Natural Incidence of Peanut Stunt Virus Infection in Hybrid Populations of *Trifolium ambiguum* × *T. repens*

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ABSTRACT

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A spaced-plant nursery was established in 1988 to evaluate the reactions to peanut stunt virus (PSV) of populations of kura (*Trifolium ambiguum*) and white (*T. repens*) clovers, F_1 hybrids H-435 and H-262, F_2 plants, first and second backcross plants of one F_1 hybrid to white clover, and first backcross intercross plants. No PSV infection was detected in one of four first backcross progenies and two kura clover populations after 11 mo of field exposure. Two F_1 hybrids and white clover checks had moderate to severe symptoms and high incidence of PSV infection. Second backcross progenies and backcross intercross progenies were apparently tolerant to PSV infection and had few or no symptoms despite very high virus incidence. Introgression of genes for PSV resistance from kura clover to white clover was not evident with the hybrid populations tested. Prospects for obtaining genes for resistance to PSV from the two interspecific hybrids tested are doubtful.

White clover (*Trifolium repens* L.) is a valuable component of many pastures in temperate regions of the world. In addition to being a high-quality forage, white clover promotes the growth of associated grasses by fixing biological nitrogen in symbiosis with the bacterium *Rhizobium trifolii* Dangeard. A limiting factor in white clover production is nonpersistence due partly to its short-lived taproot (20) and partly to its susceptibility to various virus diseases.

In a survey of pastures in southeastern United States, several viruses were found infecting white clover (3). Peanut stunt virus (PSV) was detected in 21% of all white clover plants assayed, clover yellow vein virus (CYVV) in 14%, alfalfa mosaic virus (AMV) in 11%, and white clover mosaic virus in 4%. A later study confirmed that PSV and CYVV were the two major viruses infecting white clover in southeastern United States (14). Reductions in forage yield and persistence of white clover have been shown to result from virus infection (2,7,9,13,15,17). PSV, CYVV, and AMV are transmitted in a nonpersistent manner by several aphid species (10,11,18).

Although some resistance to PSV has been found within the white clover gene pool (4,8,12), a broader spectrum of resistance to a range of viruses would be preferable. Kura clover (*T. ambiguum* M. Bieb.) has been shown to be resistant to infection by several viruses when subjected to mechanical inoculation or exposed to natural infection and appears to be the best source of genes for multivirus resistance for transfer to white clover (1,3,5,16). Although the mechanism of this resistance has not been determined, vector resistance seems unlikely, since red clover mosaic virus (a virus transmitted by some of the same aphid species that transmit PSV and CYVV) was detected by enzyme-linked immunosorbent assay (ELISA) in 16–50% of plants for six kura clover populations assayed after 1 yr of field exposure (19). Resistance to virus multiplication appears to be a possible mechanism that has not been investigated. Interspecific hybrids of white clover and kura clover have been produced (21,23).

Although the stolon nodal roots of white clover are well adapted to scavenging surface moisture, they are not effective during drought, when species with a deeper, more perennial root system would prevail. The persistence of white clover, with its short-lived taproot, may also be improved by acquisition of the rhizomatous, deep rooting habit of kura clover. Kura clover, however, has poor seedling vigor and is slow to regrow after harvest, deficiencies that may be remedied by introgression of genes from white clover, thus expediting the adaptation of kura clover to conditions in the United States. Faster establishment and increased vigor could contribute to greater forage yield, and increased seed set would reduce the number of generations needed for seed multiplication before cultivar release. This study was undertaken to evaluate interspecific hybrids of *T. ambiguum* × *T. repens* and backcross populations for susceptibility to natural infection with PSV.

MATERIALS AND METHODS

Plant material. *T. ambiguum* × *T. repens* interspecific hybrids H-435 and H-262 (23) were obtained in 1986 from Grasslands Division, DSIR, New Zealand, via E. G. Williams, University of Melbourne, Australia. The DSIR also provided F_2 plants derived from H-435 and produced by N. L. Taylor in New Zealand. White clover clones utilized included Feather Mark (PI 516413), obtained from J. van den Bosch, DSIR, New Zealand; Florida XP1, obtained from K. H. Quesenberry, University of Florida; NZ 459, a single genotype obtained from DSIR, New Zealand, via E. G. Williams, University of Melbourne; Regal (PI 516415); and one population probably originating from South Carolina designated SC 83-10, obtained by N. L. Taylor in New Zealand.

