.

Brierley (9) found that CMV from dahlia produced slight curling and vein clearing of zinnia leaves. Symptoms on tobacco varied. Some strains caused white primary lesions and others only a yellowish mottling. Diffuse mottling with only occasional vein clearing and no leaf distortion occurred in Verbesina inoculated with CMV.

Tomlinson et al. (46) reported partial purification of CMV using butanol clarification and the preparation of an antiserum. Grogan and
Kimble (15, 16) partially purified CMV by acid precipitation and differential centrifugation using borate buffer to prevent aggregation of the virus particles.

Holmes (19, p. 32) reported a thermal inactivation temperature for CMV of 74°C for 10 minutes.

Hollings (18) suggested that CMV may be eliminated from infected dahlias by growing the plants at 99°F for about 4 weeks, then striking small cuttings from the treated plants. Using this method, he eliminated the virus from 10-75% of the rooted cuttings.

**Tomato Ringspot Virus**

The virus which is designated today as tomato ringspot was first described by Price (36) in 1936 as tobacco ringspot No. 2. He showed by means of immune reaction that tobacco ringspot and tobacco ringspot No. 2 were distinct viruses.

Tomato ringspot virus was described on tomatoes in 1942 and named by Samson and Imle (38). They observed that tomato ringspot virus in tomato was characterized by intricate patterns of necrotic rings and lines on young leaves and necrotic streaks on the stems of infected plants.

Tomato ringspot virus was inactivated between 55°C and 60°C when treated for ten minutes (19, p. 102). Samson and Imle (38) reported that the virus was inactivated at 58°C when treated for a ten-minute period.

Tall et al. (45, p. 289-290) reported the preparation of an antiserum for tomato ringspot virus from partially purified extracts of
Turkish tobacco. Halliwell (17, p. 24 and 32) prepared an antiserum for tomato ringspot virus using hydrated calcium phosphate as a clarifying agent in the purification procedure.
MATERIALS AND METHODS

Culture of Dahlia Varieties

Twenty-five dahlia varieties which had shown distinct symptoms of virus diseases in 1960 were selected for study in 1961. Dormant roots were planted and grown in No. 10 cans in a greenhouse maintained at a day temperature of 70°-75°F and a night temperature of 65°F. Dormant roots of the same varieties were planted in the field for comparison of symptoms.

Isolation of Dahlia Viruses

Three viruses were transmitted from dahlia using a modification of Yarwood's leaf-disc method (51). A fourth isolate was obtained by a liquid-nitrogen technique developed by Sanger and Gold (40).

Plant Inoculation Techniques

Inoculum for all host range studies was prepared by macerating infected tissue in 0.5% Na₂HPO₄, pH 7.5. These infective preparations were applied with the forefinger to host plants dusted with 600-mesh carborundum. Following inoculation, excess carborundum and the remaining inoculum were washed from the leaves with water.

Thermal Inactivation

Thermal inactivation studies were conducted according to a modification of the procedure outlined by Bos, Hagedorn, and Quants (5, p. 334-335). Viruses were extracted in 0.01 M Na₂HPO₄-NaH₂PO₄ buffer,
pH 7.5, and the extracts were diluted 1:10 (w/v). Three ml of the inoculum were carefully pipetted into a thin-walled glass tube to avoid contaminating the sides of the tube above the level of the inoculum, then immersed in a water bath. The water level was about 3 cm above the level of the inoculum in the tube. The tubes containing the inoculum were heated for 10 minutes at a constant temperature. Following the treatment period, the tubes were immediately cooled in cold flowing water. A Magni Whirl utility bath with constant temperature control and mechanical agitation was used for all experiments. Treated inoculum was assayed on appropriate host plants. Unheated controls were also assayed at the same time to account for any aging effect on the virus activity.

Virus Type Cultures Used for Comparative Study

The following virus type cultures were used for host range, thermal inactivation, and serological studies:

(1) Cucumber mosaic virus (common type), American Type Culture Collection AC-10.
(2) Tomato ringspot virus, American Type Culture Collection AC-13.
(3) Tomato spotted wilt virus, American Type Culture Collection AC-176.

A type culture of dahlia mosaic virus was not available for comparison with the isolate obtained in this study.
Serological Tests

Cucumber mosaic virus (Imperial Strain 78) antiserum was supplied by R. G. Grogan, Department of Plant Pathology, University of California, Davis, California.

Tomato ringspot virus antiserum was prepared by R. S. Halliwell, Texas A. & M. College, College Station, Texas.

Ouchterlony double-diffusion test. Double-diffusion reactions were carried out according to the procedure described by Ball (1, p. 13-14) and Van Slogteren (49). Ionagar (0.5%) with 1:5,000 sodium azide was prepared in a physiological saline solution containing 0.01 M Na$_2$HPO$_4$-NaH$_2$PO$_4$ buffer, pH 7.0, and 0.85% NaCl. Fifteen ml of this medium were added to Petri dishes. Wells were cut in the agar with a No. 7 cork borer and the agar was removed from each well. The antigen and antibody were added to the appropriate wells in the agar medium. Antigenically related antigens and antisera formed zones of precipitation which could be analyzed by direct observation.

Microprecipitin Test. Microprecipitin reactions were also conducted according to the procedure described by Ball (1, p. 5-7) and Van Slogteren (48). Tests were carried out in disposable plastic Petri dishes which made Formvar coating unnecessary. The appropriate series of antigen-antiserum dilutions were made in a physiological saline solution containing 0.85% NaCl and neutral 0.01 M Na$_2$HPO$_4$-NaH$_2$PO$_4$ buffer. The Petri dish was divided into squares with a wax pencil and the dish was spotted with microdrops from a pipette fitted with a
22-gauge needle. After mixing the antigen and antiserum, the plates were flooded with mineral oil to prevent evaporation of the microdrops. Reactions were observed after three hours with a binocular dissecting scope under low-power magnification. The reaction plates were then placed in a cold room (4°C) and observed again after 13 and 22 hours.
Isolation of Viruses from Dahlia

Virus isolations were made from dahlia varieties grown in the greenhouse in the summer of 1961. Leaves from dahlia varieties showing distinct symptoms were macerated in 0.5% Na₂HPO₄, pH 7.5, and applied directly to herbaceous hosts dusted with 600-mesh carborundum. Inoculations of Phaseolus vulgaris L. (Bountiful bean), Vigna sinensis Endl. (black cowpea), Cucumis sativus L. (cucumber, Chicago Pickling), Vicia faba L. (horsebean), Cucurbita maxima Dcne. (buttercup squash), Cucurbita pepo L. (white scallop squash), Chenopodium amaranticolor Coste & Reyn, Gomphrena globosa L. (globe amaranth), Nicotiana tabacum L. x N. glutinosa L. (Necrotic Turk tobacco), and Nicotiana tabacum L. (Burley and Kentucky 56 tobacco) were made using the forefinger. Although repeated attempts were made to isolate viruses using this method, none was successful.

Yarwood (51) reported transmission of two unstable viruses using a leaf-disc method. Mildner (31, p. 5) reported transmission of several viruses from dahlia using this method. A modification of this procedure was used successfully to transmit three viruses from dahlias. Leaves of host plants to be inoculated were dusted with 600-mesh carborundum and sprayed with 0.5% Na₂HPO₄, pH 7.5. Several leaves from a dahlia variety showing distinct symptoms were layered (one on another), folded over once, and held with a forceps. A cut was made across the leaves, and the wounded surface was brought into contact with a leaf surface of the plant to be inoculated, then rubbed across it with a continuous
strok ing action. Inoculated leaves were then rinsed with water.

Using the modified leaf-disc method, virus isolates were obtained from the following dahlia varieties:

1) Dahlia virus 2 was isolated from dahlia variety Helen Anita on Kentucky 56 tobacco. Chlorosis along leaf midveins and chlorotic mottle were prominent symptoms in this variety (Figure 1).

2) Dahlia virus 3 was isolated from dahlia variety Amber Queen on Kentucky 56 tobacco. Symptoms developed in this variety as concentric chlorotic rings and chlorosis along the leaf veins (Figure 2).

3) Dahlia virus 4 was isolated from dahlia variety Cay on Bountiful bean and globe amaranth. Chlorotic bands developed on leaf midveins in variety Cay. These bands became diffuse as the leaves aged (Figure 1).

Another virus isolated from dahlia and designated as dahlia virus 1 was transmitted with liquid nitrogen using the procedure described by Sänger and Gold (40) to transmit the unstable form of tobacco rattle virus. Leaves from variety Jersey's Beauty (Figure 3) showing distinct vein-banding symptoms similar to those described for dahlia mosaic (7, p. 241-243) were frozen in 40 ml of liquid nitrogen and ground in a mortar in the presence of 10 ml of 0.5% Na$_2$HPO$_4$, pH 7.5, to form an ice powder. The frozen ice powder was painted on the carborundum-dusted leaves of Verbesina encelioides (Cav.) B. & H. with a moist brush. Verbesina plants with 2-4 fully expanded leaves were inoculated (Table 1).
Figure 1. Dahlia varieties Helen Anita and Cay from which dahlia virus isolates 2 and 4 were obtained.
Figure 2. Dahlia variety Amber Queen from which dahlia virus isolate 3 was obtained.
Figure 3. Dahlia variety Jersey's Beauty from which dahlia virus 1 was isolated. Unwin's hybrid seedling infected with dahlia virus 1.
Table 1. Transmission of dahlia virus 1 from dahlia variety Jersey's Beauty to Verbesina encelioides.

