Virus Diseases of Seven Species of Forage Legumes in the Southeastern United States

M. R. McLAUGHLIN, Research Plant Pathologist, U. S. Department of Agriculture, Agricultural Research Service, Crop Science Research Laboratory, Forage Research Unit, P. O. Box 5367, Mississippi State, MS 39762, and D. L. BOYKIN, Mathematical Statistician, U. S. Department of Agriculture, Agricultural Research Service, Mid South Area, Jamie Whitten Delta States Research Center, P. O. Box 225, Stoneville, MS 38776

ABSTRACT

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Researchers in 11 southeastern states conducted a regional test comparing the incidence of several virus diseases in seven species of forage legumes. The "seven species test" was conducted in 1978, 1979, and 1980 and included alfalfa, alsike clover, arrowleaf clover, crimson clover, red clover, subterranean clover, and white clover. Alfalfa mosaic virus (AMV), bean yellow mosaic virus (BYMV), clover yellow vein virus (CYVV), peanut stunt virus (PSV), red clover vein mosaic virus (RCVMV) and white clover mosaic virus (WCMV) were detected in plants of all seven species. Infections by AMV, RCVMV, and WCMV were relatively fewer than those by BYMV, CYVV, and PSV. The incidences of AMV and RCVMV did not differ significantly among the seven species. Statistically significant differences among the seven species were observed in the incidences of BYMV, CYVV, PSV, and WCMV. Relatively high incidences of BYMV infections were observed in alsike, subterranean, and red clovers and PSV infections in white clover.

In 1977, scientists in 11 southeastern states initiated Southern Regional Research Project S-127, Forage Legume Viruses. Earlier surveys identified alfalfa mosaic tricornavirus (AMV), bean yellow mosaic potyvirus (BYMV), clover yellow mosaic potexvirus (CYMV), clover yellow vein potyvirus (CYVV), peanut stunt cucumovirus (PSV), and white clover mosaic potexvirus (WCMV) from white clover (*Trifolium repens* L.) and red clover (*Trifolium pratense* L.) collected in the Southeast (5,10,12). These viruses were widely distributed in the Southeast, often infecting a high percentage of red or white clover plants in the field, and infected many other *Trifolium* species when mechanically inoculated in greenhouse tests. Several are transmitted by aphids in a nonpersistent manner (AMV, BYMV, CYVV, and PSV), while CYMV and WCMV have no known vectors. The potyviruses, BYMV and CYVV, are members of the BYMV subgroup of potyviruses and are serologically related (3).

Technical contribution to Regional Research Project S-127, Forage Legume Viruses.

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To determine the incidence of these viruses in important forage legume species under field conditions, the relative incidence of virus diseases among these species, and the geographic distribution of the viruses, cooperators in S-127 conducted a field test involving natural insect vectors in seven legume species and 11 southeastern states. Plots were replanted annually, and individual plants were indexed for virus diseases in 1978, 1979, and 1980. Results and analysis of this "seven species test" are presented here. A preliminary report of these results has been published (15).

MATERIALS AND METHODS

Seven species. Forage legume species included in the test were: alfalfa, *Medicago sativa* L. 'Apalachee'; alsike clover, *T. hybridum* L. 'Common'; arrowleaf clover, *T. vesiculosum* L. 'Yuchi'; crimson clover, *T. incarnatum* L. 'Dixie'; red clover 'Kenland'; subterranean clover, *T. subterraneum* L. 'Mt. Barker'; and white clover 'Tillman'.

Experimental design. Plantings were from seed lots maintained and distributed to cooperators by P. B. Gibson, U. S. Department of Agriculture, Agricultural Research Service, Clemson University, Clemson, SC. Individual plants were grown from seed in a greenhouse at each location, then transplanted to field plots when 6–8 wk old. Transplantings occurred in the fall in most instances, except where weather conditions precluded fall planting and spring plantings were necessary. Entries were randomized in rows with one plant of each of the seven species in each row and a minimum of 10 rows at each location. Plant spacing within and between rows varied among locations from 30 to 90 cm. New plantings were made each year for the 1978, 1979, and 1980 growing seasons. Individual plant samples for virus indexing were taken by collecting fresh leaf tissue from growing points at the four north, south, east, and west edges, and from the center of each plant. Samples were collected in late spring or early summer to correspond with the onset of flowering of the short-lived annual species, crimson and subterranean clover.