Kura clover (tetraploid = $4x$) plants used were open-pollinated clones from the cultivar Treeline (AZ 595) obtained from DSIR via E. G. Williams. The kura clover population utilized was not the same as the one providing the kura clover parents of H-435 and H-262.

Four first backcross plants of H-435 to white clover (HBC-A, HBC-C, HBC-D, and HBC-E) were produced by N. L. Taylor at DSIR, New Zealand, in 1985. Hybrid backcross-C (HBC-C) had H-435 as the female parent and Feather Mark as the male parent. The three other first backcross progenies (HBC-A, HBC-D, and HBC-E) had a single clone of SC 83-10 (whose leaves are marked with a white "v") as the male parent. Plants from second backcrosses of H-435 to white clover and backcross intercrosses were produced in 1986 by honeybee (*Apis mellifera*) pollinations of plants in isolated field cages at the University of Kentucky. The second backcross plants had HBC-A or HBC-D as the female parent and a population designated as KY Red V as the male parent. Plants of the KY Red V population were discovered in an old white clover field by N. L. Taylor at the University of Kentucky. Backcross intercross plants had either HBC-A or HBC-E as the female parent and HBC-C or another first backcross as the male parent.

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Inasmuch as the KY Red V and HBC-C clones were heterozygous for their respective leaf markings, progeny from interpollinations were recovered that did not possess the leaf marking that was needed for identification of the pollen parent. Such plants utilized were HBC-C × HBC-?, HBC-D × HBC-?, and HBC-E × HBC-?, in which the unidentified male parent represented by the question mark is HBC-A, HBC-C, HBC-D, or HBC-E, and HBC-D × (HBC-A, HBC-D, or KY Red V).

Field study, 1987. Seeds of second backcrosses of H-435 to white clover, backcross intercrosses, and Florida XP1 were germinated on moist filter paper in petri dishes in the greenhouse. After one or two trifoliolate leaves had emerged, plantlets were cultured in 10.16-cm pots containing a commercial growth medium in a greenhouse. Mature plants were transplanted to the field on 29 April 1987 in a completely randomized design with three replications, 24 rows each, with each class of plant material (Florida XP1 or plants that had a common female parent in the preceding backcross or intercross) occupying up to five rows, as allowed by seed supply. Each row consisted of up to 10 plants of each class of plant material, with 0.91 m of space between and within rows.

Plants that survived through 6 July 1988 and were analyzed consisted of 14 genotypes of Florida XP1 white clover (check cultivar), 1 genotype of HBC-A × HBC-C, 14 of HBC-E × HBC-C, 13 of HBC-D × KY Red V, 17 of HBC-C × HBC-?, 6 of HBC-E × HBC-?, and 10 of HBC-D × (HBC-A, HBC-D, or KY Red V).

Symptoms of virus disease were scored on 26 August 1988. Prior to this, portions of all plants were excavated, transplanted into 10.16-cm pots containing a commercial growth medium, and cultured in a greenhouse. Virus symptoms of the transplants were scored on a scale of 1–9, where 1–3 = mild, 4–6 = moderate, and 7–9 = severe symptoms. Infection with PSV was detected by ELISA conducted on 6 July on plants in the field and 30 August 1988 on transplants. The 10 trifoliolate leaves showing the most clearly visible virus symptoms of each plant were removed, placed in plastic bags, and stored on ice until extraction for ELISA. Healthy and PSV-infected white clover or PSV-infected cowpea (*Vigna unguiculata* (L.) Walp.) were used as negative and positive controls, respectively.

