

Virus Diseases and Stand Decline in a White Clover Pasture

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ABSTRACT

McLaughlin, M. R., Pederson, G. A., Evans, R. R., and Ivy, R. L. 1992. Virus diseases and stand decline in a white clover pasture. *Plant Dis.* 76:158-162.

Linear regression analysis was used to characterize the incidence of virus diseases in white clover, *Trifolium repens*, and associated changes in the white clover population in a grazed pasture through four growing seasons. The incidence of diseases caused by clover yellow vein virus, peanut stunt virus (PSV), and white clover mosaic virus increased linearly through the life of the white clover stand, coincident with a steady decline in the white clover population. The predominant virus detected throughout the study was PSV, which occurred in 80% of the white clover plants sampled in the spring of the fourth growing season. By that time, the clover population had declined significantly, and by the following fall, the clover had virtually disappeared from the pasture. This association provided evidence that PSV may be a major factor in the lack of persistence of white clover. A potentially important source of resistance to PSV was identified in a white clover clone exhibiting hypersensitivity to mechanical inoculation of PSV. Alfalfa mosaic virus and red clover vein mosaic virus were present in infected white clover but at relatively low incidence. Luteoviruses not previously reported from white clover in the Southeast also were detected.

Additional keywords: forage, soybean dwarf virus

Several viruses, including alfalfa mosaic virus (AMV), clover yellow vein virus (CYVV), peanut stunt virus (PSV), red clover vein mosaic virus (RCVMV), and white clover mosaic virus (WCMV), have been identified from infected white clover, *Trifolium repens* L., in the southeastern United States (5,14). These viruses also infect white clover in other parts of the United States (1,16), are

widely distributed in the Southeast, and often infect a high percentage of plants in the field (2,3). AMV, CYVV, PSV, and RCVMV are transmitted by aphids in a nonpersistent manner, but WCMV has no known vector (3). Significant yield losses attributable to virus disease in white clover have been documented (4,7-10). Virus diseases have been suggested as a major factor in the weakening of white clover plants, thus making the plants more susceptible to injury and death from other environmental stresses and diseases (10) and contributing to the lack of persistence of white clover in pastures (17).

Previous studies of disease loss, virus incidence, and virus distribution were conducted either as controlled short-term experiments in greenhouses or growth chambers or as one-time or short-term surveys. No studies have been done in the Southeast on the development and incidence with time of virus diseases and associated changes in white clover under natural

grazing conditions. In the present study, the objectives were to determine the identity and incidence of viruses infecting white clover in a grazed pasture, determine the coincident white clover plant population density, and characterize the virus incidence and white clover plant population density through the life of the clover stand in the pasture.

MATERIALS AND METHODS

Pasture establishment. The pasture study was conducted in an area comprising 10 ha located at the Forage Research Center of the Mississippi Agricultural and Forestry Experiment Station, Prairie, MS. A seedbed was prepared and fertilized with 0-20-37 kg/ha NPK according to recommendations resulting from soil tests. The soil pH was 6.7. Seed of Regal white clover was broadcast on 19 October 1982 at a rate of 4.5 kg/ha. Seed of tall fescue, *Festuca arundinacea* Schreb. 'Kentucky 31', was drilled on 0.35-m row spacing at a rate of 16.8 kg/ha on nine of the 10 ha. A white clover monoculture area of approximately 1 ha was located at the southern end of the pasture. Cattle grazing was begun on 1 May 1983 and continued throughout the study. The stocking rate during peak forage growth periods in spring and summer was two cow-calf units per hectare. The stocking rate was adjusted according to the availability of forage and soil moisture conditions.

Sample collection and clover stolon counts. Samples were collected and stand counts made on 2 August and 18 November 1983; 22 May, 21 August, and 10 December 1984; 22 March, 16 May, 5 July, and 29 November 1985; and 21 May 1986.

Sampling began in the northeast corner of the rectangular pasture and pro-

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Accepted for publication 21 August 1991 (submitted for electronic processing).

