Characteristics of Sweetpotato Whitefly-Mediated Silverleaf Syndrome and Associated Double-Stranded RNA in Squash

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ABSTRACT

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A 48-h feeding by 20 adult sweetpotato whiteflies (SPWF), *Bemisia tabaci*, resulted in 100% (63/63) of the squash (*Cucurbita pepo* 'Dixie') plants showing vein clearing and/or leaf silvering. No vein clearing and/or leaf silvering was apparent in squash plants exposed to SPWF for 8 h or less. The ability to mediate silverleaf symptoms was correlated with the density of SPWF. The percentage of plants that exhibited leaf silvering after 48-h feeding by 2, 5, 10, and 20 SPWF were 0, 13, 84, and 100%, respectively. Crude extracts from symptomatic squash leaves increased one hundredfold in RNA-dependent RNA polymerase activity when compared with extracts from asymptomatic, healthy tissue. Enzyme activity was dependent on Mg^{2+} and was insensitive to actinomycin D. Fractions of extracts with RNA polymerase activity also had distinctive 4.2- and 4.6-kb double-stranded RNA (dsRNA). Following glycerol density gradient separation of leaf extracts, dsRNA was found in fractions rich in membranes and nuclei but not in those containing chloroplasts. There were positive correlations between SPWF density, silverleaf rating, and RNA accumulation in symptomatic tissue. The dsRNA associated with silverleaf appears to be translocatable in the plant with limited synthesis in the host plant in the absence of SPWF. The presence of SPWF is necessary for continued silverleaf development. These results are consistent with the suggestion that the causal agent of silverleaf syndrome may be a virus or viruslike agent of SPWF.

Silverleaf syndrome, mediated by sweetpotato whitefly (SPWF), *Bemisia tabaci* (Gennadius), is a widespread disorder of squash in Florida (2,3,8,11,20). Silverleaf symptoms, which are similar but distinct from genetic silvering, occur throughout the growing season both in winter and summer squash with SPWF infestation. Furthermore, both young and old plants are equally susceptible, and silverleaf incidence increases markedly with increased SPWF infestation (K. R. Narayanan, *unpublished*). The nature of the silverleaf syndrome's causal agent and its association to *B. tabaci* is not yet clearly understood. Silverleaf syndrome was postulated to be caused by a toxin secreted by the SPWF during their feeding (20). However, Bharathan et al (3) have reported consistent presence of nuclease-resistant, double-stranded RNA (dsRNA) of sizes 4.2 and 4.6 kb in plants showing silverleaf syndrome, as well as in SPWF capable of mediating the syndrome. In contrast, asymptomatic plants and SPWF colonies unable to induce the syndrome did not contain the dsRNA. Bharathan et al (3) have postulated that the dsRNA could be the genome or a replicative form of a virus or viruslike agent.

The objectives of this work were to determine 1) the relationship between SPWF density, duration of SPWF feeding, and silverleaf development; 2) the quantitative relationship between dsRNA accumulation, SPWF density, and silverleaf symptom development. Because of the unusual nature of the syndrome, results from a variety of experiments aimed at elucidating the nature and ontogeny of the syndrome and its association with dsRNA and RNA-dependent RNA polymerase activity are all presented together.

MATERIALS AND METHODS

Whitefly culture and maintenance. *B. tabaci* adults collected from squash fields in the Homestead, FL, area during spring 1989 were reared and maintained in cantaloupe, *Cucumis melo* L. 'Sweet'n Early Hybrid', and/or squash, *Cucurbita pepo* L. 'Dixie', under conditions described previously (3). Unless otherwise stated, only adult SPWF were used in the experiments.

Relationship between SPWF density, feeding time, and silverleaf development. Unless stated otherwise, Dixie squash seedlings in the first true leaf stage of growth were used as test plants. In test 1, SPWF were introduced by aspiration in clip cages on leaf 1 (Fig. 1). Treatments included 1, 2, 5, 10, and 20 adults per plant. The first treatment contained 100 plants, whereas the second, third, fourth, and fifth treatments contained 45 plants each. After 48 h all SPWF eggs and adults were removed. Eggs were removed with Scotch tape under the microscope. Plants were sprayed with Thiodan before they were returned to the growth chamber. The growth chamber was maintained at 25 C with 16-h daylight and light intensity of 300 $\mu E \cdot m^{-2} \cdot s^{-1}$. Plants were periodically observed for the development of vein clearing and/or leaf silvering. Each leaf was rated 0–5 (0 = no vein clearing and/or leaf silvering, 5 = severe vein clearing and/or leaf silvering) 21 days after original infestation for severity of vein clearing and/or leaf silvering, as described previously (3). Unless stated otherwise, SPWF in groups of 20 were introduced in clip cages on leaf 1. In test 2, SPWF were allowed to feed for 2, 4, 8, 16, 24, or 48 h. Each treatment was replicated three times, and there were six plants per replication. SPWF eggs and adults were removed and the plants were sprayed with insecticides as previously described. In test 3, SPWF from test 1 (treatment 1) were transferred in groups of 20 and introduced in clip cages on leaf 1. A total of five plants were tested in this experiment. In test 4, SPWF groups of 20 were used in five successive serial transfers with 48-h feeding period each time. The insects that survived each transfer were grouped to have 20 SPWF in each clip cage on the first true leaf of each of five healthy squash seedlings.