<table>
<thead>
<tr>
<th>Transmission procedure</th>
<th>Date</th>
<th>No. of plants inoculated</th>
<th>No. of plants infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue maceration</td>
<td>March 15</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>April 23</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Yarwood's leaf-disc method</td>
<td>March 15</td>
<td>9</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>April 23</td>
<td>10</td>
<td>3</td>
</tr>
<tr>
<td>Myzus persicae (Green peach aphid)</td>
<td>April 2</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>May 12</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Liquid nitrogen + 0.5% P0₄ buffer (pH 7.5)</td>
<td>July 6</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>August 31</td>
<td>15</td>
<td>12</td>
</tr>
<tr>
<td>Liquid nitrogen + water</td>
<td>August 31</td>
<td>15</td>
<td>10</td>
</tr>
</tbody>
</table>
Earlier attempts to transmit dahlia virus 1 from dahlia to Verbesina, a known host for dahlia mosaic, by direct mechanical inoculation were unsuccessful. A low percentage of infection resulted using the leaf-disc method. Brierley (8) transferred dahlia mosaic virus, which possesses properties similar to dahlia virus 1, from dahlia variety Jersey's Beauty to Verbesina using Myzus persicae (Sulz.), the green peach aphid. In the present study green peach aphids starved four hours were fed five minutes on dahlia variety Jersey's Beauty and transferred to Verbesina for a 90-minute feeding period. Transmission of dahlia virus 1 by leaf rubbing and insects was unsuccessful, however, transmission using the liquid-nitrogen transfer method resulted in a high percentage of virus transfers (Table 1).

Symptoms on Unwin's Hybrid Seedlings Inoculated with Dahlia Virus Isolates and Cucumber Mosaic, Tomato Spotted Wilt, and Tomato Ringspot Viruses

Unwin's hybrid seedlings inoculated with each of the dahlia virus isolates expressed the following symptoms:

1) No distinct symptoms were produced by dahlia virus 1 on inoculated leaves of Unwin's hybrid seedlings. Vein-banding appeared on secondary growth (Figure 3).

2) Dahlia virus 2 caused chlorotic rings on inoculated leaves, and systemic chlorosis along leaf midveins of the new growth (Figure 4).

3) Dahlia virus 3 produced necrotic rings and spots on
Figure 4. Unwin's hybrid seedling infected with dahlia virus 2.
inoculated leaves. Necrotic spotting and systemic chlorosis were prominent secondary symptoms (Figure 5).

4) Dahlia virus 4 produced necrotic spots and rings on inoculated leaves and systemic necrotic spotting and burning of the leaf margins on the new growth (Figure 6).

Concentric chlorotic rings developed systemically on Unwin's hybrid seedlings inoculated with tomato spotted wilt type culture (Figure 5). No symptoms appeared on seedlings inoculated with cucumber mosaic and tomato ringspot type cultures.

Dahlia Virus 1 and Dahlia Mosaic Virus

Since a type culture of dahlia mosaic virus was not available, dahlia virus 1 was identified by the host range and physical properties described in the literature for dahlia mosaic virus.

Symptoms of dahlia virus 1 and dahlia mosaic virus on several hosts. Several species of the Compositae family have been infected with dahlia mosaic virus (9). Mildner (30) reported that only the genus *Dahlia* appears to be infected under natural conditions. The responses of nine test species to inoculation with dahlia virus 1 isolated from dahlia variety Jersey's Beauty are given in Table 2.

Thermal inactivation of dahlia virus 1 and dahlia mosaic virus. Brierley (8) reported that dahlia mosaic virus was partially inactivated in extracts from *Verbesina encelioides* treated at 85°C for 10 minutes and completely inactivated when treated at 90°C for 10 minutes. Therefore, *Verbesina* was used as a source of dahlia virus 1 in this thermal
Figure 5. Unwin's hybrid seedlings infected with tomato spotted wilt virus and dahlia virus 3.
Figure 6. Unwin's hybrid seedling infected with dahlia virus 4.

Local Lesions

Systemic
<table>
<thead>
<tr>
<th>Host</th>
<th>Local lesions</th>
<th>Systemic reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verbesina encelioides</td>
<td>None</td>
<td>Vein banding, chlorotic mottle of older leaves</td>
</tr>
<tr>
<td>Zinnia elegans</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>(Zinnia)</td>
<td>Diffuse chlorotic spots</td>
<td>Yellowing of the veins and distortion of the leaves</td>
</tr>
<tr>
<td>Vicia faba (Horsebean)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Vigna sinensis (Black cowpea)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Datura stramonium var. tatula</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Phaseolus vulgaris (Bountiful bean)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cucurbita maxima (Buttercup squash)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cucurbita pepo (White scallop squash)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Chenopodium amaranticolor</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>
inactivation experiment. Five g of systemically infected *Verbesina*
leaves were macerated in 0.02 M Na$_2$HPO$_4$-NaH$_2$PO$_4$ buffer, pH 7.5. The
macerated leaves were squeezed through cheesecloth and diluted to 50 ml
with the buffer. Tubes with 3-ml samples of inoculum were heated at
70°, 75°, 80°, and 85°C for 10 minutes, then cooled immediately. Each
tube of treated inoculum was assayed on four *Verbesina* plants. Systemic
symptoms were used as an indication of infection since dahlia mosaic
does not produce local lesions on *Verbesina*. Dahlia virus 1 was com-
pletely inactivated only in the sample treated at 85°C for 10 minutes
(Table 3).

Table 3. Thermal inactivation temperature of dahlia virus 1.

<table>
<thead>
<tr>
<th>Host plant</th>
<th>Treatment temperature</th>
<th>No. of leaves inoculated</th>
<th>No. of plants infected</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Verbesina</em></td>
<td>75°C</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>encelioides</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&quot;</td>
<td>80°C</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>&quot;</td>
<td>85°C</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>Control</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

**Purification of dahlia virus 1.** Dahlia mosaic virus is rapidly
inactivated in macerated dahlia tissue due to the presence of tannins
and oxidation products during extraction. However, Cornuet (11)
reported purification of dahlia mosaic virus using dahlia as a source
of the virus. He added nicotine sulfate to precipitate the tannins,
sodium bisulfite to protect the virus from oxidizing agents, and
potassium cyanide to inhibit the proteinase enzymes.

An attempt was made to partially purify dahlia virus 1 using Verbesina as a source of the virus because tannins and oxidizing agents which inactivate dahlia mosaic virus in dahlia sap extracts are apparently absent in Verbesina. Seedling Verbesina plants were inoculated with dahlia virus 1 from Verbesina. When distinct vein-banding symptoms appeared, the apical meristem was removed from each plant to induce branching. Leaves developing from lateral shoots were harvested when symptoms developed.

The virus was partially purified using the following procedure. Fifty-one g of leaves were macerated in 75 ml of 0.01 M Na₂HPO₄-NaH₂PO₄ buffer, pH 7.5. The crude extract was pressed through cheesecloth and centrifuged at 6,000 rpm for ten minutes in a Servall tabletop centrifuge. Hydrated calcium phosphate (HCP) prepared according to the procedure described by Fulton (13, 14, p. 524-528) was sedimented at 6,000 rpm. Fifty ml of HCP paste were added to the supernatant solution, and the mixture was stirred for three minutes with a Mag-Mix magnetic stirrer. This mixture was centrifuged at 6,000 rpm for ten minutes, and the supernatant solution was recentrifuged at 8,500 rpm for ten minutes. The resulting amber-colored supernatant solution was centrifuged in the Spinco Model L ultracentrifuge, No. 40 rotor, for two hours at 38,000 rpm. The supernatant solution was discarded and the pellets were resuspended in 0.01 M phosphate buffer, pH 7.5. The resuspended pellets were combined and recentrifuged at 38,000 rpm for two hours, and the resulting pellet was suspended again in phosphate buffer. This solution was centrifuged for ten minutes at 8,500 rpm, and the
supernatant solution obtained was assayed to determine virus activity. Much of the virus activity present in the crude sap extract was lost during clarification with HCP (Table 4).

Table 4. Activity of dahlia virus 1 at several stages of purification.

<table>
<thead>
<tr>
<th>Stage of purification</th>
<th>No. of plants inoculated</th>
<th>No. of plants infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude juice</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Crude juice after treatment with HCP</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>HCP plus adsorbed components</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Partially purified virus</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

**Serology.** Although the activity of dahlia virus 1 was greatly reduced after treatment with HCP, the purified virus suspension was used for rabbit immunization. This was necessary since the amount of *Verbesina* tissue was limited.

After a sample of normal serum was obtained from the rabbit to be used for immunization with dahlia virus 1, partially purified dahlia virus 1 suspended in 0.85% NaCl was injected intravenously into the rabbit at weekly intervals. Six 1-ml injections were administered. A 30-ml sample of blood was taken from the rabbit the seventh week after the first injection.

No reaction was observed in subsequent microprecipitin and gel diffusion tests when the dahlia virus 1 antiserum was tested with its homologous antigen.
Dahlia Virus 2 and Cucumber Mosaic Virus (CMV)

Symptoms of dahlia virus 2 and CMV on several hosts. Symptoms produced on several hosts by dahlia virus 2 were observed to be similar to those produced by CMV (Tables 5 and 6; Figures 7 and 8).

