Variations in Serologically Detectable Antigen of Soybean Dwarf Virus in Soybean Leaflets as a Function of Time After Inoculation and Plant Age

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ABSTRACT

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Two enzyme-linked immunosorbent assay (ELISA) systems were developed for detection of soybean dwarf virus (SDV) and compared for sensitivity. With direct double-antibody sandwich (DAS) ELISA, dilution of conjugated immunoglobulins with uninfected tissue extracts prepared with phosphate buffered saline (PBS) (1:5, w/v) improved absorbance compared with conjugates prepared with PBS alone. The apparent amplification of the positive signal occurred without changing nonspecific (background) absorbance. Indirect antigen-coated ELISA detected the dwarfing strain (SDV-D) but was much less sensitive than DAS-ELISA. DAS-ELISA was used to measure and compare the concentration of SDV antigen in canopy tissues of Wayne soybean harvested 11, 19, 26, 33, and 40 days after initiation of the inoculation access period. At all test dates, aphid-inoculated unifoliolates contained the highest concentration of SDV antigen, and a trend toward higher antigen concentrations in younger tissues was observed. SDV antigen concentration peaked at 19 days and declined thereafter. All soybean test seedlings inoculated 7 and 14 days after emergence became infected. When inoculation occurred at 28 days, only 48% of the plants became infected with the yellowing strain (SDV-Y) and 12% with the SDV-D strain.

Soybean dwarf virus (SDV) has caused widespread economic losses to soybean growers in northern Japan for more than 20 yr (25) but has not been reported in North America. During that time, SDV was purified and characterized (10,15,24) and its transmission characteristics studied in depth (4,5,13,23). The virus has a wide host range that includes mainly legumes (23). Symptom expression ranges from indiscernible to very severe, and in some cases strains can be distinguished by symptomatology on selected hosts (25). Despite years of searching, only limited resistance to the virus has been identified in soybeans (27). Another luteovirus, described as subterranean clover red leaf virus (SCRLV) but currently considered a strain of SDV (19,29), causes serious diseases of economically important legumes in Australia (14) and New Zealand (31). SDV strains (in the broad sense including SCRLV) appear to be vector-specific (12) and are transmitted by *Aulacorthum solani* (Kaltenbach) or *Acyrthosiphon pisum* (Harris). SDV has not been identified in soybeans in the United States, but a virus serologically similar to the Australian SCRLV isolate was described on other legumes in California (12) and Mississippi (19). The potential for crop loss in the United States, should SDV be in North America already or if it is introduced in the future, is being evaluated at Frederick, Maryland.

All members of the luteovirus group of plant viruses are transmitted only by aphids (20). Although visual assessment has been used to diagnose luteovirus infections for years, until recently the only acceptable method of diagnosis was an insect transmission assay. This assay is very sensitive and reliable but is time-consuming and requires secure vector-handling facilities. Luteoviruses are phloem-limited, occur in very low concentration in the plant (20), and even with the new enzyme extraction techniques (7,22,30), are arduous to purify in sufficient quantity for antiserum production. Until the development of enzyme-amplified immunoassays, serology for routine diagnosis of luteoviruses was impractical because of insufficient quantities of antiserum and low sensitivity. In 1977, the enzyme-linked immunosorbent assay (ELISA), first developed for study of animal viruses, was adapted for detection of plant virus antigens (2,16). Since then, ELISA has been the serological method of choice for rapid detection of luteoviruses (1,17). ELISA has proved valuable as a research tool to study vector relationships and epidemiology (26), to evaluate relationships among group members (6), and, here, to evaluate susceptibility and accumulation of SDV as measured by serological detection of viral coat protein antigens in the soybean host.

MATERIALS AND METHODS

Virus isolates and aphid maintenance. Two SDV strains, dwarfing (SDV-D) and yellowing (SDV-Y), and a Japanese population of the aphid vector *Aulacorthum solani* studied previously (4–6,10) were used for this work. Viruses and colonies of nonviruliferous *A. solani* were propagated and maintained on soybean (*Glycine max* (L.) Merr. 'Wayne') or curly dock (*Rumex crispus* L.) in the containment facility at Frederick (4).