Effect of SPWF density on RNA accumulation. Test plants were exposed to 2, 5, 10, or 20 SPWF for 48 h. SPWF were removed, and plants were sprayed with insecticide and transferred to the growth chamber. Tests contained five plants for each treatment with four replications. Some leaves were repeatedly sampled as the plants grew (Fig. 1). Samples included 7-mm leaf disks from leaves 1–4 sampled at 0, 2, 3, 7, 14, 21, and 35 days;

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leaves 5 and 6 sampled at 14, 21, and 35 days; and leaves 10–12 sampled on day 35. Single leaf disks from leaves 1–4, 5 and 6, 7–9, and 10–12 were grouped each time they were sampled. Samples were stored at −80 C until assayed. Two sets of single leaf disks were collected from plants on each sampling date. The first set of leaf disks from each replication of five plants were pooled and total RNA extracted as described below for dot spot nucleic acid hybridization. The remaining set was used for dsRNA extraction. Plants were scored for vein clearing and/or leaf silvering each time they were sampled.

In another test, leaf disks were collected from 10 different leaves from each plant; however, this time all of the samplings for RNA analyses were done 21 days after exposure to SPWF. Five plants were tested; leaf disks from each plant were pooled together and total RNA was extracted.

Total RNA extraction. Total RNA for dot spot nucleic acid hybridization was isolated from leaves using a combination of methods (7,10,14). The leaf disks were pulverized in liquid nitrogen and homogenized in 4.0 ml of extraction buffer (10 mM Tris-HCl, 5.0 M guanidine-HCl, 5 mM EGTA (ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid), 50 mM 2-mercaptoethanol, pH 7.5) for 30 s using a Polytron homogenizer (Brinkman Instruments, Westbury, NY). The brei was centrifuged at 10,000 g for 20 min at 4 C, and the supernatant was extracted two times with 0.5 vol of phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). After the addition of 0.10 vol 3.0 M sodium acetate, pH 5.2, and 2.5 vol ethanol, the RNA was stored at −20 C overnight.

Double-stranded RNA extraction and gel electrophoresis. Double-stranded RNA was extracted from leaf disks or 5–10 g of squash leaves by phenol extraction and cellulose column chromatography (12). Leaves were sampled at various times after SPWF feeding. The dsRNA was stored as ethanol sodium acetate suspension at −20 C and collected by centrifugation before electrophoresis. The dsRNA samples were resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM disodium ethylenediamine tetra acetic acid [EDTA]) and electrophoresed in 1% agarose gels in TAE buffer (40 mM Tris, 2 mM EDTA, 20 mM sodium acetate, pH 7.8) at 30 V/cm for 8 h. Following electrophoresis, gels were stained with ethidium bromide (50 ng/ml), and dsRNAs were visualized by UV transillumination and photographed with Polaroid (type 55) film.

Northern blot hybridization. The dsRNAs in gels were denatured in 0.05 M NaOH and 0.01 M NaCl for 30 min and neutralized in 0.1 M Tris-HCl, pH 7.5, by constant shaking at room temperature. The dsRNAs were electrophoretically transferred onto Hybond nylon membranes (Amersham Corp., Arlington Heights, IL) using a Transphor cell as recommended by the manufacturer (Hoefer Scientific Instruments, San Francisco, CA) and immobilized on the membrane by baking at 80 C for 2 h. The two dsRNA fragments (4.2 and 4.6 kb) were eluted from LGT-agarose (FMC, Rockland, ME), hydrolyzed by boiling in 100 μl of deionized formamide (nucleic acid grade, Bethesda Research Laboratories, Gaithersburg, MD) for 20 min (14), and labeled on the 5′ end with ^{32}P using T_4 polynucleotide kinase (9). The specific activity of the labeled RNA ranged from 3×10^7 to 5×10^8 cpm/μg RNA. Prehybridization, hybridization, and posthybridization conditions were as described (3). The hybridized membranes were wrapped in Saran wrap and autoradiographed using Kodak X-OMAT AR film. Exposure time ranged from overnight to several days at −80 C in the presence of an intensifying screen.

Quantitative dot spot hybridization assay. Total RNA from leaf tissue sampled at various times was denatured in 2.2 M formaldehyde and 50% formamide for 15 min at 65 C and spotted onto nylon membranes presoaked in $2 \times$ SSC ($1 \times$ SSC = 0.15 M sodium chloride and 0.15 mM sodium citrate, pH 7.0). The denatured RNA samples were applied as 10 μl spots, allowed to dry at room temperature, and immobilized on the membranes by cross-linking with UV light (302 nm) for 3–5 min. Membranes were probed with ^{32}P-labeled dsRNA as described above and, after sufficient autoradiography, individual spots were excised and counted in a Beckman 8000 scintillation counter (4,6,17).

Glycerol gradient fractionation. Thirty to forty grams of asymptomatic and symptomatic squash leaves (showing vein clearing and/or silvering) were homogenized with a mortar and pestle in 3 vol of extraction buffer (0.05 M Tris-HCl, 0.01 M KCl, 0.001 M EDTA, 0.003 M 2-mercaptoethanol, pH 7.4) (21). The homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 1,088 g for 15 min. The pellets were resuspended in 3 ml of buffer containing 0.05 M Tris-HCl, 0.01 M KCl, 0.01 M $MgCl_2$, 0.001 M EDTA, 5% glycerol (v/v), and 0.003 M 2-mercaptoethanol, pH 8.2 (21). The samples were loaded onto a discontinuous glycerol gradient (15) and centrifuged at 65,000 g on SW 28 Beckman rotor for 2 h. All of these steps were performed on ice or at 4 C. Three prominent bands (fractions), at depths of 1.5, 3.5, and 6.0 cm, and the pellet were collected; dsRNA was isolated by phenol extraction and cellulose chromatography as previously described. Nucleic acids from symptomatic tissue were treated with RNase-free pancreatic DNase I (Worthington Biochemical Corp., Freehold, NJ) as described previously (3). The fractions were analyzed for the presence of dsRNA by agarose gel electrophoresis.