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ceeded across the full width of the pasture along east-west transects that ran parallel with the northern edge of the pasture. Twenty-two transects approximately 22 m apart (20 transects in the mixed pasture and two in the clover monoculture) evenly divided the entire pasture. Five samples were collected along each transect for a total of 100 from the mixed pasture and 10 from the clover monoculture. The starting point on each transect was determined by throwing a meter stick 10–15 m into the pasture along the transect and collecting a sample where the stick fell. Subsequent samples were collected at sites 50 paces (approximately 38 m) apart along the transect. The first five samples were collected by proceeding in a westerly direction along the first transect, then the next five samples were collected by proceeding back across the pasture in an easterly direction along the next transect. This back-and-forth collection pattern continued over the full length of the pasture.

White clover stolon density measurements were made by counting the number of stolons transecting the meter stick at each site (11). Stolon density was recorded as stolons per meter for each site. A plant sample for virus testing was collected from a white clover stolon nearest to the 10-cm point on the meter stick. If no stolons transected the meter stick at a given site, no sample was collected from that site.

Samples consisted of a stolon tip with four to six attached leaves. Samples were collected individually and placed between folds in 12 × 13 cm pieces of paper toweling stacked in groups of 10 inside 18 × 21 cm plastic bags. The toweling was dampened with distilled water and the bags were placed on ice in an insulated container for transport to the laboratory.

Virus testing. Samples were held overnight at 5 C and triturated in 0.02 M sodium phosphate buffer, pH 7.3, containing 0.05 (v/v) Tween 20 (PBT) and 0.02 M 2-mercaptoethanol with a mortar and pestle. Homogenates were tested for the presence of viruses by the direct double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) as previously described (13). Samples were tested against antisera to AMV, CYVV (Pratt), PSV, RCVMV, and WCMV prepared from laboratory stocks as previously described (13).

All ELISAs were conducted with 96-well polystyrene microtiter plates coated with antibodies (60 μ l per well, 2.5 μ g/ml, in 0.5 M sodium carbonate buffer, pH 9.6, 1 hr at 5 C) and rinsed three times for 3 min each with PBT. Freshly prepared test samples were added (approximately 200 μ l per well), incubated overnight at 5 C, and rinsed from the wells with PBT as described earlier. Alkaline phosphatase-conjugated antibody preparations were added (diluted

1:400–800 in PBT, 50 μ l per well) and incubated for 6 hr at 5 C and then wells were rinsed with PBT as described earlier. Substrate solution (*p*-nitrophenylphosphate at 1 mg/ml in 10% diethanolamine, pH 9.8, containing 0.02% sodium azide) was added (100 μ l per well) and incubated at room temperature for 1 hr. Absorbance measurements were made on a BioTek Model 307 EIA Reader at 405 nm. Positive and negative control samples, consisting of fresh leaf tissue of alsike clover or white clover, were included with all tests. Test wells were rated positive if their absorbance exceeded the mean absorbance plus two standard deviations of negative control values.

Luteovirus tests. Fifty samples from the 29 November 1985 collection were divided, and the leaves were used in ELISA and the stolons were transplanted to Jiffy Mix potting medium in individual pots for growth in the greenhouse. Plants grown from these cuttings were individually labeled MS1, MS2, etc. Stolon cuttings from these 50 plants were made on 28 July 1986 and sent to V. D. Damsteegt, Frederick, MD, and J. E. Duffus, Salinas, CA, for propagation and subsequent ELISA tests for luteoviruses. Thirty of the 50 clones were propagated at Frederick and tested in October 1986 against antisera to soybean dwarf virus (SDV-dwarfing strain from Japan) and beet western yellows virus (BWYV). Thirty-two of the 50 clones were propagated at Salinas and tested in November 1986 against antisera to BWYV, legume yellows isolate of bean leaf roll virus (LYV), and a subterranean clover red leaf isolate of SDV from California. In January 1988, persistent aphid transmissions from white clover plant MS14 were conducted at Mississippi State. Pea aphids, *Acyrtosiphon pisum* (Harris), were allowed a 48-hr acquisition feeding period in groups of five aphids per leaf on 10 detached leaves of MS14. The aphids then were transferred to subterranean clover, *T. subterraneum* L. 'Geraldton', and allowed a 48-hr inoculation feeding period. Plants then were sprayed with systemic insecticide acephate (Orthene), held in the laboratory for 24 hr to ensure that all aphids were killed, and then moved to the greenhouse.