Both viruses produced small necrotic pin-point local lesions, often in the form of rings, on Necrotic Turk tobacco. Dahlia virus 2 produced systemic chlorosis along the leaf veins and chlorotic rings. Small necrotic spots similar to those expressed on inoculated leaves were also present. Older plants were symptomless and the leaves expanded normally. CMV induced severe leaf distortion with light and dark green patches on secondary leaves. Neither virus infected Bountiful bean or black cowpea. Symptoms expressed by the two viruses were similar on horsebean. Dahlia virus 2 produced a systemic mottle on the new growth of Verbesina which became symptomless with age. A marked chlorosis and distortion of the uninoculated leaves was induced on this host by CMV.

Although symptoms expressed by these viruses on a single host varied, the host range was similar.

Thermal inactivation of dahlia virus 2 and CMV. Thermal inactivation studies of dahlia virus 2 and CMV were conducted using systemically infected Necrotic Turk tobacco tissue as a source of virus. The tissue was macerated in 0.02 M Na$_2$HPO$_4$-NaH$_2$PO$_4$ buffer, pH 7.5, squeezed through cheesecloth, and diluted 1:10 (w/v). Tubes with 3-ml samples of inoculum were treated at 50°, 55°, 60°, and 65°C for ten minutes, then cooled immediately. Local lesion assays on Necrotic Turk tobacco
### Table 5. Symptoms of dahlia virus 2 on several hosts.

<table>
<thead>
<tr>
<th>Host</th>
<th>Local lesions</th>
<th>Systemic reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nicotiana tabacum x N. glutinosa</strong> (Necrotic Turk tobacco)</td>
<td>Small necrotic pin-point spots often forming ring</td>
<td>Chlorotic spots, small chlorotic stipple; older plants appear symptomless</td>
</tr>
<tr>
<td><strong>Vigna sinensis</strong> (Black cowpea)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><strong>Vicia faba</strong> (Horsebean)</td>
<td>Necrotic spots becoming diffuse, necrotic rings</td>
<td>None</td>
</tr>
<tr>
<td><strong>Phaseolus vulgaris</strong> (Bountiful bean)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><strong>Chenopodium amaranticolor</strong></td>
<td>Chlorotic spots</td>
<td>None</td>
</tr>
<tr>
<td><strong>Cucurbita maxima</strong> (Buttercup squash)</td>
<td>Chlorotic rings and spots, enlarging and fusing to form necrotic patch</td>
<td>A few chlorotic spots</td>
</tr>
<tr>
<td><strong>Cucurbita pepo</strong> (White scallop squash)</td>
<td>Not distinct</td>
<td>Chlorotic spots</td>
</tr>
<tr>
<td><strong>Cucurbita pepo</strong> (Small sugar pumpkin)</td>
<td>Chlorotic spots</td>
<td>Chlorotic spots</td>
</tr>
<tr>
<td><strong>Zinnia elegans</strong> (Zinnia)</td>
<td>Faint chlorotic spots</td>
<td>Chlorotic spots</td>
</tr>
<tr>
<td><strong>Cucumis sativus</strong> (Cucumber, var. Chicago Pickling)</td>
<td>Chlorotic spots</td>
<td>Chlorotic spots</td>
</tr>
<tr>
<td><strong>Verbesina encelioides</strong></td>
<td>None</td>
<td>Systemic mottle, no leaf distortion; symptomless with age</td>
</tr>
<tr>
<td><strong>Capsicum frutescens</strong> (Pepper)</td>
<td>Necrotic rings and spots</td>
<td>Malformed and chlorotic leaves, some veinal necrosis</td>
</tr>
</tbody>
</table>
Table 6. Symptoms of cucumber mosaic virus (common type) on several hosts.

<table>
<thead>
<tr>
<th>Host</th>
<th>Local lesions</th>
<th>Systemic reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotiana tabacum x N. glutinosa (Necrotic Turk tobacco)</td>
<td>Small necrotic pinpoint spots</td>
<td>Leaves distorted, narrow silver leaf; dark green patches on lighter field</td>
</tr>
<tr>
<td>Vigna sinensis (Black cowpea)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Vicia faba (Horsebean)</td>
<td>Necrotic spots, enlarging with age; some veinal necrosis</td>
<td>None</td>
</tr>
<tr>
<td>Phaseolus vulgaris (Bountiful bean)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cucurbita maxima (Buttercup squash)</td>
<td>Concentric chlorotic rings, becoming necrotic as they enlarge</td>
<td>A few chlorotic spots becoming necrotic</td>
</tr>
<tr>
<td>Cucurbita pepo (White scallop squash)</td>
<td>Chlorotic spots, becoming necrotic as they enlarge</td>
<td>Chlorotic spots</td>
</tr>
<tr>
<td>Cucumis sativus (Cucumber, var. Chicago Pickling)</td>
<td>Chlorotic spots, becoming diffuse</td>
<td>Chlorotic spots</td>
</tr>
<tr>
<td>Verbesina encelioides</td>
<td>None</td>
<td>Chlorosis, distortion of leaves</td>
</tr>
<tr>
<td>Capsicum frutescens (Pepper)</td>
<td>Necrotic rings, becoming diffuse</td>
<td>Some veinal necrosis</td>
</tr>
</tbody>
</table>
Figure 7. Symptoms of dahlia virus 2 on several hosts.
Figure 8. Symptoms of cucumber mosaic virus on several hosts.

Verbesina encelioides - Systemic

Cucumber (Chicago Pickling) - Systemic

Buttercup Squash - Local Lesions

Horsebean - Local Lesions
are recorded in Table 7.

Data from these experiments showed that both CMV and dahlia virus 2 are inactivated after treatment of inoculum at 60°C for ten minutes. These results indicate that CMV and dahlia virus 2 possess similar thermal-stable properties.

Partial purification of dahlia virus 2. Dahlia virus 2 was partially purified using the procedure outlined by Grogan (16) for purification of cucumber mosaic virus. This procedure is outlined in Table 8. One-hundred-forty g of small sugar pumpkin cotyledons were harvested 8-10 days after inoculation. The tissue was blended in chilled 0.1 M Na₂HPO₄-NaH₂PO₄ buffer, pH 7.5, and was then stored overnight in a cold room at 4°C. The mixture was expressed through cheesecloth and clarified at 8,500 rpm in a Servall refrigerated centrifuge. Pellets were discarded and the supernatant solution was acidified to pH 5.0 with 1.0 N HCl. After 30 minutes the pH was readjusted to 7.0 with 1.0 N NaOH. A strong precipitate developed which was sedimented at 8,500 rpm for 20 minutes. The supernatant solution was centrifuged at 40,000 rpm for 90 minutes in a Spinco Model L ultracentrifuge, No. 40 rotor. Resuspension of the resulting pellets in 0.01 M borate buffer, pH 7.5, prevented aggregation of the virus. This suspension was centrifuged at 10,000 rpm for ten minutes. The supernatant solution was recentrifuged in the No. 40 rotor at 40,000 rpm for 40 minutes, and the pellets obtained were resuspended in borate buffer. The alternate low- and high-speed centrifugation cycles were repeated, and the supernatant solution obtained after the final
Table 7. Thermal inactivation temperatures of dahlia virus 2 and cucumber mosaic virus (common type).

<table>
<thead>
<tr>
<th>Treatment temperature</th>
<th>No. of leaves inoculated</th>
<th>Average No. of lesions/leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host plant</td>
<td>for 10 minutes</td>
<td></td>
</tr>
<tr>
<td><strong>Cucumber Mosaic Virus</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Necrotic Turk tobacco</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50°C</td>
<td>8</td>
<td>Too numerous to count</td>
</tr>
<tr>
<td>55°C</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>60°C</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>Too numerous to count (200+)</td>
</tr>
<tr>
<td><strong>Dahlia Virus 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Necrotic Turk tobacco</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50°C</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>55°C</td>
<td>9</td>
<td>5</td>
</tr>
<tr>
<td>60°C</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>Too numerous to count (100+)</td>
</tr>
</tbody>
</table>


Table 8. Purification procedure for dahlia virus 2.

Infected tissue (Small sugar pumpkin)

Blend 2 minutes in chilled 0.1 M \( \text{PO}_4 \) buffer (pH 7.5)
Keep at 4°C overnight
Express through cheesecloth
Clarify at 8,500 rpm for 20 minutes
Keep supernatant

Acidify with 1.0 N HCl (pH 5.0)
Keep at 10°C for 30 minutes
Readjust to pH 7.0 with 1.0 N NaOH
Clarify at 8,500 rpm for 20 minutes
Keep supernatant

Centrifuge 90 minutes at 30,000 rpm
Keep pellets

Resuspend in 0.01 M borate buffer (pH 7.5)
Elute 1½-2 hours at 4°C
Clarify at 10,000 rpm for 10 minutes
Keep supernatant

Centrifuge 40 minutes at 40,000 rpm
Keep pellets

Resuspend pellets in 0.01 M borate buffer (pH 7.5)
Elute 1½-2 hours at 4°C
Clarify at 10,000 rpm for 10 minutes
Keep supernatant

Resuspend pellets in 0.01 M borate buffer (pH 7.5)
Elute 1½-2 hours at 4°C
Clarify 10,000 rpm for 10 minutes
Keep supernatant - partially purified preparation
low-speed centrifugation constituted the partially purified virus preparation.