Centrifugal analysis. Leaves from asymptomatic and symptomatic squash plants were pulverized in liquid nitrogen and homogenized (3 ml/g of tissue) in 0.6 M citrate phosphate buffer (pH 5.8, containing 0.001 M EDTA, 0.1% sodium diethyldithiocarbamate, and 0.5% 2-mercaptoethanol). The homogenate was filtered through two layers of Miracloth. The brei was centrifuged at 10,000 g for 10 min in a JA 20 rotor. Aliquots of the pellet (pellet 1) and the supernatant (supernatant 1) were saved for dsRNA extraction. The supernatant was centrifuged at 110,000 g for 90 min in an SW 28 rotor. Double-stranded

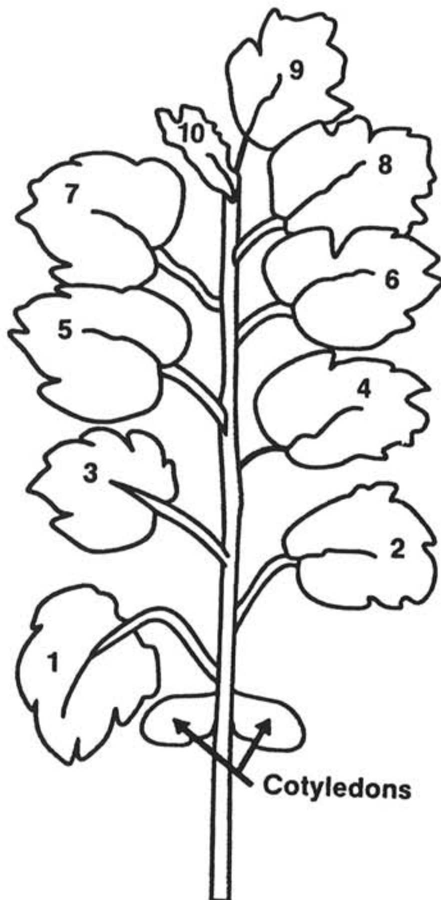

Fig. 1. Schematic representation of a squash plant. The numbers on the leaf indicate the position of the leaf on the plant. Different densities of the adult *Bemisia tabaci* in clip cages were allowed feeding access to leaf 1 for a predetermined time. Leaf disks from each of the leaves were sampled at various time intervals.

RNA was extracted from the resultant pellet (pellet 2) and the supernatant (supernatant 2), as well from pellet 1 and supernatant 1, and analyzed by agarose electrophoresis.

In vitro assay of pellet fractions for RNA polymerase activity. A typical reaction mixture in a total volume of 50 μl contained 50 mM Tris-HCl, pH 8.0; 8 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 1 mM each of adenosine triphosphate (ATP), cytidine triphosphate (CTP), guanidine triphosphate (GTP), and 0.1 mM uridine triphosphate (UTP); 1 μCi of [5,6-^{3}H] UTP (45 Ci/mmol, Amersham); and crude pellet preparations (20 μl). The reaction mixture was incubated for 1 h at 40 C and the reaction terminated by chilling on ice for 30 min, followed by addition of 10% TCA adjusted to contain 1 mM sodium pyrophosphate and 50 μg bovine serum albumin. The mixture was passed through GF/C filters (Whatman), washed successively with 10% TCA (5,18), and counted in a Beckman 8000 scintillation counter.

RESULTS

Relationship between SPWF density, duration of feeding, and development of silverleaf syndrome. In test 1, the percentages of plants that showed vein clearing and/or leaf silvering when exposed to 2, 5, 10, or 20 SPWF for 48 h were 0 (0/45), 13 (6/45), 84 (38/45), and 100% (45/45), respectively. A minimum of 48-h feeding by 20 SPWF resulted in 100% of the plants (18/18) showing leaf silvering (test 2). In the same test, SPWF feeding of 2, 4, or 8 h did not induce symptoms in any of the plants (0/18). However, longer periods of feeding (16 and 24 h) resulted in 11 (2/18) and 28% (5/18) of the plants showing vein clearing. When single SPWF were allowed a 48-h feeding, none of the plants (0/100) tested showed vein clearing and/or leaf silvering. However, when the same SPWF were allowed to feed for 48 h in groups of 20 (test 3), all of the plants (5/5) showed vein clearing followed by leaf silvering. In test 4 all of the plants (5/5) in each of five successive transfers showed vein clearing and/or leaf silvering.