Tests for resistance in virus-free plants. Seven plants propagated from stolons collected in November 1985 were apparently free of all viruses for which they had been tested at Mississippi State, Frederick, and Salinas. Three of these produced vigorous plants and were selected for further testing. The selected clones, designated clones 22, 31, and 48, were propagated by stolon cuttings and tested for possible resistance to AMV, CYVV, PSV, and WCMV. Plants with two different leaf markings were noted within vegetatively propagated increases of what was considered to be the single

clone 31. One plant had a white leaf mark, or water mark (WM), on the leaflets and the other plant did not. Therefore, they were separated and maintained as different clones designated clone 31 (no leaf mark) and clone 31WM (with water mark). The four clones were increased by stolon cuttings in the greenhouse in the winter of 1988–1989. Fifty plants were produced from each clone, 10 each for inoculation with AMV, CYVV, PSV, and WCMV, and 10 as noninoculated controls. All inoculated plants were mechanically inoculated three times each at 2-day intervals and evaluated for symptoms over the following 4–5 wk and then tested by ELISA. Clones 22 and 31WM were inoculated in January and tested in March 1989. Clones 31 and 48 were inoculated in May and tested in June 1989.

Data analysis. Data on virus incidence (numbers of virus-infected samples from each sampling date) were analyzed as percentages. Data on white clover plant density at each sampling date were analyzed as the mean number of stolons per meter. Regression analysis was conducted using the SAS general linear model (GLM) procedure (19) for total and individual virus incidence and stolon density as functions of time (months) after seeding. Linear, quadratic, and cubic models were tested for all regressions. In all cases, only linear coefficients had a significant *F* value ($P < 0.05$). Data from the mixed and monoculture areas of the pasture were analyzed separately.

Analysis of variance was conducted on the number of stolons per meter for 10 sampling dates. Each set of five consecutive samples along a transect was considered a replication, giving 20 and two replicates in the mixed and monoculture areas of the pasture, respectively. After a significant *F* test ($P = 0.05$), a least significant difference (LSD) analysis was performed for mean separations.

RESULTS

Sample collection and clover stolon density. One hundred samples were collected from the mixed pasture at each sampling date except 22 May 1984 (99 samples), 10 December 1984 (97 samples), 29 November 1985 (77 samples), and 21 May 1986 (72 samples). Samples were used entirely for virus testing in ELISA, except for those collected 29 November 1985, which were also propagated for luteovirus tests as described earlier. Ten samples were collected from the white clover monoculture area of the pasture at each sampling date except 22 March 1985 (eight samples), 16 May 1985 (nine samples), 29 November 1985 (four samples), and 21 May 1986 (five samples).

Mean clover stolon density in the mixed pasture area was highest at the first sampling date in August 1983, at 20.3 stolons per meter. Stolon density

declined (LSD = 1.8) through 1983 and 1984 to 15.7 stolons per meter in November 1983, 10.8 in May 1984, 10.7 in August 1984, and 8.0 by December 1984. Stolon density increased in spring 1985 to 13.7 stolons per meter in May, declined to 8.7 in July, and remained essentially unchanged at 8.8 in November. The final measurement in May 1986 of 4.4 stolons per meter was lower than all previous measurements in the mixed pasture area. No additional measurements were made, but a final inspection was conducted in October 1986 and virtually no clover remained in the pasture. Stolon density in the white clover monoculture area was highest at the first sampling date in August 1983 at 21.8 stolons per meter, then declined (LSD = 5.0) to 16.6 in November 1983, and to 8.9 in May 1984. Stolon density showed a slow upward trend over the next four sampling dates to 13.0 in August 1984, 14.6 in December 1984, 16.3 in March 1985, and 17.0 in May 1985, decreased to 10.2 in July 1985, decreased to 2.5 in November 1985, and then reached a low of 1.7 at the final sampling date in May 1986. Linear regressions of stolon count means are shown in Figure 1.