Ultraviolet absorption of partially purified dahlia virus 2. The ultraviolet absorption spectra of dahlia virus 2 and a healthy preparation purified according to the same procedure are given in Figure 9. (A) shows the absorption spectrum of the extracts from diseased plants following acidification and subsequent low-speed centrifugation. (B) shows the absorption spectrum following the first high-speed centrifugation. (C) shows the absorption spectrum after the final high-speed centrifugation. The absorption spectrum of the healthy preparation was determined after the final high-speed centrifugation. Both curves show typical absorption for nucleoprotein with a maximum at 263 μ and minimum at 243 μ. Although the partially purified virus was highly infectious, no differences were noted in the absorption spectra of preparations from diseased and healthy plants.

Serology. Immunization of rabbits followed the same procedure as outlined for dahlia virus 1.

Activity of the antiserum was determined using the Ouchterlony agar double-diffusion test. The test was conducted using the absorbed dahlia virus 2 antiserum and its homologous antigen. The antiserum was absorbed 1:2 with partially purified healthy small sugar pumpkin cotyledons and tested against crude sap extracts squeezed from Necrotic Turk tobacco systemically infected with dahlia virus 2. An extract squeezed from healthy Necrotic Turk tobacco was used as a control. No reaction was observed in the healthy control
Figure 9. Ultraviolet absorption spectra of partially purified dahlia virus 2 and healthy small sugar pumpkin. Recordings were made using a Beckman Model DB spectrophotometer.

Diseased Preparation

A = spectrum of the suspension after acidification and low-speed centrifugation

B = spectrum of the suspension after first high-speed centrifugation

C = spectrum of the suspension after final high-speed centrifugation

Healthy Preparation

Spectrum of suspension following final high-speed centrifugation
Figure 10. Ouchterlony double-diffusion test of CMV (Imperial Strain 78) antiserum with dahlia virus 2 and CMV antigens and healthy control.

Plate A
1 = crude sap from Necrotic Turk tobacco infected with dahlia virus 2
2 = CMV (Imperial Strain 78) antiserum
3 = crude sap from healthy Necrotic Turk tobacco

Plate B
1 = saline
2 = partially purified healthy small sugar pumpkin
3 = partially purified small sugar pumpkin infected with dahlia virus 2
4 = crude sap extract from healthy Necrotic Turk tobacco
5 = crude sap extract from Necrotic Turk tobacco infected with dahlia virus 2
6 = crude sap extract from Necrotic Turk tobacco infected with CMV (common type)
7 = CMV (Imperial Strain 78) antiserum
but a zone of precipitation developed between the dahlia virus 2 antiserum and its homologous antigen. Crude juice squeezed from Necrotic Turk tobacco systemically infected with CMV was tested with the dahlia virus 2 antiserum, but no reaction occurred.

An agar double-diffusion test was conducted using Grogan's CMV antiserum with dahlia virus 2 (Figure 10, Plate A) and CMV in adjacent wells (Figure 10, Plate B). The viruses were in crude sap squeezed from systemically infected Necrotic Turk tobacco. A strong zone of precipitation developed between the CMV antiserum and the CMV antigen. The dahlia virus 2 precipitation zone was connected with the zone from CMV, indicating that the antigens of these two viruses possess similar diffusion rates. No reaction was present opposite wells containing crude sap extracts from healthy Necrotic Turk tobacco.

Dahlia Virus 3 and Tomato Spotted Wilt Virus (TSWV)

Symptoms of dahlia virus 3 and TSWV on several hosts. Dahlia virus 3 and TSWV gave similar reactions on several herbaceous hosts (Tables 9 and 10; Figures 11 and 12). Necrotic spot local lesions were present on leaves of Necrotic Turk inoculated with TSWV and dahlia virus 3. Both viruses induced severe systemic veinal necrosis. Chlorotic local lesions were formed on leaves of black cowpea inoculated with these viruses. Neither virus produced symptoms on Bountiful bean, squash, or Chenopodium. Both viruses produced similar symptoms on zinnia, and Bonny Best tomato was infected by both viruses. Host range studies indicated a possible relationship between TSWV and
Table 9. Symptoms of dahlia virus 3 on several hosts.

<table>
<thead>
<tr>
<th>Host</th>
<th>Local lesions</th>
<th>Systemic reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Nicotiana tabacum</em> x <em>N. glutinosa</em> (Necrotic Turk tobacco)</td>
<td>Necrotic spots enlarging with age</td>
<td>Severe systemic necrosis of the veins</td>
</tr>
<tr>
<td><em>Vigna sinensis</em> (Black cowpea)</td>
<td>Chlorotic spots, necrotic centers developing with age</td>
<td>Systemic chlorotic spotting and vein banding</td>
</tr>
<tr>
<td><em>Vicia faba</em> (Horsebean)</td>
<td>Necrotic spots becoming diffuse with age</td>
<td>None</td>
</tr>
<tr>
<td><em>Datura stramonium</em> var. <em>tatula</em></td>
<td>None</td>
<td>Leaf distortion and puckering, light and dark green patches</td>
</tr>
<tr>
<td><em>Phaseolus vulgaris</em> (Bountiful bean)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><em>Chenopodium amaranticolor</em></td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><em>Cucurbita maxima</em> (Buttercup squash)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><em>Cucurbita pepo</em> (White scallop squash)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td><em>Zinnia elegans</em> (Zinnia)</td>
<td>Chlorotic spots</td>
<td>Mottle and chlorotic patches</td>
</tr>
<tr>
<td><em>Lycopersicon esculentum</em> (Bonny Best tomato)</td>
<td>Necrotic spots</td>
<td>Purpling and necrosis</td>
</tr>
<tr>
<td><em>Gomphrena globosa</em> (Globe amaranth)</td>
<td>Necrotic rings</td>
<td>Necrosis and distortion of the leaves</td>
</tr>
</tbody>
</table>
Table 10. Symptoms of tomato spotted wilt virus on several hosts.

<table>
<thead>
<tr>
<th>Host</th>
<th>Local lesions</th>
<th>Systemic reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotiana tabacum x N. glutinosa</td>
<td>Necrotic spots enlarging with age</td>
<td>Severe systemic necrosis of the veins</td>
</tr>
<tr>
<td>(Necrotic Turk tobacco)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vigna sinensis (Black cowpea)</td>
<td>Chlorotic spots with necrotic centers in some lesions</td>
<td>Chlorotic mottle</td>
</tr>
<tr>
<td>Vicia faba (Horsebean)</td>
<td>Small necrotic rings and spots, spots becoming diffuse</td>
<td>None</td>
</tr>
<tr>
<td>Datura stramonium var. tatula</td>
<td>Not distinct</td>
<td>Chlorosis, leaves puckered, light and dark green blotches</td>
</tr>
<tr>
<td>Phaseolus vulgaris (Bountiful bean)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Chenopodium amaranticolor</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cucurbita maxima (Buttercup squash)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cucurbita pepo (White scallop squash)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Zinnia elegans (Zinnia)</td>
<td>Not distinct</td>
<td>Puckering of leaves, chlorosis</td>
</tr>
<tr>
<td>Lycopersicon esculentum (Bonny Best tomato)</td>
<td>Necrotic spots</td>
<td>Purpling and necrosis</td>
</tr>
<tr>
<td>Gomphrena globosa (Globe amaranth)</td>
<td>Necrotic rings</td>
<td>Necrosis and distortion of the leaves</td>
</tr>
</tbody>
</table>
Figure 11. Symptoms of dahlia virus 3 on Kentucky 56 and Necrotic Turk tobacco.
Figure 12. Symptoms of tomato spotted wilt virus on several hosts.
dahlia virus 3.

**Thermal inactivation of dahlia virus 3 and TSWV.** Thermal inactivation temperatures of dahlia virus 3 and TSWV were determined (Table 11). Two g of systemically infected Necrotic Turk tobacco were macerated in 0.02 M Na$_2$HPO$_4$-NaH$_2$PO$_4$ buffer, pH 7.5. The extract was squeezed through cheesecloth and diluted 1:10 (w/v). Tubes containing three-ml samples of inoculum were treated at 36°, 38°, 40°, 42°, and 44°C for ten minutes and cooled immediately. Local lesion assays were made on Necrotic Turk tobacco. TSWV was inactivated when treated at 44°C for ten minutes, and dahlia virus 3 was inactivated in the sample treated at 42°C for ten minutes. Thus, dahlia virus 3 possesses a low temperature of inactivation, which is characteristic of tomato spotted wilt virus.

**Partial purification of dahlia virus 3.** Partial purification of TSWV has been reported by Black (3). He extracted the virus in 0.01 M sodium bisulfite and 0.1 M neutral phosphate buffer but lost approximately 50% of the virus activity during the extraction procedure. In the present study an attempt was made to increase the stability of TSWV. The virus was completely inactivated during purification when Necrotic Turk tobacco systemically infected with dahlia virus 3 was macerated in phosphate buffer, pH 7.5, containing 0.1% sodium thioglycollate. A buffer containing 0.1 M glycine, 0.05 M K$_2$HPO$_4$, and 0.03 M NaCl (GPS buffer), adjusted to pH 9.5, reported by Diener (12) for extraction of TMV infectious RNA increased virus activity in the first 30 minutes, however, the virus was inactivated after one hour.
<table>
<thead>
<tr>
<th>Host plant</th>
<th>Treatment temperature</th>
<th>No. of leaves inoculated</th>
<th>Average No. of lesions/leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tomato Spotted Wilt (Type)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Necrotic Turk tobacco</td>
<td>38°C</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>40°C</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>42°C</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>44°C</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>9</td>
<td>Too numerous to count (100+)</td>
</tr>
<tr>
<td><strong>Dahlia Virus 3</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Necrotic Turk tobacco</td>
<td>36°C</td>
<td>8</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>38°C</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>40°C</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>42°C</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>9</td>
<td>35</td>
</tr>
</tbody>
</table>
Control suspensions in phosphate buffer were inactivated after 30 minutes. Due to the instability of TSWV in vitro, the virus was not successfully purified and an antiserum was not prepared.