Cooperators and locations. Scientists from State Agricultural Experiment Stations and the U. S. Department of Agriculture, Agricultural Research Service (USDA-ARS) in 11 southeastern states participated in the seven species test. Test locations, years of testing, location cooperators, and their respective affiliations were as follows: Tallassee, AL (1978–1980), E. M. Clark and W. C. Johnson, Auburn University; Gainesville, FL (1979), C. E. Dean, R. G. Christie, J. R. Edwardson, S. R. Christie, D. E. Purcifull, and F. W. Zettler, University of Florida; Experiment, GA (1978–1979), J. W. Demski and M. A. Khan, University of Georgia; Tifton, GA (1978–1979), H. D. Wells and J. D. Miller, USDA-ARS; Lexington and Springfield, KY (1978–1979), T. P. Pirone, University of Kentucky; Baton Rouge, LA (1979–1980), B. G. Harville and K. S. Derrick, Louisiana State University; Beltsville, MD (1978–1979), J. P. Meiners, USDA-ARS; Mississippi State, MS (1978–1980), W. E. Knight, R. G. Pratt, and M. M. Ellsbury, USDA-ARS; Raleigh, NC (1978–1979), W. A. Cope, USDA-ARS, and L. T. Lucas and C. L. Campbell, North Carolina State University; Clemson, SC (1978–1980), P. B. Gibson, USDA-ARS, and O. W. Barnett, M. R. McLaughlin, and R. H. Baum, Clemson University; Knoxville, TN (1980), M. R. McLaughlin, University of Tennessee; and Blacksburg, VA (1978–1980), S. A. Tolin, S. Boatman, Virginia Polytechnic Institute and State University, and J. D. Miller, USDA-ARS.

Virus indexing. Plant tissue samples at most locations were indexed for AMV, BYMV, CYMV, CYVV, PSV, red clover vein mosaic carlavirus (RCVMV), and

WCMV by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) using either antibody-sensitized plates distributed and processed by cooperators at Clemson University (16,17), or antisera prepared for ELISA and distributed from Clemson University. In both cases, the same sources of antisera were used as published and positive reactions were determined by visual comparison with control wells containing appropriate infected and healthy plant leaf tissue samples prepared either from fresh tissue or from freeze-dried tissue mailed to cooperators (17).

Reactions in ELISA were generally easily interpreted for most of the viruses, although cross reactivity of field isolates of BYMV and CYVV with both the BYMV-KY204-1 and CYVV-Pratt antisera used in the study presented occasional problems. Whenever possible in such cases, additional samples were collected and tested from the plants in question, or the viruses were isolated by mechanical inoculation to indicator host plants and ELISA was done on the indicator hosts. In instances where subsequent testing either was not possible, or again resulted in positive reactions to both antisera, the results were interpreted as positive for both viruses.

Cooperators in Florida used light microscopy of viral inclusion bodies (7) and SDS-immunodiffusion serology (19) to detect virus infections, and cooperators in Georgia used a latex agglutination test (8). Mechanical inoculation of indicator host plants (5), serologically specific electron microscopy (9), and immunodiffusion serology (10,22) were occasionally used to supplement or verify test results.

Table 1. Incidence of virus infections among seven species of forage legumes from uniform trials in the southeastern United States, 1978–1980

Virus[w]	Observations[x]	Species	Infected[y] (%)
AMV	1,886	Red clover	5.5
		Crimson clover	5.2
		Alsike clover	4.4
		Alfalfa	4.1
		Subterranean clover	4.1
		White clover	3.9
		Arrowleaf clover	3.4
		ANOVA F, P = NS[z]	
CYVV	2,184	Alsike clover	16.7 a
		White clover	15.5 ab
		Crimson clover	10.8 abc
		Subterranean clover	8.5 bcd
		Arrowleaf clover	6.2 cd
		Red clover	3.9 cd
		Alfalfa	2.0 d
		ANOVA F, $P = 0.0001$	
RCVMV	1,251	Crimson clover	6.5
		White clover	4.9
		Subterranean clover	3.3
		Alfalfa	2.1
		Alsike clover	2.1
		Arrowleaf clover	2.1
		Red clover	2.1
		ANOVA F, P = NS	
BYMV	2,253	Alsike clover	27.8 a
		Subterranean clover	22.2 ab
		Red clover	16.7 bc
		Arrowleaf clover	15.5 bc
		Crimson clover	14.5 bc
		White clover	9.4 cd
		Alfalfa	2.7 d
		ANOVA F, $P = 0.0001$	
PSV	2,067	White clover	23.8 a
		Arrowleaf clover	14.0 b
		Alsike clover	13.7 b
		Subterranean clover	9.7 bc
		Crimson clover	9.5 bc
		Red clover	7.3 bc
		Alfalfa	1.7 c
		ANOVA F, $P = 0.0001$	
WCMV	2,047	Crimson clover	4.8 a
		White clover	4.1 ab
		Red clover	3.2 abc
		Alsike clover	2.2 abc
		Arrowleaf clover	1.6 abc
		Alfalfa	1.3 bc
		Subterranean clover	0.7 c
		ANOVA F, $P = 0.01$	

[w] AMV = Alfalfa mosaic virus, BYMV = bean yellow mosaic virus, CYVV = clover yellow vein virus, PSV = peanut stunt virus, RCVMV = red clover vein mosaic virus, and WCMV = white clover mosaic virus.