Virus testing. A total of 945 samples were tested for viruses from the mixed culture area of the pasture and 86 from the clover monoculture area. Of these, 351 (37%) and 53 (62%), respectively, were infected by one or more viruses. The total number of virus infections detected in the mixed pasture area was 431 and in the monoculture area was 86. The incidence of virus-infected samples ranged from 3 to 82% in the mixed culture area and 0 to 100% in the monoculture area. Peanut stunt virus accounted for the highest number of infections in both areas, 290 (67.3%) in the

mixed culture area and 38 (44.2%) in the monoculture area. The contributions of other viruses to the total number of infections in the mixed culture and monoculture areas, respectively, were WCMV, 80 (18.6%) and 36 (41.9%); AMV, 29 (6.7%) and 9 (10.5%); CYVV, 21 (4.8%) and 3 (3.5%); and RCVMV, 11 (2.6%) and 0.

The percentage of the total number of plant samples found to be infected by the viruses in the mixed and monoculture areas, respectively, were PSV, 30.7 and 44.2; WCMV, 8.5 and 41.8; AMV, 3.1 and 10.5; CYVV, 2.2 and 3.5; and RCVMV, 1.2 and 0. Single infections accounted for 29 of 80 WCMV infections in the mixed area and eight of 36 in the monoculture area. The ratio of the number of virus infections to the number of infected plants was calculated as a measure of the incidence of mixed infections. These ratios were 1.23 and 1.62 in the

mixed pasture and monoculture areas, respectively. When WCMV infections were subtracted from the total number of infected plants and from the total number of infections, the ratios were 1.09 and 1.11 in the mixed and monoculture areas, respectively.

The incidence of total virus-infected white clover plants among those sampled in the mixed pasture area was best described by a linear model defining a straight line from 0 to 80% infected plants between 10 and 43 mo after seeding (Fig. 2A). The incidence of total virus-infected white clover plants in the monoculture area was described by a linear model for the period from 10 to 33 mo after seeding, during which time the incidence reached 100% (Fig. 2B). Data from collections made 37 and 43 mo after seeding were not included in the models shown in Figure 2B, because the incidence of infected samples had reached 100% and the white clover population had declined markedly (only four and five samples, respectively, were collected).

In regression analysis of the incidence of individual viruses, only PSV, WCMV, and CYVV increased linearly in the mixed pasture area (Fig. 2A), and only PSV and WCMV increased linearly in the monoculture area (Fig. 2B). The incidence of AMV in the mixed pasture area, although higher in total number than CYVV, remained at a low level (about 3%) throughout the study. CYVV occurred late in the study in the monoculture area at 33 and 37 mo after seeding and accounted for only three infections. The incidence of RCVMV was low and not fitted to a regression model.

Luteovirus tests. Six plants tested positive for SDV, one plant tested positive for SDV and BWYV, and three plants tested positive for LYV. Five of the luteovirus-positive plants also were infected with PSV, two with WCMV, and two with PSV and WCMV. One SDV-positive plant, designated MS14, was apparently free of nonpersistently and mechanically transmitted viruses. One of 10 Geraldton indicator plants, inoculated in January 1988 by persistent aphid transmission from white clover plant MS14, developed red leaf symptoms typical of luteovirus infection.

Tests for resistance in virus-free plants. All clones were susceptible to one or more of the viruses, but infection rates (number of infected plants out of 10 inoculated) ranged from three to 10 and symptoms were not always reliable indicators of infection. Clone 22 was susceptible to AMV (three plants had yellow mosaic symptoms and positive ELISA) and WCMV (10 plants with mosaic symptoms and positive ELISA) but apparently resistant to CYVV (no symptoms and negative ELISA) and hypersensitive to PSV. Leaves of clone 22 inoculated with PSV developed chlorotic lesions that rapidly became necrotic within