**Dahlia Virus 4 and Tomato Ringspot Virus**

**Symptoms of dahlia virus 4 and tomato ringspot virus on several hosts.** Dahlia virus 4 produced local necrotic spots and rings on Bountiful bean suggestive of tomato ringspot virus. The systemic movement and severe systemic necrosis caused by the tomato ringspot type culture on Bountiful beans were not common on Bountiful beans inoculated with dahlia virus 4. Although severe systemic necrosis appeared on some of these plants, many exhibited normal secondary growth. Necrotic Turk tobacco was infected infrequently by dahlia virus 4, but this host was readily infected with tomato ringspot virus, which produced necrotic local lesions. The hosts tested and local and systemic reactions produced by dahlia virus 4 and tomato ringspot virus are given in Tables 12 and 13 and in Figures 13, 14, and 15. Although host symptoms differed, the range of hosts infected by the two viruses was similar.

**Thermal inactivation of dahlia virus 4 and tomato ringspot virus.** Thermal inactivation temperatures of dahlia virus 4 and tomato ringspot virus were compared (Table 14). Primary leaves of Bountiful bean plants inoculated with dahlia virus 4 were harvested 7-8 days after inoculation. Inoculum for the tomato ringspot thermal inactivation study was from systemically infected Bountiful bean. The tissue was
Table 12. Symptoms of dahlia virus 4 on several hosts.

<table>
<thead>
<tr>
<th>Host</th>
<th>Local lesions</th>
<th>Systemic reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicotiana tabacum x N. glutinosa (Necrotic Turk tobacco)</td>
<td>Necrotic rings</td>
<td>Some veinal necrosis</td>
</tr>
<tr>
<td>Vigna sinensis (Black cowpea)</td>
<td>Necrotic rings and spots and veinal necrosis</td>
<td>Usually symptomless, occasionally chlorotic spots</td>
</tr>
<tr>
<td>Vicia faba (Horsebean)</td>
<td>Necrotic spots and rings</td>
<td>Occasional necrotic spots</td>
</tr>
<tr>
<td>Datura stramonium var. tatula</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Phaseolus vulgaris (Bountiful bean)</td>
<td>Necrotic and chlorotic rings and spots</td>
<td>May or may not develop severe necrosis; secondary growth may be symptomless</td>
</tr>
<tr>
<td>Chenopodium amaranticolor</td>
<td>Chlorotic spots</td>
<td>Distortion of leaves and chlorosis</td>
</tr>
<tr>
<td>Cucurbita maxima (Buttercup squash)</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>Cucumis sativus (Cucumber, var. Chicago Pickling)</td>
<td>Chlorotic spots</td>
<td>Some veinal chlorosis and chlorotic spots</td>
</tr>
<tr>
<td>Verbesina encelioides</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>
Table 13. Symptoms of tomato ringspot virus on several hosts.

<table>
<thead>
<tr>
<th>Host</th>
<th>Local lesions</th>
<th>Systemic reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nicotiana tabacum x N. glutinosa</strong></td>
<td>Necrotic spots</td>
<td>Some necrotic spots, necrotic streaks in irregular patterns</td>
</tr>
<tr>
<td>(Necrotic Turk tobacco)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vigna sinensis</strong></td>
<td>Necrotic spots and rings with veinal necrosis</td>
<td>Chlorotic spots and vein banding</td>
</tr>
<tr>
<td>(Black cowpea)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vicia faba</strong></td>
<td>Large necrotic spots</td>
<td>Necrotic spots on some leaves, most are symptomless</td>
</tr>
<tr>
<td>(Horsebean)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Datura stramonium var. tatula</strong></td>
<td>Necrotic spots, death of inoculated leaves</td>
<td>Systemic chlorosis and stunting of the plant</td>
</tr>
<tr>
<td><strong>Phaseolus vulgaris</strong></td>
<td>Necrotic and chlorotic rings and spots</td>
<td>Severe necrosis and death of the secondary growth</td>
</tr>
<tr>
<td>(Bountiful bean)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Chenopodium amaranticolor</strong></td>
<td>Chlorotic spots</td>
<td>Distortion of leaves and chlorosis</td>
</tr>
<tr>
<td><strong>Cucurbita maxima</strong></td>
<td>Necrotic ringspots enlarging to develop necrotic patches</td>
<td>None</td>
</tr>
<tr>
<td>(Buttercup squash)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cucurbita pepo</strong></td>
<td>Pin-point chlorotic spots, becoming necrotic</td>
<td>None</td>
</tr>
<tr>
<td>(White scallop squash)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cucumis sativus</strong></td>
<td>Chlorotic spots</td>
<td>Veinal chlorosis and some necrotic spots</td>
</tr>
<tr>
<td>(Cucumber, var. Chicago Pickling)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Verbesina encelioides</strong></td>
<td>Chlorotic ringspots</td>
<td>None</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Host plant</th>
<th>Treatment temperature (°C)</th>
<th>No. of leaves for 10 minutes inoculated</th>
<th>Average No. of lesions/leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tomato Ringspot Virus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bountiful bean</td>
<td>54°C</td>
<td>10</td>
<td>92</td>
</tr>
<tr>
<td>&quot;</td>
<td>56°C</td>
<td>10</td>
<td>46</td>
</tr>
<tr>
<td>&quot;</td>
<td>58°C</td>
<td>10</td>
<td>22</td>
</tr>
<tr>
<td>&quot;</td>
<td>60°C</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>Control</td>
<td>10</td>
<td>120</td>
</tr>
<tr>
<td><strong>Dahlia Virus 4</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bountiful bean</td>
<td>54°C</td>
<td>10</td>
<td>172</td>
</tr>
<tr>
<td>&quot;</td>
<td>56°C</td>
<td>10</td>
<td>97</td>
</tr>
<tr>
<td>&quot;</td>
<td>58°C</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>60°C</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>&quot;</td>
<td>Control</td>
<td>10</td>
<td>157</td>
</tr>
</tbody>
</table>
Figure 13. Symptoms of dahlia virus 4 on Bountiful bean.
Figure 14. Symptoms of dahlia virus 4 on horsebean and black cowpea.
Figure 15. Symptoms of dahlia virus 4 on Chenopodium amaranticolor, cucumber (Chicago Pickling) and Necrotic Turk tobacco.
macerated in 0.02 M Na$_2$HPO$_4$-NaH$_2$PO$_4$ buffer, pH 7.5, squeezed through cheesecloth, and diluted 1:10 (w/v). Tubes containing 3-ml samples of inoculum were treated at 54°, 56°, 58°, and 60°C for ten minutes, then cooled immediately. Local lesion assays were made on Bountiful bean. The dahlia virus 4 isolate was inactivated in samples treated at 60°C, and tomato ringspot virus was inactivated in samples treated at 58°C for ten minutes. These results suggest a possible relationship between the two viruses.

**Partial purification of dahlia virus 4.** An experiment was conducted to determine the pH at which the virus extraction buffer would yield maximum virus activity. Two g of inoculated primary Bountiful bean leaves showing prominent veinal necrosis were harvested eight days after inoculation. The tissue was macerated in Na$_2$HPO$_4$-NaH$_2$PO$_4$ buffer, pH 6.5-8.0, strained through cheesecloth, and diluted 1:10 (w/v). Extracts were assayed on Bountiful bean and local lesions were counted (Table 15). Results indicated that maximum virus activity was obtained with an extraction buffer of pH 7.5.

Table 15. Local lesion assays of dahlia virus 4 extracts at pH values from 6.5-8.0.

<table>
<thead>
<tr>
<th>pH</th>
<th>No. of Bountiful bean leaves inoculated</th>
<th>Average No. of lesions/leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>20</td>
<td>67</td>
</tr>
<tr>
<td>7.0</td>
<td>20</td>
<td>104</td>
</tr>
<tr>
<td>7.5</td>
<td>20</td>
<td>132</td>
</tr>
<tr>
<td>8.0</td>
<td>20</td>
<td>78</td>
</tr>
</tbody>
</table>
The dahlia virus was partially purified by the following procedure (Table 16). Sixty g of inoculated primary Bountiful bean leaves were harvested 8-10 days after inoculation, chilled overnight at 4°C, and macerated in 0.02 M Na₂HPO₄-NaH₂PO₄ buffer, pH 7.5. Hydrated calcium phosphate (HCP) prepared according to the procedure described by Fulton (13, 14, p. 524-528) was sedimented at 6,000 rpm. The sedimented HCP paste was added to the crude extract, and the mixture was stirred in a Mag-Mix magnetic stirrer for three minutes. After thorough mixing, the HCP was sedimented by centrifuging at 6,500 rpm for 15 minutes. If the resulting supernatant solution was not completely clarified, it was recentrifuged at 7,500 rpm for ten minutes. The pellet was discarded and the supernatant solution was sedimented at 38,000 rpm in the No. 40 rotor of a Spinco Model L ultracentrifuge. Two types of pellets were present. One of these pellets was on the bottom of the tube, while the other pellet was sedimented on the side of the tube. Active virus was present in both pellets. All but 0.5 ml of the supernatant solution was removed from the tubes with a syringe, leaving the two pellets in the tube. The pellets were resuspended in

0.02 M Na₂HPO₄-NaH₂PO₄ buffer, pH 7.5, and centrifuged at 7,500 rpm for ten minutes. The supernatant solution was centrifuged at 38,000 rpm for 120 minutes. The pellets obtained from this centrifugation were resuspended in phosphate buffer, pH 7.5, yielding the partially purified virus suspension.