Extractions were prepared for ELISA by homogenizing 10–12 leaf disks (9 mm diameter, approximately 120 mg total) with 600 μl of carbonate coating buffer, pH 9.6, (6) in 1.5-ml tubes. The extract, filtered by pipetting it through glass wool into clean 1.5-ml tubes, was stored overnight at 4 C. An indirect ELISA procedure was used for detection of PSV. First, 100 μl of antigen preparation in coating buffer was added to each well of microtiter plates (Elkay Products, Inc., Shrewsbury, MA), two wells per sample, and incubated for 1 hr at 37 C. The wells were rinsed three times after this step and between subsequent steps by filling each with PBS-Tween and incubating at least 3 min per rinse. Then, 100 μl of blocking solution (0.5% bovine serum albumin [BSA] in phosphate buffered saline [PBS]) was added to each well and incubated 1 hr at 37 C. This was followed by addition of 100 μl of anti-PSV IgG (2 μg/ml in PBS containing 0.5% BSA) per well and incubation for 1 hr at 37 C. Goat antirabbit IgG-alkaline phosphatase conjugate (1 μg/ml in PBS containing 0.5% BSA) was then added (100 μl per well) and incubated for 1 hr at 37 C. This was followed by addition of *p*-nitrophenylphosphate, 1 mg/ml in diethanolamine buffer, pH 9.6 (6). The enzyme-substrate reaction was measured spectrophotometrically at 405 nm in a plate reader (Titertek Multiskan) at appropriate intervals (usually 15, 30, and 60 min after incubation).

Sample A_{405nm} values that exceeded the mean plus two standard deviations of A_{405nm} values for the healthy control were classified as positive. Relative virus concentrations were not compared because of the semiquantitative nature of this indirect assay. A chi-square test was used to test for independence of visual virus scores and PSV ELISA results.

Field study, 1988. A 26-entry, spaced-plant nursery was established in 1988, consisting of clonal propagules of NZ 459 white clover, H-435 and H-262, four first backcross plants of H-435 to white clover, and genotypes propagated from the 1987 white clover study, including Florida XP1, one genotype of HBC-A × KY Red V, three genotypes of HBC-D × KY Red V, three of HBC-A × HBC-C, three of HBC-E × HBC-C, and two of HBC-D × HBC-?. Clonal propagules were made of a single genotype of Treeline kura clover obtained from a nearby field, also established in 1987. Entries of seed-propagated genotypes included open-pollinated Treeline kura clover, F_2 plants from H-435, and white clover genotypes from Feather Mark, Regal, and SC 83-10.

The experimental design was a randomized complete block with four replications. Each replication consisted of 26 entries, with up to five plants per entry, depending on availability of plant material. Plants were transplanted to the field on 31 May 1988 and spaced on 0.91-m centers. In June 1988, cowpea plants infected with a white clover isolate of PSV were transplanted between replications. Experimental plots were maintained weedfree by regular cultivation. The field was irrigated periodically from June through mid-July. Survival of the clover was poor despite periodic irrigation, and virus symptom data of only two replications were analyzed.

Observations on symptoms of virus diseases were recorded on 1 October 1988 and 4 May 1989. Virus disease symptoms were recorded on a scale of 1–9, as outlined earlier. Harvest dates for ELISA testing were 19, 20, and 29 September and 14 October 1988 (classified as 4 mo of field exposure) and 4 and 8 May 1989 (classified as 11 mo of field exposure). Plants from only those genotypes within each entry with the most clearly visible symptoms were assayed for PSV after 4 mo of field exposure. After 11 mo of field exposure, some of the plants classified as not infected were retested along with additional plants not tested after 4 mo. The leaf tissue harvest, ELISA procedure, and data analysis were as used for the 1987 field study. Visual symptom-score data were analyzed from two replications and 21 and 18 entries for 1988 and 1989 data, respectively. Treatment means were then compared by computing an LSD (0.05). Chi-square tests for independence

Table 1. Number and percentage of plants from 1987 field study infected with peanut stunt virus as indicated by ELISA

	First observation		Second observation[a]		
Genotype	No. tested	No. infected	No. tested	No. infected	Percent infected[b]
White clover check					
Florida XP1	14	5	6	3	57
Backcross intercrosses					
HBC-A × HBC-C	1	0	···	···	0
HBC-E × HBC-C	14	4	7	1	36
HBC-C × HBC-?	17	9	5	3	71
HBC-E × HBC-?	6	1	4	0	17
Second backcrosses					
HBC-D × KY Red V	13	12	1	0	92
HBC-D × (HBC-A, HBC-D, or KY Red V)	10	9	···	···	90

[a] Only surviving plants that tested negative in first observation were retested in second observation.
[b] Percent infected = [(no. infected in first observation + no. infected in second observation)/no. tested in first observation] × 100.

were applied to reveal any correlation between ELISA results and symptom scores.