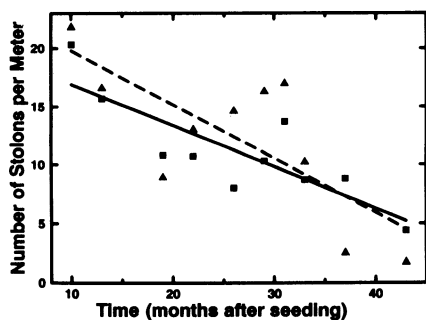


Fig. 1. The white clover stand, (number of stolons per meter) plotted as a function of time (months after seeding) in a grazed pasture. In mixed culture with tall fescue (■, —), the regression model described the relationship as a linear function: number of stolons per meter = $20.5 - 0.4$ (months after seeding), $r^2 = 0.69^{**}$. In monoculture (▲, - - -), the regression model described the relationship as a linear function: number of stolons per meter = $24.4 - 0.5$ (months after seeding), $r^2 = 0.56^*$. (* = *F* test for linear coefficient significant at 0.05 level, ** = significant at 0.01 level.)

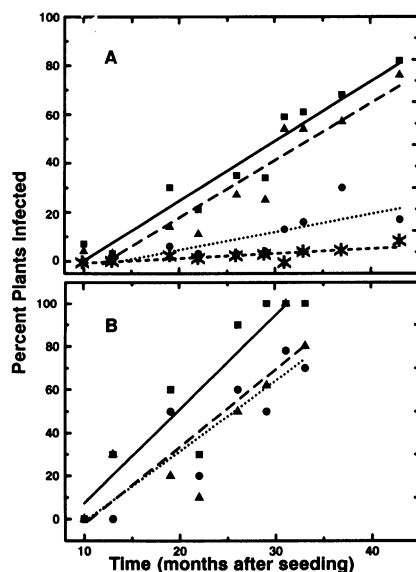


Fig. 2. The incidence of virus infections (percent infected plants among those sampled) in white clover plotted as a function of time (months after seeding) in a grazed pasture. (A) In mixed culture with tall fescue, regression models described the relationships as linear functions: percent plants infected with one or more viruses (■, —) (total) = $-24.1 + 2.4$ (months after seeding), $r^2 = 0.93^{**}$; percent plants infected with peanut stunt virus (PSV) (▲, - - -) = $-29.2 + 2.3$ (months after seeding), $r^2 = 0.90^{**}$; percent plants infected with white clover mosaic virus (WCMV) (●, - - -) = $-10.1 + 0.7$ (months after seeding), $r^2 = 0.64^{**}$; and percent plants infected with clover yellow vein virus (CYVV) (*, - - -) = $-2.8 + 0.2$ (months after seeding), $r^2 = 0.65^{**}$. (B) In monoculture, regression models described the relationships as linear functions: percent plants infected with one or more viruses (■, —) (total) = $-36.3 + 4.4$ (months after seeding), $r^2 = 0.86^{**}$; percent plants infected with PSV (▲, - - -) = $-38.2 + 36$ (months after seeding), $r^2 = 0.74^{**}$; and percent plants infected with WCMV (●, - - -) = $-34.1 + 3.3$ (months after seeding), $r^2 = 0.82^{**}$. (** = *F* test for linear coefficient significant at 0.01 level.)

7–10 days after inoculation. Noninoculated leaves grown after inoculation of clone 22 were negative in ELISA. Clone 31WM was susceptible to AMV (three plants with mosaic symptoms and positive ELISA), PSV (four plants with mosaic and positive ELISA) and three symptomless plants with positive ELISA), and WCMV (10 symptomless plants with positive ELISA) but was not infected by CYVV (no symptoms and negative ELISA). Clone 31 was not infected by CYVV or PSV (no symptoms and negative ELISA), and only one plant was infected by WCMV (no symptoms and one positive ELISA). Clone 48 produced no symptoms to CYVV, PSV, or WCMV but was infected by all three (five, two, and 10 positives in ELISA, respectively). The AMV tests of clones 31 and 48 were inconclusive, possibly because of low virus titer in the inoculum.