Ultraviolet absorption of partially purified dahlia virus 4.

The ultraviolet absorption spectra of partially purified dahlia virus 4
Table 16. Purification procedure for dahlia virus 4.

**Infected tissue** (Bountiful beans, inoculated leaves harvested 8-10 days following inoculation)

Macerate in chilled 0.02 M PO₄ buffer (pH 7.5) - 1 ml of buffer per gram of tissue - express through cheesecloth and clarify at 6,500 rpm for 10 minutes
Keep supernatant

Add hydrated calcium phosphate paste 1:1 (original tissue weight: HCP volume)
Stir with Mag-Mix magnetic stirrer for 2-3 minutes and clarify at 6,500 rpm for 15 minutes
Keep supernatant

Centrifuge 120 minutes at 38,000 rpm
Keep pellets

Resuspend in 0.02 M PO₄ buffer (pH 7.5)
Elute 2-3 hours at 4°C
Clarify at 7,500 rpm for 10 minutes
Keep supernatant

Centrifuge 120 minutes at 38,000 rpm

Resuspend pellet in 0.02 M PO₄ buffer (pH 7.5)
Elute 2-3 hours at 4°C
Clarify at 7,500 rpm for 10 minutes
Keep supernatant - partially purified preparation
and a healthy extract from Bountiful beans prepared by the same purification procedure are presented in Figure 16. Although the purified virus was highly infectious, producing 85-125 lesions per Bountiful bean leaf, the typical nucleoprotein absorption spectrum with maximum and minimum absorption at 260 μm and 240 μm was not observed. The flat portion of the absorption curve between 250 μm and 280 μm may be due to interference from non-virus components.

**Serology.** Sixty g of inoculated primary leaves of Bountiful beans were harvested 8-10 days after inoculation and prepared for immunization of rabbits using the following procedure. The partially purified virus was suspended in 0.85% saline for injection into rabbits. A series of seven weekly intramuscular injections were administered. Two ml of the partially purified virus suspended in saline were emulsified 1:1 with Freund's incomplete adjuvant. Two ml were injected into each back leg of the rabbit. One-ml intravenous injections were administered three days apart at the beginning of the seventh week. A serum sample was obtained one week after the last intravenous injection.

The antiserum was absorbed with two volumes of healthy plant sap which was prepared by the same procedure as the virus antigen.

Microprecipitin tests were conducted according to the procedure outlined by Ball (1, p. 5-7) and Van Slogteren (48). Appropriate dilutions of antigen and antiserum were made in physiological saline solutions. Microdrops were spotted on plastic Petri dishes, and the dishes were flooded with mineral oil after the reaction was established.
Figure 16. Ultraviolet absorption spectra of partially purified dahlia virus 4 and healthy Bountiful bean. Recordings were made using a Beckman Model DB spectrophotometer.

Absorption spectrum of partially purified dahlia virus 4 after final high-speed centrifugation

Absorption spectrum of partially purified healthy Bountiful bean after final high-speed centrifugation
Reactions were observed after 3.5 hours. The dishes were placed in a refrigerator and were observed again after 13 and 22 hours.

The antiserum prepared for dahlia virus 4 reacted in a micro-precipitin test with its homologous antigen giving a dilution end point of 1/128 after 3.5 hours (Table 17). In a reaction between the dahlia virus antiserum and the tomato ringspot antigen, no reaction was observed after 3.5 hours, but a reaction of 1/16 was present after 22 hours (Table 18). When the tomato ringspot antiserum was reacted with its homologous antigen, a dilution end point of 1/64 was obtained after 22 hours (Table 19). The tomato ringspot antiserum was tested with the dahlia virus antigen in a microprecipitin test. No reaction was apparent after 3.5 hours, however, a dilution end point of 1/16 was present after 22 hours (Table 20).

Results of microprecipitin reactions indicated that the viruses possessed antigenic properties in common.

Ouchterlony double-diffusion tests were conducted according to the procedure described by Ball (1, p. 13-14).

The test of the dahlia virus antiserum and the homologous antigen at each stage of purification is shown in Figure 17. Crude sap squeezed from black cowpea infected with dahlia virus 4 reacted with the homologous antiserum forming two distinct zones of precipitation. The single reaction zone formed with sap squeezed from healthy black cowpea joined one of the two reaction zones formed with the diseased sap, indicating that one of the two precipitation zones from well 2 represented a healthy component in the reaction. The second zone of precipitation from well 2 corresponded to the zone produced by the
Table 17. Microprecipitin reaction of dahlia virus 4 antiserum with partially purified dahlia virus. Read after 3.5 hours.

<table>
<thead>
<tr>
<th>Antigen prepared from</th>
<th>Antiserum</th>
<th>Diseased plants</th>
<th>Healthy plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
<td>2+</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>3</td>
<td>2+</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>2+</td>
<td>2</td>
<td>1+</td>
</tr>
<tr>
<td>32</td>
<td>1+</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>64</td>
<td>1</td>
<td>1</td>
<td>t</td>
</tr>
<tr>
<td>128</td>
<td>t</td>
<td>t</td>
<td>0</td>
</tr>
<tr>
<td>256</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>512</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

No reaction occurred when diseased and healthy preparations were tested with normal serum.

<sup>a</sup> Dilution factor

<sup>b</sup> Saline (0.85%)

<sup>c</sup> 0 = no precipitation; 1 = slight; 2 = moderate; 3 = strong; t = trace

<sup>d</sup> The dahlia virus 4 antiserum was absorbed with 2 volumes of healthy black cowpea prepared by the same procedure as the virus antigen.
Table 18. Microprecipitin reaction of dahlia virus 4 antiserum with partially purified tomato ringspot. Read after 22 hours.

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Antigen prepared from</th>
<th>Healthy plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>1+ t</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>1+ t</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>t</td>
<td>0</td>
</tr>
<tr>
<td>32</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>64</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>128</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>500</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

No reaction occurred when diseased and healthy preparations were tested with normal serum.

---

<sup>a</sup> Dilution factor

<sup>b</sup> Saline (0.85%)

<sup>c</sup> 0 = no precipitation; 1 = slight; 2 = moderate; 3 = strong; t = trace

<sup>d</sup> The dahlia virus 4 antiserum was absorbed with 2 volumes of healthy black cowpea prepared by the same procedure as the virus antigen.
Table 19. Microprecipitin reaction of tomato ringspot antiserum with partially purified tomato ringspot virus. Read after 22 hours.

<table>
<thead>
<tr>
<th>Antigen prepared from</th>
<th>Diseased plants</th>
<th>Healthy plants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>16</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>32</td>
<td>t</td>
<td>t</td>
</tr>
<tr>
<td>64</td>
<td>t</td>
<td>0</td>
</tr>
<tr>
<td>128</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>S</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

No reaction occurred when diseased and healthy preparations were tested with normal serum.

---

<sup>a</sup> Dilution factor

<sup>b</sup> Saline (0.85%)

<sup>c</sup> 0 = no precipitation; 1 = slight; 2 = moderate; 3 = strong; t = trace

<sup>d</sup> The antiserum was absorbed with healthy plant antigen.
Table 20. Microprecipitin reaction of tomato ringspot antiserum with partially purified dahlia virus 4. Read after 22 hours.

<table>
<thead>
<tr>
<th>Antigen prepared from</th>
<th>Diseased plants</th>
<th>Healthy plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>l&lt;sup&gt;c&lt;/sup&gt;</td>
<td>l</td>
</tr>
<tr>
<td>4</td>
<td>l</td>
<td>1</td>
</tr>
<tr>
<td>8</td>
<td>t</td>
<td>t</td>
</tr>
<tr>
<td>16</td>
<td>t</td>
<td>0</td>
</tr>
<tr>
<td>32</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>64</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>128</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

No reaction occurred when diseased and healthy preparations were tested with normal serum.

---

<sup>a</sup> Dilution factor

<sup>b</sup> Saline (0.85%)

<sup>c</sup> 0 = no precipitation; l = slight; 2 = moderate; 3 = strong; t = trace

<sup>d</sup> The antiserum was absorbed with healthy plant antigen.
Figure 17. Ouchterlony double-diffusion test of dahlia virus 4 antiserum and dahlia virus 4 antigen at several stages of purification.

1 = partially purified healthy black cowpea

2 = crude sap from black cowpea infected with dahlia virus 4

3 = crude sap from healthy black cowpea

4 = crude sap from black cowpea infected with dahlia virus 4 after HCP

5 = saline

6 = partially purified dahlia virus 4 from black cowpea

7 = antiserum to dahlia virus 4 absorbed 1:2 with partially purified healthy black cowpea
partially purified dahlia virus 4 in well 6.

The test of the dahlia virus antiserum with the homologous antigen and tomato ringspot virus is shown in Figure 18. Reaction zones were present between the dahlia virus 4 antigen in wells 3, 5, and 6 and the homologous antiserum. Three weak zones of precipitation were observed between the dahlia virus 4 antiserum and the tomato ringspot antigen in wells 1 and 2. Therefore, tomato ringspot and dahlia virus 4 possess similar antigenic properties.