Antiserum production and ELISA procedures. SDV-D was purified using an enzyme extraction method (10). Antisera were made in two New Zealand white rabbits from approximately 200 μg of virus in total, administered in a course of three injections at approximately triweekly intervals. The virus and an equal volume of Freund's complete adjuvant were mixed and administered the first time as an injection into the large muscle of the hind leg, the second time with Freund's incomplete adjuvant in a series of injections under the skin at six locations near the lymph nodes, and the third time with Freund's incomplete adjuvant by two injection routes, via an

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ear vein and intradermally. Antisera were collected at 5, 8, 12, 13, 14, and 17 wk after the first injection. Immunoglobulins (Ig) collected from one rabbit at 14 wk and cross-absorbed with soybean plant extracts were used for this study. Extracts for cross-absorption were prepared as follows: Uninfected 21-day-old Wayne soybean leaves were ground in 0.5 M sodium phosphate buffer, pH 6 (1:2, w/v), treated with 2% Rohamet-P (no longer available but other enzymes from Finnsugar Biochemicals, Schaumburg, IL, appear equally effective in our laboratory) (w/v) for 6 hr, clarified with 30% chloroform followed by centrifugation in a DuPont-Sorvall GSA rotor at 7,500 rpm (9,000 *g*) for 15 min. Final concentration of the extract was by centrifugation in a Beckman 60 Ti rotor for 3 hr at 28,000 rpm (55,220 *g*). The pellets were resuspended (1 ml/50 g of tissue) in phosphate buffered saline (PBS) (0.137 M NaCl, 0.0015 M KH_2PO_4, 0.008 M Na_2HPO_4, 0.003 KCl, 0.003 M NaN_3), pH 7.4. Antiserum was mixed with soybean extracts (1:1, w/v) and stirred for 1 hr at room temperature, then held overnight at 4 C. After centrifugation in a DuPont-Sorvall SS-34 rotor at 10,000 rpm (12,000 *g*) for 10 min, the Ig was purified as described earlier (11) except that the final purification was by chromatography with a bifunctional affinity gel containing diethylaminoethyl groups and Cibachron Blue F3GA (DEAE Affi-Gel Blue, Bio-Rad Laboratories, Richmond, CA). Type VII-T alkaline phosphatase (Sigma Chemical Company, St. Louis, MO), was conjugated to the purified Ig as follows: 2 mg of alkaline phosphatase, 1 mg of purified Ig, and glutaraldehyde to a final concentration of 0.2% were mixed, allowed to stand at room temperature for 2 hr, then dialyzed sequentially in three 1-L volumes of PBS, one of which was overnight.

The ELISA procedure adopted was modified from one developed for beet western yellows virus (11). Tissue was harvested, weighed, and placed into chilled (−20 C for several hours or overnight to minimize cracking) thick-walled 40-ml glass sample bottles. A few milliliters of liquid nitrogen were added, and the samples were pulverized with a Teflon rod and ground for 30 sec in 0.05 M sodium phosphate buffer, pH 7, with a Tissumizer (Tekmar, Cincinnati, OH). A 3 × 3 cm square of coarse absorbent paper was pushed to the bottom of the sample bottle such that the plant debris was trapped at the bottom while the extract passed through the paper. Tissue-to-buffer (TB) ratios were 1:3 or 1:5 (w/v) except for detection tests, where TB ratios were 1:5, 1:25, 1:50, 1:100, 1:200, and 1:300.

For direct double-antibody sandwich (DAS) ELISA, round-bottom Immulon I microtiter plates (Dynatech, Chantilly VA) were coated with 2 μg/ml of anti-SDV-D Ig in 0.05 M sodium carbonate coating buffer, pH 9.6, for 2 hr at 30 C. A 100-μl sample of tissue extract was added and allowed to stand at 4 C overnight. Conjugated Ig diluted 1:400 in PBS was added and held overnight at 4 C.

Samples for indirect antigen-coated (IND) ELISA were ground in carbonate coating buffer (1:5, w/v), filtered through coarse absorbent paper, and transferred to a microtiter plate for 1–3 hr at 30 C. During the coating period, uninfected Wayne soybean extracts were prepared by grinding tissue in PBS (1:5, w/v), pelleting the debris in the SS-34 rotor for 10 min at 10,000 rpm (12,000 *g*), and filtering the supernatant through Miracloth (Chicopee Mills, Inc., Miltown, NJ). Anti-SDV-D Ig was mixed with the uninfected tissue extracts such that the final Ig concentration was 2 μg/ml, and the mixture was stirred for 1–3 hr at room temperature. Cross-adsorbed Ig and goat antirabbit alkaline phosphatase-conjugated IgG (Sigma) diluted 1:250 was sequentially incubated, each overnight at 4 C.