DISCUSSION

This study was the first done in the Southeast to document the development of virus diseases and associated changes in stand density in a white clover pasture under natural grazing conditions. PSV accounted for more infections in the mixed culture area than all of the other viruses combined. Incidence of PSV also was high in the monoculture area, followed closely by WCMV. Transmission of WCMV was presumably by mechanical means and accomplished by mowing machines during periodic clipping or by the treading and grazing of cattle. The intercropped grass may have had a diluting effect on WCMV inoculum in the mixed pasture, thus, the spread of WCMV in the mixed culture was slower than in the monoculture. Furthermore, the higher mixed infection ratio (the total number of virus infections divided by the total number of infected plants) for the monoculture area was attributable almost entirely to WCMV infections. When infections of WCMV were factored out, mixed infection ratios for the two areas were nearly equal. Without WCMV, the presence or absence of grass apparently had little effect on the mixed infection ratios, which were just slightly higher than 1.0, indicating that most infected plants had single virus infections.

The relative contribution of WCMV to total infections was nearly five times greater in the monoculture area than in the mixed area, and that of AMV about three times greater, whereas CYVV and PSV differed little between the two areas. Incidence of RCMV was low and contributed little to the virus disease incidence. These findings corroborate those of an earlier study (6) in which interplanting with tall fescue resulted in one-third as many infections by AMV and WCMV as when white clover was grown in monoculture. These observations suggest that interplanting white clover with tall fescue may offer some control of the

spread of AMV and WCMV but little or no control of the spread of CYVV or PSV.

The occurrence of luteoviruses in white clover was not unexpected, because a subterranean clover red leaf virus strain of SDV was isolated earlier from naturally infected subterranean clover at Mississippi State (15), less than 80 km away. Furthermore, these viruses are common in white clover in Japan, New Zealand, and Australia (12). Although the incidence of luteovirus infections was low in the present study, relatively few samples were tested. The extent of luteovirus infections in white clover, the identity of the viruses, and their possible roles as contributing factors in decline of white clover need further investigation.

The high incidence of PSV measured in this study was consistent with earlier published work on the incidence of viruses infecting white clover in the Southeast (14). The increasing and predominant incidence of PSV and the decline of white clover documented in the present study (Figs. 1 and 2) led us to conclude that PSV infection is a significant factor that should be evaluated in relationship to lack of persistence of white clover in this region.

Attempts to obtain direct evidence for the role of PSV in white clover decline through controlled experiments in the open field have been largely unsuccessful because of rapid natural spread of the virus from inoculated treatment plots into noninoculated control plots (18). Filtered-air enclosures to exclude vectors have proven useful in measuring effects of viruses on white clover in the field (9) but are highly artificial environments requiring extensive maintenance. Near isogenic lines of white clover, with and without resistance to PSV, are needed as experimental tools to provide the control needed for definitive field experiments under grazing.

During periods of favorable environmental conditions, increased secondary stolon development and rapid stolon elongation resulted in increased plant density as measured by stolons per meter. This factor and the loss of weakened plants during periods of unfavorable environmental conditions, such as drought, which frequently occurs in late summer, probably contributed toward seasonal fluctuations in white clover plant density. Over the long term of this study, however, virus disease pressure on the white clover continued to increase. This provided an effective natural screen that allowed identification of white clover genotypes as possible sources of resistance to virus infections, notably hypersensitive clone 22. Hypersensitivity to virus infection has proved valuable in breeding for resistance to bean yellow mosaic virus in red clover (20). Further research to determine the value and genetic inheritance of the hypersensitive reaction to

PSV found in white clover clone 22 is underway. This clone has possibilities for development of the near isogenic experimental lines of white clover needed for definitive field study of the effects of PSV on white clover persistence and may also be important in the improvement of white clover germ plasm and in cultivar development.

ACKNOWLEDGMENTS

We gratefully acknowledge the cooperation and encouragement of the late Robert E. Coats, former superintendent of the Prairie Research Center, in establishing the experimental pasture. We thank V. D. Damsteegt and J. E. Duffus for their cooperation in making possible the ELISA for luteoviruses. We also thank Carol Trevathan, Quinnia Yates, Mary Hardy, and Charlotte Freeman for technical assistance.

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