

Soybean Dwarf, Bean Leaf Roll, and Beet Western Yellows Luteoviruses in Southeastern U.S. White Clover

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ABSTRACT

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Leaf samples collected from white clover plants in several eastern and southeastern states between 1986 and 1991 were tested for the presence of soybean dwarf virus (SbDV), bean leaf roll virus (BLRV), and beet western yellows virus (BWYV) by aphid transmission and serology. The majority of the 991 samples were from randomly selected, asymptomatic, white clover plants in or near established clover stands. We report here the widespread occurrence of SbDV and BLRV in the southeastern United States. Nearly 25% of all white clover samples contained SbDV, BLRV, or both, while less than 10% contained BWYV.

Forage legumes are hosts to many plant viruses that are transmitted mechanically and nonpersistently by aphids or by seeds (18). In contrast, few phloem-limited, circulative, aphid-transmitted viruses (luteoviruses) have been isolated from forage legumes in the United States because of difficulty in extraction, purification, and identification (8,9,25).

The best known legume luteovirus in the U.S. is beet western yellows virus (BWYV) (7,8). Soybean dwarf luteovirus (SbDV) was described from soybean in Japan (23) and a closely related virus, subterranean clover red leaf virus (SCRLV) was described in New Zealand (27) and Australia (16). Because SCRLV is serologically indistinguishable from SbDV, the former has been reclassified

as SbDV (13). Bean leaf roll virus (BLRV) (1), synonymous with legume yellows virus (LYV) (9), pea leaf roll virus (1,10,22), and Michigan alfalfa virus (25), was first described in legumes in Germany in 1954 (22).

The dwarfing strain (SbDV-D) and yellowing strain (SbDV-Y) of the soybean dwarf virus were described by Tamada (24), based on symptomatology induced in soybean. Both strains are transmitted by the foxglove aphid, *Aulacorthum solani* (Kaltenbach) (23) and have not been reported outside Japan. Isolates of SbDV from New Zealand (SbDV-NZ) and Tasmania (SbDV-T) (= original SCLRV) were described from legumes other than soybean and are transmitted by *A. solani* although *Acyrtosiphon pisum* (Harris) is a vector of some isolates (13). An SbDV-like pathogen isolated from legumes in California (14) was transmitted by *A. pisum* and not by *A. solani*.

Soybean dwarf virus-D infects red clover (*Trifolium pratense* L.) but does not infect white clover (*T. repens* L.) (4,24), whereas SbDV-Y, -NZ, and -T asymptotically infect white clover but not red clover. The objective of the work reported here was to survey white clover in several eastern and southeastern states for the presence of SbDV, BLRV, and BWYV.

METHODS AND MATERIALS

Virus strains and antisera. Soybean dwarf strains SbDV-D and -Y, and the aphid vector *A. solani*, were obtained from T. Tamada, Naganuma, Hokkaido, Japan. Australasian strains of SbDV (-NZ and -T) were obtained from J. W. Ashby, Christchurch, N.Z., and G. R. Johnstone, Hobart, Tasmania, respectively. Bean leaf roll virus was obtained from R. Hampton, USDA, ARS, Oregon State University, Corvallis, OR, and BWYV was available in our laboratory. All virus strains and aphid vectors were acquired through quarantine permits from APHIS and the Maryland Department of Agriculture and have been maintained in the USDA Foreign Disease containment facility at Frederick, MD (21).

Polyclonal antibodies to SbDV-D and -Y were produced in New Zealand white rabbits at the Virginia Polytechnic Institute & State University, Laboratory of Animal Science, by S. A. Tolin, as described previously (12). Polyclonal antibodies to purified BLRV were produced by the Animal Resources Division, United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, MD, by the procedure described for SbDV (12). Bean leaf roll virus had been purified from *Pisum sativum* L. cv. Puget using an enzyme extraction method similar to that described for SbDV (11). Polyclonal antiserum for BWYV was purchased from AGDIA, Inc., Elkart, IN.

Plant sampling. A preliminary study was initiated in 1986 in conjunction with the Technical Committee of the Southern Regional Research Project (S-127/S-228) Forage Legume Viruses to determine which luteoviruses were present in symptomless white clover in the southeast. Researchers in several states randomly selected up to 50 white clover samples from established plantings in their state,

Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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propagated cuttings in insect-free areas, and sent samples of one to five grams to Frederick, MD, (A. D. Hewings and V. D. Damsteegt) and Salinas, CA, (J. E. Duffus) to test for the presence of luteoviruses (20).