Finally, for both DAS- and IND-ELISA, freshly prepared substrate, 1 mg/ml of *p*-nitrophenyl phosphate disodium in 10% diethanolamine buffer, pH 9.8, was added, the plates were allowed to develop, and absorbance (405 nm) of each well was determined with a Bio-Tek Model EL-307 EIA Reader (Winooski, VT). From previous experience (6,11) and the work of Sutula et al (21), a positive threshold was designated as the mean plus four standard deviations of at least five wells containing extracts from uninfected plants (21,28). Because SDV-D and SDV-Y antigens are indistinguishable in polyclonal DAS- or IND-ELISA systems, the SDV-D system was used for these studies.

Effect of extraction method on detection by DAS-ELISA. Combinations of techniques for extracting antigen from leaf tissue were tested for absorbance and ease of preparation. Matched sets of half leaves of equal weight were prepared from fresh tissue in 0.05 M sodium phosphate buffer, pH 7 (1:3, w/v).

Effect of uninfected tissue extracts on DAS-ELISA. Uninfected tissue extracts were prepared as for IND-ELISA and mixed with alkaline-phosphatase conjugated anti-SDV-D Ig. The preparation was stirred for 1–3 hr and added to the plate. All other steps were as described above except that each treatment was replicated four times.

Virus transmission. Virus sources were detached leaflets of the youngest one or two trifoliolates (three-fourths to fully expanded) harvested from plants inoculated 18–24 days earlier. Acquisition access periods were 48 hr in 150-mm petri dishes in the dark at 20 C. Five aphids were transferred with camel's-hair brushes to the emerging unifoliolates of each 6- to 8-day-old Wayne soybean seedling at the cotyledon stage (8) and confined with tubular cellulose butyrate cages. Following a 48-hr inoculation access period, the cages were removed and the aphids were killed with dilute Malathion (0.2 g a.i./L). Test plants were rinsed with tap water, placed in the greenhouse, and harvested at various times according to the experimental plan.

Antigen concentration in the canopy. To determine and compare the concentration of SDV-D and SDV-Y antigens at various levels in the canopy, five viruliferous aphids (SDV-D or SDV-Y) were transferred to each of 50 Wayne soybean test seedlings; 2 days later, the aphids were killed. Individual 1-g samples of unifoliolates from five randomly selected plants were harvested every 7 or 8 days starting on the 11th day after initiation of the inoculation access period and ending on the 40th day. Harvests of the youngest trifoliolate (approximately three-fourths expanded) and each fully

Table 1. Effects of tissue preparation on absorbance at 405 nm in double-antibody sandwich enzyme-linked immunosorbent assay of soybean dwarf virus, dwarfing strain, from matched sets of Wayne soybean half leaves of equal fresh weight prepared in 0.05 M sodium phosphate buffer, pH 7 (1:3, w/v)

Expt. no.[a]	Tissue preparation[b]	Absorbance	
		Infected	Uninfected
1A	Mortar and pestle, Tissumizer	1.348 ± 0.182[c]	0.034 ± 0.004
1B	Mortar, pestle, and Carborundum, Tissumizer	1.041 ± 0.256	0.027 ± 0.006
2A	Liquid nitrogen, Tissumizer and chloroform	0.992 ± 0.273	0.062 ± 0.008
2B	Liquid nitrogen, Tissumizer	1.180 ± 0.369	0.032 ± 0.008
3A	Liquid nitrogen, mortar and pestle	1.188 ± 0.235	0.031 ± 0.008
3B	Liquid nitrogen, mortar and pestle, Tissumizer	1.007 ± 0.310	0.025 ± 0.005
4A	Chopping, mortar and pestle	1.353 ± 0.232	0.028 ± 0.008
4B	Chopping, mortar and pestle, Tissumizer	0.998 ± 0.144	0.033 ± 0.012
5A	Liquid nitrogen, Tissumizer	0.432 ± 0.136	0.034 ± 0.001
5B	Chopping, Tissumizer	0.442 ± 0.065	0.040 ± 0.006

[a] One preparation variable was tested in each of five separate experiments, each run at different times using different sources of tissue.