Double-stranded RNA analysis and northern blot hybridization. Results of dsRNA analysis by gel electrophoresis from plants sampled at various time intervals after exposure to SPWF is shown in Figure 2. Typically, 4.2- and 4.6-kb dsRNA were detected by both gel electrophoresis and northern analysis only in plants sampled at 14, 21, and 35 days after initial SPWF infestation (Fig. 2A and B). Larger nucleic acid bands (23.1 kb) that are occasionally seen in some lanes have no sequence homology to 4.2- and 4.6-kb dsRNA when used as a probe (Fig. 2B) and are DNA. Double-stranded RNA or SPWF-related RNA was not detectable until 14 days. The dsRNA analysis of squash plants exposed to different SPWF densities and sampled at various time intervals is shown in Figure 3. There were considerable differences in the detectable levels of dsRNA not only from plants exposed to various densities of SPWF but also from position of the leaf relative to leaf 1 and time of sampling. No detectable levels of dsRNA were observed in leaves sampled at 0, 2, 3, and 7 days in any of the treatments (Fig. 3A and B, lanes 1–8). No dsRNA was detected in leaves 1–4 exposed to 2, 5, 10, or 20 SPWF and sampled at 14, 21, and 35 days (Fig. 3C and D, lanes 1–4). dsRNA was first detected on leaves 5 and 6 (Fig. 3C, lanes 6–8) sampled on day 14, but on plants exposed to 5, 10, and 20 SPWF, not on plants exposed to only 2 SPWF (Fig. 3C, lane 5). No dsRNA was detected from any of the leaves (5–12) exposed to 2 SPWF and sampled on days 21 and 35 (Fig. 3D, lanes 5 and 9; Fig. 3E, lanes 1, 5, and 9). Double-stranded RNA was detected up to the last sampling day (35) in leaves 10–12 exposed to 20 SPWF (Fig. 3E, lane 12), but not in plants exposed to 5 and 10 SPWF (Fig. 3E, lanes 10 and 11).

Influence of SPWF density on silverleaf development and accumulation and translocation of RNA. One or two SPWF failed to induce vein clearing and/or leaf silvering. In dot spot hybridization assays, leaves from such plants sampled at various times after SPWF infestation (0–35 days) revealed no detectable RNA. However, in experiments with 5, 10, or 20 SPWF, silverleaf ratings and levels of RNA increased with increasing numbers

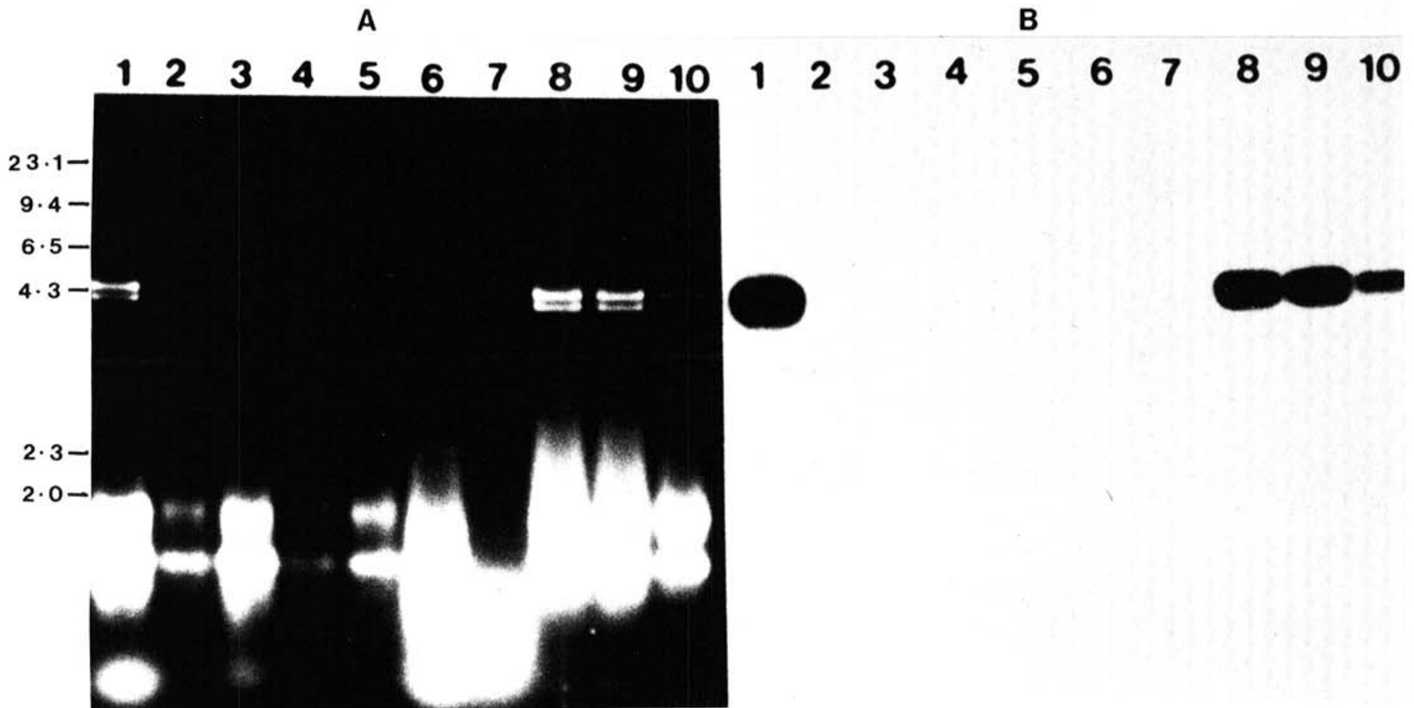

Fig. 2. Detection of silverleaf syndrome associated dsRNA by agarose gel electrophoresis and northern blotting. **A,** Double-stranded RNA from squash leaves sampled at various time intervals (after 48-h feeding) in 1% agarose gel stained with ethidium bromide. **B,** Northern blotting. Nucleic acids were transferred electrophoretically to a nylon membrane and the blot was hybridized to 4.2- and 4.6-kb dsRNA from silverleaf tissue. Lane 1, positive control; lane 2, asymptomatic control; lane 3, 0 days after sweetpotato whitefly (SPWF) feeding access; lane 4, 1 day after SPWF feeding access; lane 5, 2 days after SPWF feeding access; lane 6, 3 days after SPWF feeding access; lane 7, 7 days after SPWF feeding access; lane 8, 14 days after SPWF feeding access; lane 9, 21 days after SPWF feeding access; lane 10, 35 days after SPWF feeding access. The sizes (kb) of the lambda DNA *Hind*III-digested molecular weight markers are shown on the left.