Upon completion of these studies in 1989, a standardized sampling procedure was initiated for 1990–1991 through the cooperative efforts of the S-228 Committee members. Surveyors were asked to collect at least five samples from each of five fields per county and five counties per state. Five clover samples were selected at 10- and 50-m intervals along a line originating at the right front corner (road side) of the field, running at an angle toward the center rear of the field, and returning to the left front corner (an inverted V). Distances between sampling sites along the line varied depending on the field size and shape. The number of sites and samples collected varied among states but the sampling procedure was standardized for all collections. White clover plants grown in Frederick, MD, greenhouses served as healthy controls.

Serological assays. Samples were logged in immediately upon arrival by overnight carrier and held at 4 C until processing (never more than 4 days). Samples were pulverized in liquid nitrogen and triturated 45 sec with a Tissumizer (Tekmar, Cincinnati, OH) in 0.05 M phosphate buffered saline + 0.05% Tween 20 (PBST) + 0.2% nonfat dried milk, pH 7.4, in a 1:5 (w/v) ratio. Samples were analyzed by double antibody sandwich-enzyme-linked immunosorbent assay (DAS-ELISA) (2,3). If samples were in poor condition or if some antisera were in short supply, the preference of testing was for SbDV followed by BLRV and BWYV. Absorbance (A405 nm) values at least 2× the mean of those obtained from extracts from healthy plants were designated as positive. Round-bottom Immulon I microtiter plates (Dynatech, Chantilly, VA) were read with a Bio-Tek

Model EL-307 EIA Reader (Winooski, VT) or a Molecular Devices V-Max (Molecular Devices Corp., Menlo Park, CA 94025). All ELISA tests were done with alkaline phosphatase (P-5521, Sigma Chemical Company, St. Louis, MO) conjugated immunoglobulin as previously described (4).

Biological assays. Aphid transmissions were attempted from 50% of the white clover samples with positive ELISA reactions to SbDV and BLRV, and from randomly selected white clover samples that tested negative by ELISA. A minimum of 20 *A. pisum* and *A. solani* individuals were used per test sample. Aphids were given a 48-hr acquisition access period on detached leaflets on moist filter paper maintained in the dark at 10–15 C. Aphid-infested leaflets were then placed on healthy *T. subterraneum* cv. Mt. Barker seedlings in 5 × 15-cm cellulose butyrate tube cages for an inoculation access period (IAP) of 48 hr at 20 C. Healthy aphids, given a 48-hr IAP on test seedlings, served as a negative control. Following the IAP the cages were removed, seedlings sprayed with a solution of Malathion (0,0-Dimethyl phosphorodithioate of diethylmercaptosuccinate) (0.2 g a.i./L), and the plants placed on a glasshouse bench for a 30-day observation period.

Symptoms of SbDV infection in Mt. Barker clover seedlings were usually evident by 14 days after inoculation. Initial symptoms began as leaf reddening, starting at the margins and progressing inward and accompanied by reduced plant vigor. Reddening was more vivid on the upper leaf surface. Symptoms of BLRV in subterranean clover (Mt. Barker) are similar to SbDV with more intense reddening, leaf curling, stunting, and production of tiny new leaves. Symptoms of BWYV infection in Mt. Barker consisted of mild leaf reddening beginning along the midrib and progressing outward with the most intense

coloration on the lower leaf surface (15). Confirmatory ELISA assays were performed on test clovers with and without symptoms to verify symptom diagnosis.

RESULTS

In the preliminary studies, 491 white clover samples were processed for luteovirus detection by ELISA and aphid transmission. Samples were collected each year between 1986 and 1989 from 10 different states, although not in every state each year. There were no standardized methods for sampling among or within different states or years. Total samples collected by state and the data (ELISA, bioassay, or both) for SbDV, BLRV, or BWYV were, respectively: AL (66)—0/66, 2/26, 10/59; FL (57)—6/57, 11/57, 8/57; KY—(68) 5/68, 0/68, 8/68; LA (1)—0/1, 0/1, 0/1; MD (41)—16/41, Nt, 5/20; MS (46)—12/46, 3/32, 3/39; NY (4)—0/4, 0/4, 0/4; SC (82)—7/80, 6/33, 6/39; TX (9)—0/9, Nt, 1/9; and, VA (117)—20/117, 6/49, 2/49.

During 1990–1991, 500 samples were processed representing 100 fields in 40 counties in nine states. Using a standardized sampling method, SbDV was detected by ELISA in 32 and 47%, BLRV was detected in 47 and 21%, and BWYV was detected in 4 and 9% of the samples, respectively (Table 1). Samples co-infected by SbDV and BLRV accounted for 17% of the total.

Bioassays were conducted on 219 white clover samples in 1990 and 1991. Not all samples that were ELISA positive gave positive bioassays (mean 70%); however, all samples that were positive in bioassay tests gave strong ELISA reactions. The only successful transmissions from white clover were with *A. pisum*.