[b] Mortar and pestle = manual pulverization, liquid nitrogen = samples frozen and powdered in liquid nitrogen, chopping = samples cut into 1-mm pieces with a razor blade, Tissumizer = rotary homogenizer.

[c] Data are means and standard deviations of five replications.

expanded trifoliolate, again from five randomly selected plants, began 19 days after initiation of the inoculation access period and continued for the duration of the experiment. A parallel set of control plants was prepared in the same way except that the aphids were not given an acquisition access period on infected Wayne soybean. Tissue samples from each harvest were weighed and frozen at −80 C until they were tested for antigen concentration by DAS-ELISA in a randomized complete block design. Absorbance data were subjected to analysis of variance (ANOVA), and means were separated using least significant differences (LSD). The experiment was done three times with five replications per treatment. Because the trends observed from all experiments were similar, data presented are from a single experiment.

Effect of plant age on susceptibility to SDV. To determine and compare the susceptibility of Wayne soybean to SDV-D and SDV-Y, 10 viruliferous aphids were transferred to each of 10 seedlings aged 7, 14, 21, and 28 days. The aphids were confined to the unifoliolates with leaf cages made from discarded Beckman Ultraclear SW 40 centrifuge tubes, Saran netting, and a 0.5-cm-thick ring of plastic foam cut to size with cork borers. The cages were attached to the leaf with hair clips and supported with plant stakes. After the inoculation access period, the plants were sprayed with Malathion and moved to the greenhouse for symptom development. A 1-g sample was harvested 16 days after inoculation from the youngest trifoliolate of each plant of each age and tested by DAS-ELISA. The experiment was done three times and the data pooled for a total of 30 test seedlings for each virus strain at each plant age.

RESULTS

Effect of extraction on detection by DAS-ELISA. All techniques for extraction of SDV-D antigen from infected soybean tissue were effective, and although absorbance of paired half leaves varied, no method within a paired comparison (A vs. B) was appreciably better than any other (Table 1). Chopping the samples with a razor blade and grinding the tissue in a mortar and pestle was arduous and time-consuming, especially when a large number of samples were processed, and was no more effective than powdering the tissue in liquid nitrogen, then grinding with the Tissumizer (Table 1, experiment 5). Addition of Carborundum to the mortar to facilitate extraction did not improve absorbance (Table 1, experiment 1), and clarification of samples with chloroform doubled the background absorbance (Table 1, experiment 2).

Effect of uninfected tissue extracts on DAS-ELISA. Dilution of conjugated Ig with uninfected tissue extracts did not decrease background absorbance when compared with Ig diluted with PBS, but absorbance of infected tissue samples was enhanced, especially at low TB ratios (Fig. 1). SDV-D was detected by the DAS-ELISA system at all TB ratios. Low ratios resulted in faster development with higher final absorbance values than did high ratios, but detection was still possible at 1:300 (w/v) when the mean of the healthy controls plus four standard deviations was used as the positive threshold, especially if, as Lister (17) noted, healthy soybean extracts are incubated with conjugated antibody to reduce undesired reactions.

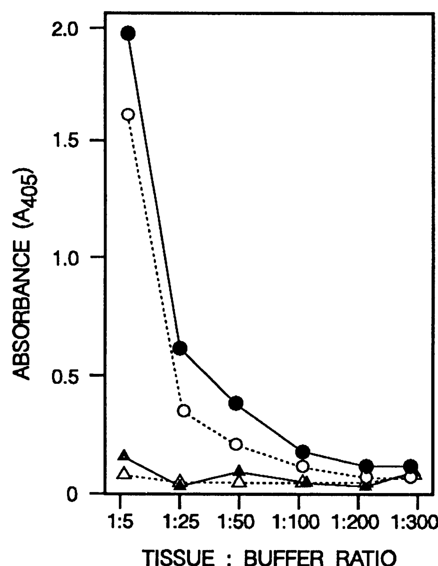

Fig. 1. Comparative absorbance of soybean dwarf virus dwarfing strain in double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) at different tissue-to-buffer ratios and with conjugate diluted with phosphate buffered saline (PBS) only or with uninfected tissue extracts (tissue ground in PBS, 1:5, w/v). Conjugate diluted with PBS: ○ = infected, △ = uninfected; conjugate diluted in uninfected tissue extracts: ● = infected, ▲ = uninfected. The absorbance of infected tissue extracts is the mean of four replicates; the absorbance of uninfected tissue extracts is the mean of four replicates plus 4 SD.