of SPWF (Fig. 4). Quantitatively, levels of RNA were highest in leaves sampled between 14 and 21 days for each treatment (Fig. 4), at which time 50% of the leaves (5/10) showed vein clearing and/or silvering. Little or no RNA was detected in leaves 1–4 (Fig. 5) and leaves 9 and higher. However, in leaves 5–8 comparatively high RNA levels were observed (Fig. 5). There was a very high correlation ($r^2 = 0.98$) between the levels of RNA and silverleaf rating (Fig. 4). The RNA levels started to decrease from leaf 7 onwards, reaching undetectable levels in leaf 9 and beyond. However, such a decrease in RNA levels was not as rapid in plants that were continuously infested with SPWF (Fig. 5).

Glycerol density fractionation and differential centrifugation analysis. Analysis of fractions from glycerol density gradient revealed the presence of dsRNA in fractions rich in membranes and nuclei but not in chloroplasts (Fig. 6). Differential centrifugal analysis of cellular fractions showed the consistent presence of dsRNA in the pellet fractions (Fig. 7). However, no dsRNA was detected in any of the fractions from asymptomatic control plants (Figs. 6 and 7).

In vitro RNA polymerase activity in pellet fractions. The pellet fractions from symptomatic and asymptomatic squash were assayed for RNA polymerase activity (Table 1). RNA polymerase activity in the pellet fraction from symptomatic squash increased 100× that from asymptomatic squash. The RNA polymerase activity was insensitive to actinomycin D but required $MgCl_2$ (Table 1).

DISCUSSION

The SPWF-mediated silverleaf syndrome in squash is unique and differs from virus-incited diseases that are vectored by SPWF. Leaves that emerge after the removal of SPWF do not show silverleaf symptoms (3,20). SPWF nymphs are reportedly more efficient than adults in inducing silverleaf (20), and conventional plant virological techniques such as graft transmission and mechanical inoculation are incapable of reproducing this syndrome (3,20). Additionally, efforts to visualize virions or virus particles in symptomatic tissue have been unsuccessful (3).

However, there is an unequivocal relationship between the presence of two dsRNAs (4.2 and 4.6 kb) and the silverleaf syndrome (2,3). Additionally, colonies of SPWF capable of inducing silverleaf syndrome contained the two species of dsRNA while SPWF, which did not contain the dsRNA, did not mediate silverleaf syndrome (3). These results prompted Bharathan et al (3) to hypothesize that the dsRNA may be the replicative form of a virus or viruslike particles causing the silverleaf syndrome. Hence, the current work was undertaken to study the biological relationship of SPWF and the silverleaf syndrome and to further