[x] Number of plants on which observations (virus tests) were conducted.

[y] Percentages are least squares means of infected plants for each species. Means followed by the same letter were not significantly different using the LSD test for $P = 0.05$.

[z] Analysis of variance F test. Mean comparisons were not made when F tests were not significant (NS) ($P = 0.05$).

Data analysis. Data were recorded separately for each plant and virus combination tested. The exact number of plants tested varied among locations and years. Not all plants were tested for all viruses at all locations. Tests for RCVMV were not conducted in 1978. Thus, incidence of RCVMV was based on tests of fewer plants than for the other viruses. The occurrence of a virus infection in a test plant was recorded as "1" if present or "0" if absent. If a given plant was indexed several times during a growing season, the cumulative total of virus infections for that plant was used for statistical analysis, but the maximum cumulative incidence of any given virus infection was still recorded as "1," even though the virus may have been detected several times during repeated tests. Data were combined over years and locations using year-state combinations (environments) as replications. Least squares estimates of means were obtained from an analysis of variance using a general linear models procedure for a randomized complete block design. In order to adjust for unequal numbers of plants, separate analyses for each virus comparing bait species (bait species were considered treatments, and environment the block) and separate analyses for each bait species comparing viruses (viruses were considered treatments, and environment the block) were performed. Because the data were unbalanced, least significant differences between all possible combinations of paired least squares means were used for separating means. The standard errors of the means needed for these calculations were based on numbers of plants and environments contributing to the estimated means.

In the analysis of the data, year and location had no separate fixed meaning. Each year-location combination represented a randomly selected environment in which each species-virus effect was measured. For each analysis performed, the environment variance component was also estimated as a year, location, and year-location variance component. The year-location variance component

was nearly always more important than the individual year and location components. Therefore, virus incidence was not broken down by states.

RESULTS

Over 2,000 individual plants from 11 locations were evaluated for virus infections over 3 years. A total of 731 infected plants were identified. Of these, 612 (84%) had single virus infections and 119 (16%) had multiple virus infections. The incidence of virus-infected plants within each of the seven species of forage legumes was lowest for alfalfa and highest for alsike and white clovers. Differences in incidence of virus infections among the seven species (Table 1) were not statistically significant ($P = 0.05$) by ANOVA F tests for AMV and RCVMV, but were statistically significant for BYMV, CYVV, PSV, and WCMV. The differences in incidences of the six viruses within each of the seven forage legume species (Table 2) were not statistically significant ($P = 0.05$) by ANOVA F tests for alfalfa, arrowleaf clover, and crimson clover, but were statistically significant for alsike, red, subterranean, and white clovers.

The percentages of all test plants infected by the respective viruses varied considerably among the test plot locations. The arithmetic means (percentages of test plants infected) for each virus across all locations and all species were 2.3% for WCMV, 2.4% for RCVMV, 3.9% for AMV, 8.7% for CYVV, 9.9% for PSV, and 12.9% for BYMV. Although positive ELISA tests occurred occasionally with CYMV antiserum (one each from alsike and arrowleaf clovers in Alabama in 1978 and crimson, red, and subterranean clovers in Mississippi in 1980), no infections by CYMV could be verified by subsequent ELISA or by inoculation of indicator hosts.

DISCUSSION

Three viruses, AMV, RCVMV, and WCMV were detected from relatively low percentages of plants. The detection of WCMV, a mechanically transmitted virus with no known vector (6), raises the possibility that at some locations handling procedures used in growing, transplanting, and maintaining plants may have resulted in their inoculation with WCMV. An alternative explanation based on transmission of WCMV through seed is a possibility made remote by the facts that common seed lots were used, the virus occurred in fewer than half of the locations, and two locations accounted for 87% of the WCMV-positive plants. Whether local handling procedures may have affected the incidence of the other viruses, either directly by inoculation or indirectly due to prior infection with WCMV, is unknown. Unless precautions against WCMV contamination are taken, vegetatively propagated white clover plants in the greenhouse and mechanically clipped white clover plants in small field plots, especially plants that are more than 1 year old, are often 100% infected with WCMV (M. R. McLaughlin, *unpublished data*). Although CYMV is very similar to WCMV and is also mechanically transmitted and lacks a known vector, it is much less prevalent in the Southeast and was not detected in the present study.