Detection by IND- and DAS-ELISA. When tested on the same plate with the same source of tissue, SDV-D antigen was detected in both IND- and DAS-ELISA at all concentrations of specific antibody tested (Table 2). The IND-reactions were slower to develop, taking up to 3 hr compared with DAS-ELISA plates, in which positive reactions were easily determined in 15–60 min, when TB ratios were 1:10 or 1:5.

Other tests (data not shown) with TB ratios of 1:5, 1:10, 1:100, and 1:300 were done on the same plate with the homologous antibody at 2 μg/ml. SDV-D was detected at all ratios in DAS-ELISA but only at 1:5 and 1:10 in IND-ELISA. Mean absorbance of DAS wells was as much as 10 times that in IND wells (1.757 and 1.489, for 1:5 and 1:10 TB ratios, respectively, compared with 0.174 and 0.160), whereas nonspecific absorbance, determined by adding the mean plus four standard deviations, was similar in both systems (0.089 and 0.107 for DAS and 0.103 and 0.119 for IND).

For quantitative estimation of SDV in the canopy and in different aged plants, DAS-ELISA was used with the conjugate absorbed with uninfected tissue extracts at a 1:5 TB ratio and all samples were frozen with liquid nitrogen, crushed, and ground with a Tissumizer.

Antigen concentration in the canopy. The greatest concentration of both viral antigens occurred at the earlier harvest dates and, in general, in the inoculated unifoliolates and the youngest expanding trifoliolates.

Except for the SDV-Y on day 26, there was no statistically significant difference in antigen concentration between these two leaves at a given harvest date. Antigen concentration was lower in the expanded than in the corresponding young, expanding trifoliolates. Antigen concentration dropped in all leaves as the plant aged and in each leaf as it aged (Fig. 2).

Effect of plant age on susceptibility to

Table 2. Effects of homologous antibody concentration on absorbance of tissue extracts infected with soybean dwarf virus, dwarfing strain (SDV-D), and prepared in 0.05 M sodium phosphate buffer, pH 7 (1:3, w/v), in indirect antigen-coated enzyme-linked immunosorbent assay (IND-ELISA) and double-antibody sandwich ELISA (DAS-ELISA)

Treatment	Absorbance at 405 nm at indicated homologous antibody concentration (μg/ml)[a]			
	1	2	4	6
IND-ELISA				
SDV-D infected	0.187[b]	0.194	0.245	0.210
Uninfected	0.101[c]	0.103	0.118	0.122
DAS-ELISA				
SDV-D infected	1.557[b]	**[d]	**	**
Uninfected	0.094[c]	0.130	0.114	0.171

[a] First antibody layer for IND-ELISA and coating antibody for DAS-ELISA.
[b] Data are means of absorbance values of five replications at A_{405nm}. IND-ELISA absorbances are at 3 hr after the addition of substrate and DAS-ELISA absorbances are at 30 min.
[c] Data represent the positive threshold (treatment mean plus 4 SD).
[d] ** = Absorbance greater than accuracy of ELISA reader.

SDV-D and SDV-Y. Visual observation and ELISA ratings gave the same results at all plant ages. All plants inoculated at 7 and 14 days after emergence were rated positive (Fig. 3). Infection percentages dropped to 53 and 68% in 21-day-old plants and to 12 and 48% in 28-day-old plants for SDV-D and SDV-Y plants, respectively.

DISCUSSION

Preparation of samples is the most labor-intensive step in analysis by ELISA. The investment of time increases if the antigen is in low concentration in the plant, if it is difficult to extract, or if a quantitative assay is needed. Thorough extraction is required for detection of luteoviruses and their quantitative assay to prevent underestimation of antigen concentration because of incomplete extraction from tough phloem tissue.