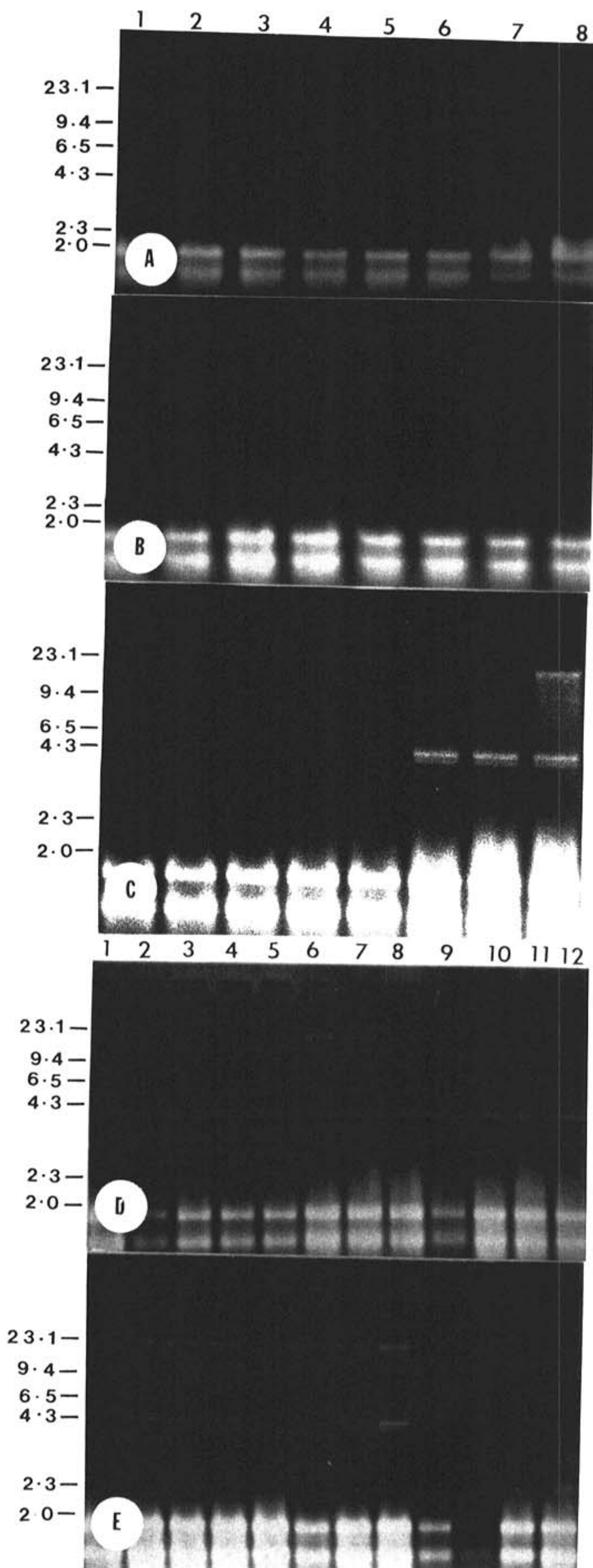

Fig. 3. Effect of sweetpotato whitefly (SPWF) density on dsRNA. Leaf disks (7 mm) from leaves 1–4, 5 and 6, and 7–9 from each of the SPWF density treatments were pooled individually and dsRNA extracted. **A,** Lanes 1–4, sampling done 0 days after whitefly infestation. Lanes 1–4, dsRNA from leaves (1–4) exposed to 2, 5, 10, and 20 SPWF, respectively; lanes 5–8, dsRNA from leaves (1–4) sampled 2 days after whitefly infestation. **B,** Lanes 1–4, sampling done 3 days after whitefly infestation. Lanes 1–4, dsRNA from leaves (1–4) exposed to 2, 5, 10, and 20 SPWF, respectively; lanes 5–8, dsRNA from leaves (1–4) sampled 7 days after whitefly infestation. **C,** Sampling done 14 days after SPWF infestation. Lanes 1–4, dsRNA from leaves (1–4) exposed to 2, 5, 10, and 20 SPWF, respectively. Lanes 5–8, dsRNA from leaves (5 and 6) exposed to 2, 5, 10, and 20 adult SPWF, respectively. **D,** Samplings done 21 days after SPWF infestation. Lanes 1–4, dsRNA from leaves (1–4) exposed to 2, 5, 10, and 20 SPWF, respectively. Lanes 5–8, dsRNA from leaves (5 and 6) exposed to 2, 5, 10, and 20 SPWF, respectively. Lanes 9–12, dsRNA from leaves (7–9) exposed to 2, 5, 10, and 20 SPWF, respectively. **E,** Samplings done 35 days after SPWF infestation. Lanes 1–4, dsRNA from leaves (5 and 6) exposed to 2, 5, 10, and 20 SPWF, respectively. Lanes 5–8, dsRNA from leaves (7–9) exposed to 2, 5, 10, and 20 adult SPWF, respectively. Lanes 9–12, dsRNA from leaves (10–12) exposed to 2, 5, 10, and 20 adult SPWF, respectively.

characterize the association of dsRNA with the syndrome.

A minimum feeding period of 48 h and a SPWF density of five adults were necessary to induce vein clearing and/or leaf silvering. Up to 100% of the plants exhibited leaf silvering when exposed for 48 h to 20 SPWF adults. Yokomi et al (20) had reported that adults of *B. tabaci* are incapable of mediating silverleaf syndrome in *C. pepo* 'Senator'. However, Hoelmer et al (8) reported that adults of *B. tabaci* do induce silverleaf in both Senator and Dixie cultivars of *C. pepo*. No differences in symptom development were found in the current study between Senator and Dixie after a 48-h feeding with 20 adult SPWF.

Yokomi et al (20) also found two species of dsRNA in Senator plants showing silverleaf syndrome. However, the dsRNA reported by Yokomi et al (20) may not be the same dsRNA reported by Bharathan et al (2,3). Occasionally, larger dsRNA are found in extracts of symptomatic tissue (Fig. 2A) using both agarose and acrylamide gels, and these fragments have no sequence homology with the 4.2- and 4.6-kb dsRNAs associated with silverleaf syndrome (Fig. 2B). This may explain the lack of correlation between silverleaf syndrome and dsRNA in the studies reported by Yokomi et al (20), while there is an unequivocal correlation

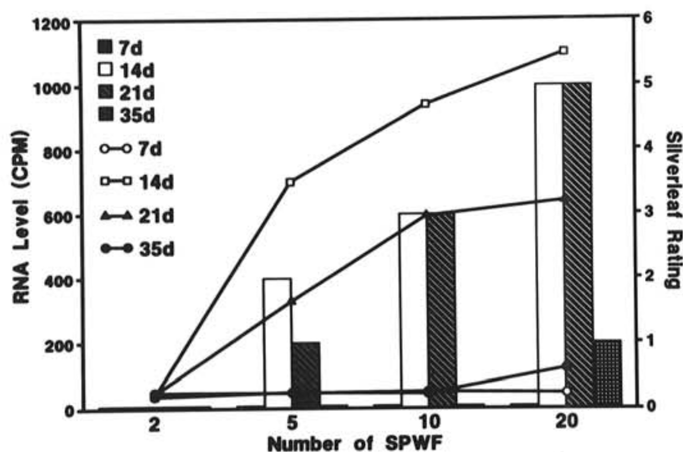

Fig. 4. Effect of sweetpotato whitefly (SPWF) density on silverleaf rating and RNA accumulation. Different densities of adult SPWF were introduced on leaf 1. Leaf disks were collected at various time intervals as described in materials and methods. Total RNA was extracted, spotted onto a nylon membrane, and hybridized to 4.2- and 4.6-kb dsRNA. Individual spots were excised and counted in a scintillation counter. Each time leaves were sampled, they were rated for vein clearing and/or leaf silvering. Line, RNA level (CPM); bars, silverleaf rating.