Consideration of the distributions of virus infections across species (Table 1) and within species (Table 2) led to some associations. Relatively high incidences of BYMV occurred in alsike, subterranean, and red clovers, and a relatively high incidence of PSV occurred in white clover. Associations of BYMV with alsike and red clovers and of PSV with white clover have been reported in several other studies (1,10,11,14). The recognition of these virus diseases as important factors in clover production has led to new research efforts aimed at finding resistance to these viruses in these important forage legume species (2,20,21).

Subterranean clover is an annual species used extensively in Australia, New Zealand, parts of Europe and South America, and in Oregon and northern California in the United States. Although not extensively used in the southeastern United States, subterranean clover is increasing in acreage and importance in this region (13). The association of BYMV with subterranean clover made in this study might lead researchers to expect BYMV to be an important factor in adoption of this species in the Southeast. Since subterranean clover is grown as a winter annual in the Southeast, it may complete most of its growth before the major spring peak in aphid populations and consequential spread of BYMV occur. At Mississippi State, MS, for example, the spring peak in aphid flight activity associated with forage legumes occurs in mid-April to early May (18) as subterranean clover is flowering and setting seed. The effects of late-season BYMV infection on seed production in subterranean clover are unknown but potentially important, since subterranean clover is often selected and managed for its natural reseeding ability.

Species associations with CYVV were less distinct, although CYVV occurred more frequently in white and alsike clovers than in red clover. An apparent association of BYMV with red clover and CYVV with white clover, almost to the exclusion of the reverse associations, was suggested in previous work (14) but this was not as clearly demonstrated in the present data. Although the relative incidences of these two closely related viruses observed in the present data were consistent with those expected for red clover, an unexpectedly high incidence of BYMV was observed in white clover, considered by many to be a nonhost for BYMV (3,4,8,14). In a related study, Barnett and co-workers (3) tested isolates

Table 2. Incidence of virus infections within seven species of forage legumes from uniform trials in the southeastern United States, 1978–1980

Species	Virus[y]	Infected (%)[z]
Alfalfa	AMV	3.9
	RCVMV	3.1
	BYMV	2.7
	CYVV	1.9
	PSV	1.4
	WCMV	1.1
	ANOVA F, $P =$ NS	
Arrowleaf clover	BYMV	14.2
	PSV	11.6
	CYVV	7.3
	RCVMV	4.5
	AMV	4.3
	WCMV	1.4
	ANOVA F, $P =$ NS	
Red clover	BYMV	16.7 a
	PSV	7.1 b
	AMV	4.5 b
	CYVV	3.5 b
	WCMV	3.1 b
	RCVMV	1.4 b
	ANOVA F, $P = 0.0041$	
White clover	PSV	23.5 a
	CYVV	15.2 ab
	BYMV	9.4 bc
	WCMV	3.4 c
	AMV	2.7 c
	RCVMV	2.3 c
	ANOVA F, $P = 0.0009$	
Alsike clover	BYMV	28.2 a
	CYVV	16.9 ab
	PSV	13.4 bc
	AMV	3.7 c
	WCMV	2.1 c
	RCVMV	1.3 c
	ANOVA F, $P = 0.0002$	
Crimson clover	BYMV	11.6
	CYVV	11.4
	RCVMV	5.3
	PSV	6.3
	WCMV	5.7
	AMV	5.0
	ANOVA F, $P =$ NS	
Subterranean clover	BYMV	18.3 a
	CYVV	6.9 b
	RCVMV	6.2 b
	PSV	6.0 b
	AMV	4.1 b
	WCMV	0.7 b
	ANOVA F, $P = 0.0204$	

[y] AMV = Alfalfa mosaic virus, BYMV = bean yellow mosaic virus, CYVV = Clover yellow vein virus, PSV = peanut stunt virus, RCVMV = red clover vein mosaic virus, and WCMV = white clover mosaic virus.

[z] Percentages are least squares means for infected plants of the total plants tested. Mean separation comparisons were not done if F tests were not significant (NS) ($P = 0.05$) in ANOVA. Means followed by the same letter within a species group were not significantly different by LSD test ($P = 0.05$).

within the BYMV subgroup of potyviruses and reported a range of heterologous reactions in ELISA with the same BYMV-KY204-1 and CYVV-Pratt antisera used in the present study. They also reported that BYMV-KY204-1 did not infect white clover (3). Therefore, the BYMV incidence in white clover reported here, based on reactions with BYMV-KY204-1 antiserum, probably represents infections of CYVV and other serologically cross-reactive strains of BYMV rather than infections by BYMV-KY204-1-like viruses.

Although variation in virus incidence among locations contributed to a broader and more representative view of the forage legume virus situation in the Southeast, the data from one or two locations within a given state may not be representative of the virus situation within that state. More locations would need to be sampled within each state before such data could be interpreted as fully representative of any individual state.

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