The reaction of dahlia virus 4 and a healthy control with the tomato ringspot antiserum is shown in Figure 19. A reaction zone was observed between the tomato ringspot antiserum and the dahlia virus 4 antigen in well 3. A slight halo of precipitation was observed in the healthy control which did not correspond with the reaction to the dahlia virus 4 antigen.

The reaction of the tomato ringspot antiserum and the homologous antigen and a healthy control is shown in Figure 20. The tomato ringspot antigen in well 3 reacted to form two zones of precipitation which were absent in the healthy control. A slight halo of precipitation was observed in this reaction which did not correspond to the reaction between the tomato ringspot antiserum and its homologous antigen.

The test of the tomato ringspot antiserum with the homologous antigen and dahlia virus 4 is shown in Figure 21. A single zone of precipitation was observed when tomato ringspot antiserum, wells 1 and 3, was tested with the dahlia virus 4 antigen, well 4. Two zones of precipitation were observed between the tomato ringspot antiserum and
Figure 18. Ouchterlony double-diffusion test of dahlia virus 4 anti-
serum, dahlia virus 4 and tomato ringspot antigens, and healthy control.

1 & 2 = partially purified tomato ring-
spot virus from black cowpea

3 = partially purified dahlia virus
from black cowpea

4 = partially purified healthy
black cowpea

5 & 6 = partially purified dahlia virus
from black cowpea

7 = dahlia virus antiserum
absorbed 1:2
with healthy black cowpea
Figure 19. Ouchterlony double-diffusion test of tomato ringspot antiserum, dahlia virus 4 antigen and healthy control.

1 = crude sap from healthy black cowpea
2 = tomato ringspot antiserum
3 = crude sap from black cowpea infected with dahlia virus 4
Figure 20. Ouchterlony double-diffusion test of tomato ringspot antiserum, tomato ringspot antigen and healthy control.

1 = crude sap from healthy black cowpea
2 = tomato ringspot antiserum
3 = crude sap from black cowpea infected with tomato ringspot
Figure 21. Ouchterlony double-diffusion test of tomato ringspot antiserum and tomato ringspot and dahlia virus 4 antigens.

1 = tomato ringspot antiserum
2 = crude sap from black cowpea infected with tomato ringspot virus
3 = tomato ringspot antiserum
4 = crude sap from black cowpea infected with dahlia virus 4
its homologous antigen in well 2. Therefore, a single antigenic component is common to both viruses.

The antigenic component common to dahlia virus 4 and tomato ringspot virus was also present in diffusion reactions with partially purified preparations of the two viruses.

**Electron microscopy.** Electron micrographs were taken of a partially purified preparation of dahlia virus 4 (Figure 22). The preparation was diluted 1:1 with a suspension of polystyrene latex diluted 1:500. Latex spheres of 264 μm were used as a standard in measuring the size of particles in the virus suspension. The virus suspension with added latex was sprayed on a Formvar-coated copper grid with a nebulizer. Preparations were shadowed in a Mikros vacuum evaporator at approximately 25° with an 80% platinum-20% palladium alloy. An RCA EMU-3 electron microscope was used to photograph the preparations.

Particles ranging in size from 36.5 to 73.6 μm were observed by direct measurement. Calculations were made to compensate for the effect of flattening on the particles in the partially purified preparation.

A measurement of the shadow cast by the polystyrene latex indicated that the length of the latex shadow was 1.5 times greater than the diameter of the latex sphere. The shadows cast by the particles in the partially purified virus suspension were 1.2 times greater than the particle diameters. Thus, the shadows cast by the particles in the virus suspension are 0.7-0.8 as long as the shadows cast by the
Figure 22. Electron micrograph of partially purified dahlia virus 4.
polystyrene sphere. Therefore, the actual diameters of the particles in the partially purified virus suspension would range from 25.5-58.8 μm.

Dahlia Virus Disease Therapy

Heat therapy. Holmes (21) reported that dormant dahlia roots did not stand heating. However, he presented no data and the heat tolerance of the roots was not reported.

In the present study dormant roots were treated in hot water in an attempt to inactivate the viruses present. Roots were coated with paraffin wax to prevent water-soaking injury during the hot-water treatment. Wax-coated roots were treated at 40°C and 45°C for one, two, and four hours. Temperature measurements were made to determine how rapidly the temperature in the center of the roots reached the equilibrium temperature of the water. A thermocouple was inserted into the center of the root at 5-minute intervals after immersion of the root in water. The equilibrium temperature of 40°C was reached after 15 minutes (Figure 23). Treated tubers were planted in No. 10 cans and placed in the greenhouse for further observation. Many roots were killed by the treatment, and surviving roots expressed virus symptoms in all cases (Table 21).

Chemotherapy. The use of substituted purines and pyrimidines possessing antiviral activity has been reviewed by Matthews (28, p. 74-90). These compounds may be incorporated into the virus nucleic acid and prevent multiplication of the virus.
Figure 23. Time required for dahlia roots to reach the equilibrium temperature in hot-water treatment.
Table 21. Hot-water treatment of dormant dahlia roots.

<table>
<thead>
<tr>
<th>Dahlia variety</th>
<th>Treatment temperature</th>
<th>Length of treatment</th>
<th>Growth of the root</th>
<th>Symptom expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue River</td>
<td>104°F</td>
<td>60 min.</td>
<td>+</td>
<td>Chlorotic mottle</td>
</tr>
<tr>
<td>Challenge</td>
<td>104°F</td>
<td>60 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>D'Arcy Sainsbury</td>
<td>104°F</td>
<td>60 min.</td>
<td>+</td>
<td>Vein chlorosis</td>
</tr>
<tr>
<td>Challenge</td>
<td>104°F</td>
<td>120 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Golden Heart</td>
<td>104°F</td>
<td>120 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Jap Bishop</td>
<td>104°F</td>
<td>120 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Joanna Pettit</td>
<td>104°F</td>
<td>120 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lily Belle</td>
<td>104°F</td>
<td>240 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Pink Sutherland</td>
<td>104°F</td>
<td>240 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>White Superior</td>
<td>104°F</td>
<td>240 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Jersey's Beauty</td>
<td>104°F</td>
<td>240 min.</td>
<td>+</td>
<td>Vein banding and mottle</td>
</tr>
<tr>
<td>Gay</td>
<td>113°F</td>
<td>60 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Challenge</td>
<td>113°F</td>
<td>60 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>D'Arcy Sainsbury</td>
<td>113°F</td>
<td>60 min.</td>
<td>+</td>
<td>Vein chlorosis</td>
</tr>
<tr>
<td>Jersey's Beauty</td>
<td>113°F</td>
<td>60 min.</td>
<td>+</td>
<td>Vein banding</td>
</tr>
<tr>
<td>Dutch Purple</td>
<td>113°F</td>
<td>120 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Golden Heart</td>
<td>113°F</td>
<td>120 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Helen Anita</td>
<td>113°F</td>
<td>120 min.</td>
<td>+</td>
<td>Vein chlorosis</td>
</tr>
<tr>
<td>Golden Drop</td>
<td>113°F</td>
<td>240 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lila's Superbe</td>
<td>113°F</td>
<td>240 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Pink Sutherland</td>
<td>113°F</td>
<td>240 min.</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Amber Queen</td>
<td>113°F</td>
<td>240 min.</td>
<td>+</td>
<td>Chlorosis along the midvein</td>
</tr>
</tbody>
</table>

\[1\]  + = growth  
- = no growth  
\[2\]  2 roots
Experiments were designed to obtain virus-free cuttings from apical meristems treated with 2-thiouracil. A 0.01 M solution of 2-thiouracil was applied to dahlia shoots about 3.0 cm in length, which were growing from roots planted in the greenhouse. The compound was made up in 0.01 N NaOH at pH 12.2, and the pH reduced to 9.0 with hydrochloric acid. Spray applications were made with an atomizer to sprouting shoots until runoff occurred. The compound was applied in three successive treatments at 3-day intervals beginning May 10. A marked chlorosis of the treated foliage developed after three days. After the second application, veinal necrosis and necrotic spotting were evident. Growth of the shoots was inhibited and the internodes failed to elongate. A second experiment was conducted using 0.001 M 2-thiouracil. The procedure used was the same as that described in the previous experiment. Treated leaves showed marked chlorosis three days after the first spray application. In both experiments terminal cuttings were taken 14 days after the last treatment and placed in sand under intermittent mist until rooted. Rooted cuttings were planted in No. 10 cans and placed in the greenhouse for observation. Symptoms were expressed on some cuttings taken from plants treated at both concentrations of 2-thiouracil, but other plants were symptomless. Cuttings from control plants also showed variation in symptom expression. Thus, 2-thiouracil did not inhibit the translocation of viruses into the apex of dahlia stems under conditions of these experiments.
Inhibitors present in macerated dahlia leaves rapidly inactivate viruses \textit{in vitro}. For this reason direct maceration of dahlia tissue followed by mechanical inoculation of several herbaceous hosts was not a successful method for transmission of viruses from dahlia. Therefore, Yarwood's leaf-disc method \textit{(51)}, which Mildner \textit{(31, p. 5)} reported to be a successful means of transmitting viruses from dahlia, was used. Three viruses were transmitted from dahlia using this technique, including tomato spotted wilt, cucumber mosaic, and a ringspot virus possessing host range and physical properties similar to those of tomato ringspot virus. The fourth isolate, dahlia mosaic virus, was obtained using Sänger and Gold's \textit{(40)} liquid-nitrogen transmission technique. These workers have suggested that enzymatic destruction of virus infectivity may be inhibited by low temperature. Liquid-nitrogen transmission of dahlia mosaic virus yielded a high percentage of transfers from dahlia to \textit{Verbesina encelioides} in August. Late-season transmission of the virus is considered to be more difficult due to an increase in inhibitors in older tissues. Brierley \textit{(9)} had limited success in transferring dahlia mosaic virus from dahlia to \textit{Verbesina} by mechanical inoculation in March and April.