Quantitative detection studies with carnation mottle virus and carnation ringspot virus indicate that contaminating host proteins do not affect detection when those proteins are added to known antigen concentrations in DAS-ELISA (18). Thus, the concentration of those viruses in tissue extracts can be estimated by comparing the absorbance of tissue extracts to a standard curve of known purified virus concentrations. In the SDV system there is an apparent amplification of SDV absorbance when conjugate stocks are diluted in uninfected tissue extracts rather than in PBS. Although we have no data to demonstrate that the cross-absorbance went to completion, background absorbance in tests using both conjugate preparations was similar, suggesting that this difference is not due to a reduction in nonspecific reactions. The reason for the amplification is not immediately obvious, but it appears that sap can affect detection sensitivity even when the serological reagents are reasonably free from antiplant antibodies. It would be prudent to characterize the effects of diluting conjugates with uninfected sap and PBS when a new DAS-ELISA system is established to assay tissue samples, particularly if a standard curve of known virus concentration will be used to estimate virus concentration in infected tissue extracts.

IND-ELISA is four to eight times more sensitive than DAS-ELISA in homologous reactions with three purified luteoviruses, including SDV (6). However, IND-ELISA with tissue extracts is approximately 10 times less sensitive than DAS-ELISA. Until a method to amplify SDV absorbance without increasing background can be found, IND-ELISA is not practical for detection of this virus in plant sap. IND-ELISA may be useful for studying serological relationships of viruses (6) or for preliminary determination of a luteovirus infection.

In the greenhouse, the unifoliolates showed a trend toward higher SDV antigen concentrations than the other leaves. The virus was introduced into the plant by the aphid vector feeding on the unifoliolates, and the resulting virus concentrations may have reflected a relationship similar to that seen with barley yellow dwarf virus, in which the virus appears to replicate locally in the phloem before translocation to other parts of the plant (9). As replication continued, antigen appeared to accumulate in the youngest trifoliolates, leaves that were growing rapidly. The concentration of antigen dropped dramatically as the time after inoculation increased. If this pattern of concentration is found to be