DISCUSSION

Based on ELISA data and transmission to indicator hosts, we have provided evidence that SbDV commonly infects white clover asymptotically in 11 southern and eastern states. We also have identified BLRV in white clover in seven states (AL, FL, KY, MD, NC, SC, VA) where BLRV had not been reported previously. Beet western yellows virus, though present, was not as common as either SbDV or BLRV and could not be confirmed by aphid transmission to indicator plants. Positive ELISA reactions to BWYV were always weak indicating that the virus titer was probably low.

Our serological assays (1986–1989) were by DAS-ELISA with polyclonal antisera produced against SbDV-D, SbDV-Y, BLRV, and BWYV. Because SbDV-D and SbDV-Y are indistinguishable in DAS-ELISA (12), we used SbDV-D polyclonal antisera for our detection assays. Luteoviruses are known to be serologically interrelated (6,26). Many of our samples gave a strong ELISA reaction (more than 2× healthy

Table 1. Summary of 500 white clover samples analyzed by enzyme-linked immunosorbent assay (ELISA) for soybean dwarf, bean leaf roll, and beet western yellows luteoviruses from nine eastern states in 1990–1991

| State | Year | Samples (n) | % Positive | | |
|----------------|------|-------------|-----------------|------|-----------------|
| | | | SbDV | BLRV | BWYV |
| Alabama | 1991 | 30 | 30 ^a | 23 | 30 |
| Kentucky | 1990 | 79 | 25 | 59 | 0 |
| | 1991 | 39 | 50 | 41 | 0 |
| Maryland | 1990 | 24 | 60 | 69 | 0 |
| | 1991 | 30 | 54 | 50 | 4 |
| New York | 1991 | 15 | 54 | 0 | 0 |
| North Carolina | 1990 | 152 | 31 | 58 | 0 |
| Pennsylvania | 1991 | 12 | 29 | 0 | 0 |
| South Carolina | 1990 | 2 | 50 | 0 | 0 |
| | 1991 | 31 | 71 | 21 | 11 |
| Texas | 1990 | 7 | 0 | 0 | Nt ^b |
| | 1991 | 5 | 0 | 0 | 0 |
| Virginia | 1990 | 64 | 35 | 16 | 0 |
| | 1991 | 10 | 20 | 10 | 0 |

^aPercentage of samples that tested positive by ELISA (O.D. reading at least 2× the reading for samples from healthy plants [A405 nm]).

^bNot tested.

control) for one virus and a borderline positive reaction (less than 2× control) for a second virus. We did not determine if these borderline positive reactions were caused by shared coat protein epitopes, reactions to common host proteins (non-specific background), or lower titer of the second virus. Cross-absorbance of the conjugated antibodies to healthy plant sap in the assay wells removed much of the nonspecific background (17).

Prior to 1983, the literature on the natural distribution of persistent-aphid-transmitted viruses of legumes in the U.S. was limited to BWYV (7), BLRV (10), LYV in California and the Pacific Northwest (9), and a report of alfalfa in Michigan (25). Our research on SbDV as an exotic virus of soybean in the U.S. had demonstrated the susceptibility of white clover to the yellowing strain of SbDV and the close serological relatedness of SbDV-Y to the SCRLV strain of SbDV, both of which produced symptomless infections in white clover (4). Our luteovirus survey in white clover was initiated after the report of a SCRLV-like (SbDV-like) virus in California (14). During the course of this survey, reports by McLaughlin et al (19,20) demonstrated the presence of SbDV, BWYV, and LYV (BLRV) in white clover and subterranean clover in Mississippi. Our results confirm these preliminary reports and determine conclusively that one or more strains of SbDV, transmitted by *A. pisum*, are endemic in the United States and that BLRV also is common in white clover in the southeast.

In this study we were interested only in identifying SbDV and other luteoviruses in white clover in the eastern United States. We did not attempt to identify strains of SbDV although evidence from previous studies had indicated that SbDV-Y infected white clover but SbDV-D did not (4,5,23). Therefore, we believe the white clover isolates may be related more closely to SbDV-Y.

The agronomic importance of SbDV and BLRV infections in asymptomatic white clover has not been determined. Future studies should investigate the effects of SbDV infection on stand longevity, host physiology, white clover nutritional value, vector/virus relationships, and interactions with other patho-

gens. The presence of SbDV and BLRV in asymptomatic white clover has epidemiological significance for other legume crops. Should an aphid vector biotype arise that colonizes clover and soybeans, a residual endemic inoculum source would be available for potential spread into soybeans.

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