Dahlia virus 1 was identified as dahlia mosaic virus based on host range and thermal inactivation studies. No new host was found for this virus and only members of the Compositae were infected. An Unwin's hybrid seedling inoculated with the virus expressed vein banding symptoms similar to those described for dahlia mosaic virus.
A strain of cucumber mosaic virus was isolated from dahlia variety Helen Anita. The virus was identified on the basis of host range and thermal inactivation properties. In gel diffusion tests a positive reaction was observed when dahlia virus 1 was tested with the CMV antiserum prepared by Grogan. Brierley (8) was unable to infect Unwin's hybrid seedlings with several strains of CMV. Dahlia virus 2, identified in this study as a strain of CMV, apparently differs from Brierley's isolates since Unwin's hybrids were infected with this isolate. Chlorotic banding along the midvein and mottle were prominent systemic symptoms in infected seedlings.

Tomato spotted wilt has been widely reported in dahlias (30). Dahlia virus 3 isolated from dahlia variety Amber Queen was identified as a strain of TSWV. Identification of dahlia virus 3 isolate was based on host range, temperature of inactivation, and instability in vitro. The virus isolate from Amber Queen and TSWV expressed different symptoms on Unwin's hybrid seedlings. The dahlia isolate produced necrotic local lesions, while TSWV produced concentric chlorotic rings on inoculated leaves. This isolate was not purified due to the unstable nature of the virus. Attempts to increase stability by the addition of various reducing substances to the extraction buffer were not successful.

Dahlia virus 4 isolate from variety Cay apparently differs from other viruses previously described on dahlia. This dahlia variety was hybridized in Oregon. Disease symptoms were recognized during field testing before introducing the variety. Severe virus-like symptoms were observed under field conditions and the variety was discarded. Dahlia virus 4 produced symptoms on several hosts suggestive of tomato
ringspot virus. Comparison of the heat stability of the dahlia isolate and tomato ringspot virus indicated that the viruses are inactivated at similar temperatures.

A dilution end point reaction of 1/16 was obtained when an antiserum prepared to dahlia virus 4 was tested against tomato ringspot virus and when the tomato ringspot antiserum was tested against dahlia virus 4. These dilution end points were observed after a 22-hour period of incubation. No reaction was observed between each antiserum and the heterologous antigen after 3.5 hours. The dahlia virus 4 antiserum reacted strongly with the homologous antigen after 3.5 hours with a dilution end point of 1/128, while tomato ringspot virus showed no reaction after the same period of time. A dilution end point of 1/64 was observed after 22 hours when tomato ringspot antiserum was tested with its homologous antigen. These reactions indicate that the two viruses possess antigens in common.

In gel diffusion tests, tomato ringspot antiserum tested against its homologous antigen and dahlia virus 4 produced a single reaction zone which was common to both viruses. This zone of precipitation could be the result of an antigenic component common to both viruses, which is induced in the host as a result of virus infection. The component could be partially formed virus possessing antigenic properties similar to both viruses or possibly a non-specific antigen.

Electron micrographs of partially purified dahlia virus 4 showed that diameters of particles in infective preparations varied from 25.5-58.5 μm. The occurrence of more than one particle size suggests that partially formed virus of a different size than the infective particles,
or possibly non-specific particles formed in response to host infection, may be present. Kahn et al. (23) reported a particle size of 43 x 13.5 mp for purified tomato ringspot virus. Most of the particles present in preparations of dahlia virus 4 were larger than those reported for tomato ringspot virus. However, freeze-dried preparations must be made to confirm this conclusion and to reduce the possibility of particle flattening.

Hot-water treatment of dormant dahlia roots was unsuccessful in eliminating viruses from naturally infected dahlias. About 70% of the treated roots were killed, and virus symptoms developed on new growth of all surviving roots. Wax coatings used to protect the dahlia roots from water-soaking injury were not completely impermeable, and water was in contact with the roots during the treatment period. These roots could have been injured by water soaking rather than by heat.

A 0.01 M solution of 2-thiouracil produced severe stunting and necrosis when sprayed on young dahlia sprouts. Plants treated at a concentration of 0.001 M 2-thiouracil showed less necrosis but leaf chlorosis was observed. Cuttings taken from untreated shoots of plants on which terminal growth was treated also showed virus symptoms. Although some plants did not express virus symptoms, it cannot be assumed that they were virus free until they are grown and tested under varied environmental conditions. 2-Thiouracil apparently did not inhibit the translocation of viruses into the apical meristem of dahlia plants under the conditions of these experiments.

Virus symptoms on naturally infected dahlia varieties differed from those which developed on Unwin's hybrid seedlings inoculated with
isolates from these varieties. Dahlia variety Cay showed chlorotic banding along the leaf midveins, but dahlia virus 4 isolated from this variety produced necrotic ring local lesions and systemic necrotic spotting with necrosis on the leaf margins on inoculated seedlings. Concentric chlorotic rings and chlorosis along the leaf midveins were observed on variety Amber Queen. Seedlings inoculated with dahlia virus 3 isolated from this variety developed necrotic ring local lesions, systemic necrotic spots, and chlorosis. Symptoms on seedlings inoculated with dahlia isolates 1 and 2 were similar to those on the varieties from which the isolations were made.

Symptom differences on naturally infected varieties and seedlings inoculated with the virus isolates from these varieties may be attributed to the presence of more than one virus in the naturally infected host or to differences in host response to infection by a single virus. Therefore, attempts to diagnose and identify dahlia viruses on the basis of symptoms are of limited value.

Virus diseases were widespread in the commercial dahlia plantings surveyed during the course of this work. Detection of virus diseases in the field was difficult because symptoms were often masked. Prominent symptoms developed on dahlia varieties grown in the greenhouse during the summer months, but the same varieties were symptomless when grown in the field. Therefore, it is important that reliable diagnostic tests be developed to detect virus-infected dahlias.
SUMMARY

1. Three viruses were transmitted from naturally infected dahlias using a modification of Yarwood's leaf-disc method (51).

2. A fourth isolate was obtained using liquid nitrogen in a transmission procedure described by Sänger and Gold (40).

3. Local and systemic symptoms of dahlia virus isolates 2, 3, and 4 on several hosts were similar to symptoms produced by cucumber mosaic, tomato spotted wilt, and tomato ringspot type cultures, respectively. A type culture of dahlia mosaic virus was not available for comparison with dahlia virus isolate 1.

4. Dahlia virus isolates 2, 3, and 4 possessed thermal inactivation properties similar to cucumber mosaic, tomato spotted wilt, and tomato ringspot viruses. Heat stability of dahlia virus isolate 1 was similar to that described in the literature for dahlia mosaic virus.

5. Because purification of dahlia virus 1 was only partially successful, an active antiserum was not prepared for this virus. Clarification of crude sap extracts from diseased Verbesina plants using hydrated calcium phosphate resulted in low virus activity.

6. Dahlia virus 2 (CMV) was partially purified using the procedure described by Grogan (16). This antiserum did not react positively with CMV in gel diffusion tests. An antiserum prepared by Grogan and Kimble (15) to CMV (Imperial strain 78) gave a positive reaction in gel diffusion tests indicating that dahlia virus 2 and CMV are antigenically related.
7. Dahlia virus 4 was partially purified using hydrated calcium phosphate as an adsorbing agent in the removal of host materials during clarification. An antiserum was prepared from the partially purified virus which reacted with tomato ringspot virus in micro-precipitin tests. Dahlia virus 4 also reacted with an antiserum prepared to tomato ringspot virus. In gel diffusion tests the major zone of precipitation present in the reaction between tomato ringspot antiserum and its homologous antigen was absent in the reaction between the tomato ringspot antiserum and dahlia virus 4. However, a single zone of precipitation was present in this reaction which corresponded to a second precipitation zone formed in the reaction between the tomato ringspot antiserum and its homologous antigen. Therefore, tomato ringspot virus and dahlia virus 4 possess common antigens.

8. Particles ranging in size from 25.5-58.8 nm were observed in air-dried preparations of partially purified dahlia virus 4 photographed with an electron microscope. Most of the particles observed were larger than the particle size reported for tomato ringspot virus (23).

9. Procedures used in heat and chemical therapy were not successful in eliminating viruses from naturally infected dahlias.

10. Virus diseases were widespread in dahlia plantings surveyed during the course of this work. Dahlias grown in the greenhouse showed distinct symptoms, but the same varieties were symptomless when grown in the field.
11. Virus symptoms on naturally infected varieties differed from those which developed on Unwin's hybrid seedlings inoculated with isolates from these varieties.

12. Since virus disease symptoms are often masked in dahlias, it is important to develop reliable methods for the diagnosis and identification of viruses in naturally infected dahlias.
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