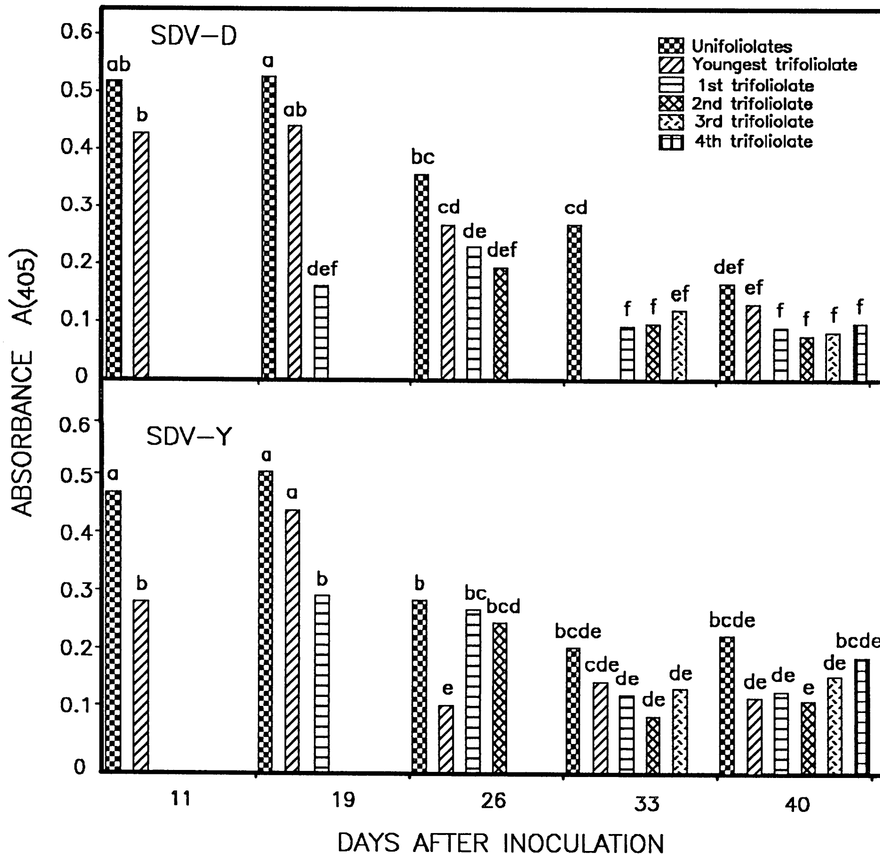

Fig. 2. Absorbance by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) of tissue extracts from Wayne soybean seedlings inoculated with soybean dwarf virus, dwarfing strain (SDV-D) or yellowing strain (SDV-Y), at the cotyledon stage. Tissue samples from five randomly selected plants (five replicates) were harvested as follows: unifoliolates and the youngest trifoliolates (approximately three-fourths expanded) at indicated days (x-axis) after the start of the inoculation access period. Counting from the base of the plant, the first trifoliolates were not fully expanded until day 19, at which time they were harvested. The second fully expanded trifoliolates were harvested for the first time on day 26, the third trifoliolates on day 33, and the fourth trifoliolates on day 40. Once harvests of a given tissue type commenced, they continued until the 40th day. After each harvest, tissue samples were weighed and frozen at −80 C until the experiment was completed. Means of five randomly selected uninfected control plants (five replicates) were 0.05 or less. Absorbances associated with bars labeled with the same letters are not significantly different ($P = 0.05$) based on least significant differences (LSD).

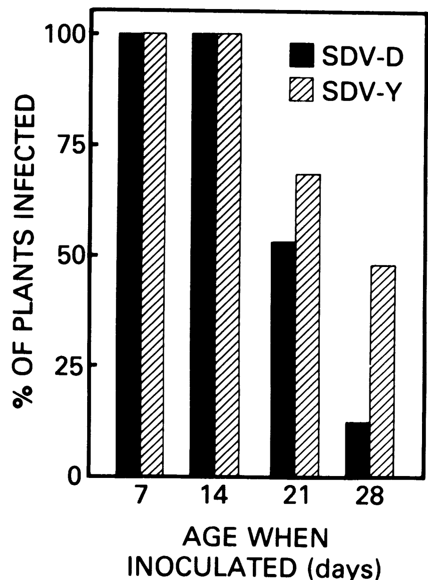

Fig. 3. Percent infection of Wayne soybean seedlings after aphid inoculation with soybean dwarf virus, dwarfing strain (SDV-D) or yellowing strain (SDV-Y), 7, 14, 21, and 28 days after emergence. Ten seedlings were inoculated with each virus strain. Data from three trials were pooled, making a total of 30 seedlings for each date. Infection data were obtained by symptomatology and confirmed by double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) analysis.

typical of luteoviruses in general, it may have implications for spread in the field and also for successful experimental virus transmission. Even though the concentration of a luteovirus in the host may be low overall, the concentration in the phloem in some plant tissues may be quite high (11,26) and transmission may be correspondingly efficient. In controlled feeding studies in the greenhouse with young susceptible soybeans, with SDV-D and SDV-Y the *A. solani* aphids from Japan used in these studies prefer feeding on the partially expanded trifoliolates and the unifoliolates (3). Transmission in the field and in the greenhouse is very efficient and may be due, in part, to aphids obtaining a greater charge of virus by preferentially feeding on tissues with the highest concentration of virus.

In Japan, the primary hosts for *A. solani* are clovers that are often infected with SDV. In late spring, alates migrate to their secondary host (young soybeans), initiating the primary spread of the virus (14). Studies in Aomori Prefecture indicate that chemical control of aphids from the time of soybean emergence until the third week of June gives adequate protection against SDV (32). The greater incidence of SDV infection in very young soybean seedlings than in older plants in this study is consistent with the Aomori data. In contrast to the Japanese populations of *A. solani*, populations of *A. solani* studied from California, New Zealand, and Nova Scotia utilize clover or potatoes as secondary hosts but do survive and reproduce on soybean in the greenhouse (4). The secondary host preference of *A. solani* may reduce the threat of SDV to soybean but perhaps increase the threat to clovers and other susceptible legumes. Reports from California (12) and Tasmania (14) of luteoviruses closely related serologically to SDV but vectored by *Acyrthosiphon pisum* suggest that SDV is widely distributed.

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