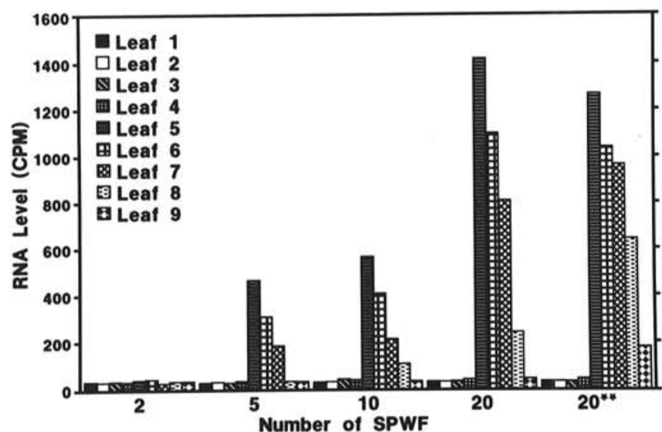

Fig. 5. Effect of sweetpotato whitefly (SPWF) density on RNA translocation. Single leaf disks were collected from leaves 1–9 21 days after 48-h SPWF feeding access. Five plants were used, and leaf disks from each were pooled together and total RNA extracted. Total RNA was applied as 10 μl dots and hybridized to 4.2- and 4.6-kb dsRNA. Individual spots were excised and counted in a scintillation counter. ** = Continuous exposure to SPWF.

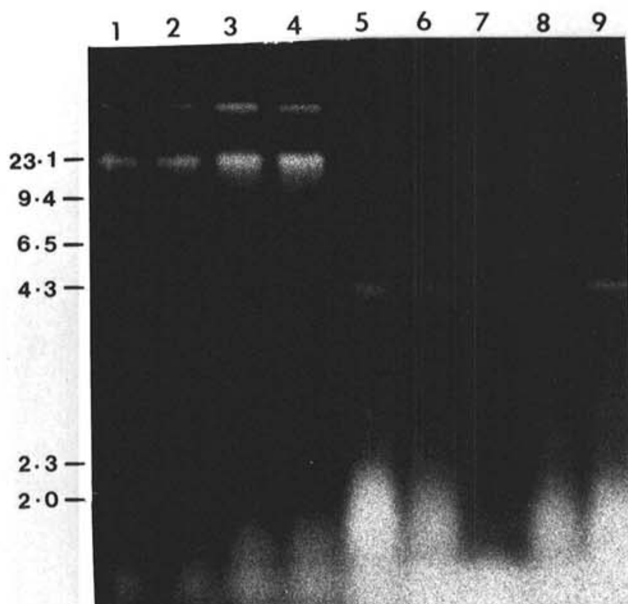

Fig. 6. Intracellular localization of dsRNA by glycerol density gradient fractionation. Cell fractions rich in vesicles, chloroplasts, and nuclei were separated on glycerol density gradient and collected, and dsRNA was extracted by CF-11 cellulose column chromatography. Fractions from symptomatic leaves were further treated with DNase followed by proteinase K treatment for 30 min at 37 C. Samples were analyzed for the presence of dsRNA by agarose gel electrophoresis. Lanes 1–4, fractions from asymptomatic leaf tissue: 1, rich in nuclei (pellet); 2, fraction at depth of 6 cm (nuclei and chloroplast); 3, fraction at depth of 3.5 cm (rich in chloroplast); 4, fraction at depth of 1.5 cm (rich in membranes). Lanes 5–9, fractions from symptomatic tissue: 5, rich in nuclei (pellet); 6, fraction at depth of 6 cm (nuclei and chloroplast); 7, fraction at depth of 3.5 cm (rich in chloroplast); 8, fraction at depth of 1.5 cm (rich in membranes); and lane 9, interphase between 1.5 and 3.5 cm. The numbers on the left indicate the size (kb) of lambda DNA *Hind*III digest.

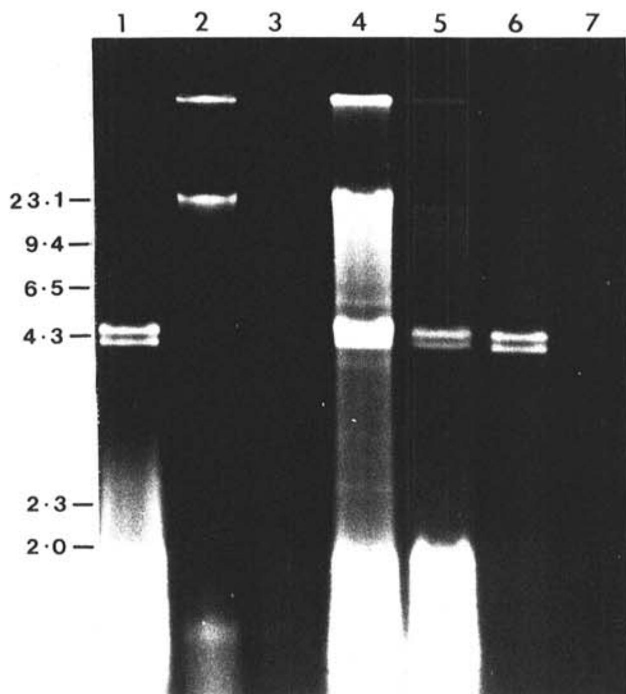

Fig. 7. Intracellular localization of dsRNA by centrifugal analyses. Different pellet and supernatant fractions from asymptomatic and symptomatic squash plants after low-speed, high-speed pelleting, and passage through CF-11 cellulose column were analyzed for the presence of dsRNA by agarose gel electrophoresis. Lane 1, positive control dsRNA; lane 2, pellet 1, asymptomatic; lane 3, supernatant 1, asymptomatic; lane 4, pellet 1; lane 5, supernatant 1, symptomatic; lane 6, pellet 2, symptomatic; lane 7, supernatant 2, symptomatic.

between silverleaf symptoms and the presence of 4.2- and 4.6-kb fragments. The 23.1-kb fragment found occasionally in gels (Figs. 2A and 6) is DNA as shown by its sensitivity to DNase treatment (Fig. 6, lanes 5–9), confirming earlier observations (3).

Several phenotypically normal cucurbit cultivars are known to contain dsRNAs, which are apparently not the products of RNA virus infection (13). More recently, virus-nonspecific 12-kb dsRNA in the chloroplast fraction, but not in nuclear or cytoplasmic fractions, of tissue extracts from pepper was reported (19). In the present study, lack of dsRNAs in the chloroplast fraction of glycerol density gradient and presence of dsRNA in the membrane fractions of silverleaf tissue suggest that the detected dsRNAs may be membrane-bound. Consistent detection of dsRNA in the pellet fractions after differential centrifugation further lends support for viral origin of the 4.2- and 4.6-kb dsRNA. Association of dsRNA with the nuclei would suggest that the dsRNA is the genome or the replicative form of a virus. Higher levels of hybridizable RNA have been reported in vesicle and nuclei fractions of pea enation mosaic virus (16).

Increased RNA-dependent RNA polymerase activity has been associated with viruses (1,16,18). Although no virions or viruslike particles have been unequivocally associated with silverleaf syndrome, an increased RNA polymerase activity with pellet fractions in silverleaf tissue (Table 1) is consistent with a viral etiology of silverleaf syndrome. Insensitivity to actinomycin D would suggest that the observed increase in activity is due to RNA-dependent RNA polymerase activity (16).

Dot spot hybridization assays using leaf disks sampled at various time intervals show that the degree of silverleaf syndrome and RNA levels are directly correlated with the SPWF density. Increased levels of RNA with higher SPWF density, the quantitative relationship between RNA level and silverleaf rating, and the lack of dsRNA and silverleaf symptoms in newly emerging leaves after removal of SPWF would suggest that the dsRNA is merely transported in the plant in the absence of SPWF. This is further substantiated by the appearance of the syndrome and significant levels of dsRNA only in leaves 5, 6, and 7, with no or very few symptoms in leaves 2, 3, 4, 8, and 9 (Fig. 4). Apparently, primordia of leaves 5, 6, and 7 were being formed at the time of SPWF exposure. Leaves 2, 3, and 4 were already present or formed at that time. Leaves 8 and 9 emerged after the removal of SPWF.

Five successive 48-h feeding periods with the same adult SPWF resulted in 100% development of the silverleaf syndrome in five sets of plants. This may suggest that the causal agent of the silverleaf syndrome is able to replicate in SPWF. Continued presence of SPWF is necessary for continued symptom development. Even in field conditions, when populations of SPWF are significantly reduced by pesticide applications or cold weather conditions, the silverleaf syndrome is virtually absent. It is tempting to speculate that the causal agent of silverleaf may be a viruslike agent of SPWF because of the obligate requirement of SPWF for continued symptom development in the host plant, persistent ability of nymph and adult SPWF to induce silverleaf symptoms, and the high correlation between RNA levels, severity of symptoms, and SPWF density.

TABLE 1. RNA polymerase activity in pellet fractions from symptomatic and asymptomatic squash and the effect of various cofactors on RNA polymerase activity

Reaction mixture	[^{3}H]UTP incorporation (CPM)[a]
Asymptomatic, standard reaction[b]	2,880
Symptomatic, standard reaction	384,512
Symptomatic, + actinomycin D[c]	294,236
Symptomatic, − actinomycin D	242,618
Symptomatic, + creatine phosphate (20 mM)	147,114
Symptomatic, − $MgCl_2$	8,126
Symptomatic, − NTPs	3,440

[a] A standard reaction mixture in a 50-μl final volume consisted of 50 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 1 mM each of the NTPs, 1 μCi of [^{3}H]UTP, and 20 μl of crude pellet preparation from asymptomatic and symptomatic leaf tissue. The pH of the reaction mixtures was adjusted at 40 C.

[b] The effect of different cofactors on RNA polymerase activity in pellet fractions from symptomatic tissue was tested in the presence and absence of actinomycin D (0.125 mg/ml), presence of creatine phosphate (20 mM), and absence of $MgCl_2$ (8 mM) and NTPs (1 mM each of ATP, CTP, and GTP, and 0.1 mM of UTP).

[c] After incubation at 40 C for 1 h, the reaction mixture was precipitated with 10% TCA by passing through GF/C filters and tested for TCA-precipitable [^{3}H]UTP. Counts per minute (CPM) were measured in a 20-μl sample of the TCA-precipitable radioactivity of each reaction mixture.

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