RESULTS

Most surviving clones from the 1987 study were found to be infected with PSV (Table 1), although virus incidence was lower in genotypes with HBC-E as the female parent. Chi-square tests for independence between visual ratings of viruslike symptoms and PSV ELISA data were significant ($P < 0.01$). Plants in the 1988 white clover field study had a high incidence of PSV infection (Table 2). Most propagules of H-435 and H-262 were infected with PSV. No virus was detected in kura clover, HBC-E plants, and three of four F_2 plants of H-435 (Table 2). The fact that not all propagules of susceptible genotypes showed positive ELISA values suggests that some plants may have escaped virus infection.

Plants from the 1988 study were identified that showed severe symptoms (>6 on a 1–9 scale) but tested negative for PSV infection according to ELISA. These plants included one each of H-262 and SC 83-10 and one F_2 of H-435 identified after 4 mo of field exposure and one each of H-435, HBC-C, HBC-D, and Regal and three of SC 83-10 identified after 11 mo of field exposure. These observations suggest the presence of more than one virus, although PSV was widespread and was likely the predominant virus present in both 1988 and 1989, as indicated by chi-square tests for independence between viruslike symptoms and PSV ELISA ($P < 0.01$). Tests were not conducted to identify other viruses. Scores for visual viruslike symptoms of backcross intercross and second backcross plants were relatively low, considering that most of these entries were 100% infected with PSV.

Table 2. Scores of visual symptoms of virus infection and percentage of plants from 1988 field study infected with peanut stunt virus as indicated by ELISA conducted after 4 and 11 mo of field exposure

	Field exposure						
	4 mo			11 mo			
Entry	Visual symptoms	No. tested	No. infected	Visual symptoms	No. tested	No. infected	Percent infected[a]
Trifolium ambiguum							
Treeline	1.0[b]	5	0	2.1[b]	6 (5)[c]	0 (0)[c]	0
Open-pollinated Treeline	1.2	3	0	···	6	0	0
T. repens							
Feather Mark	5.0	5	3	6.4	6	6	82
Florida XP1	6.9	6	5	8.7	4 (1)	4 (1)	100
NZ 459	7.8	5	3	···	··· (2)	··· (0)	60
Regal	6.3	4	3	5.0	8	7	83
SC 83-10	3.1	7	3	4.7	5 (4)	4 (0)	58
F_1 hybrids							
H-262	7.0	6	4	3.8	4 (2)	3 (0)	70
H-435	6.0	7	6	6.2	8 (1)	5 (0)	73
F_2 plants of H-435	2.7	4	1	···	··· (1)	(0)	25
First backcrosses							
HBC-A	3.0	6	1	2.4	··· (4)	··· (2)	50
HBC-C	···	2	1	···	··· (1)	··· (0)	50
HBC-D	2.8	4	3	2.5	··· (1)	··· (0)	75
HBC-E	···	4	0	···	··· (3)	··· (0)	0
Second backcrosses							
HBC-A × KY Red V	2.8	6	5	3.0	4	4	90
HBC-D × KY Red V (1)[d]	3.0	4	4	5.4	1	1	100
HBC-D × KY Red V (2)	2.3	5	3	3.4	··· (1)	··· (1)	80
HBC-D × KY Red V (3)	2.8	4	4	3.3	2	2	100
Backcross intercrosses							
HBC-A × HBC-C (1)	4.4	3	3	4.0	2	2	100
HBC-A × HBC-C (2)	···	2	2	···	1	1	100
HBC-A × HBC-C (3)	···	2	2	···	0	0	100
HBC-D × HBC-? (1)	2.8	3	3	4.9	2	2	100
HBC-D × HBC-? (2)	4.7	2	2	5.3	3	3	100
HBC-E × HBC-C (1)	3.6	5	1	5.0	2 (3)	2 (0)	43
HBC-E × HBC-C (2)	3.3	4	4	3.5	2	2	100
HBC-E × HBC-C (3)	···	2	2	···	0	0	100
LSD (0.05)	1.9			1.5			

[a] Percent infected = [no. infected (4 mo + 11 mo + retested after 11 mo)]/[no. tested (4 mo + 11 mo)] × 100.

[b] Analyzed on the basis of two replications. Plants were scored on a scale of 1–9, with 9 indicating the most severe symptoms. Missing values reflect entries that had no plants remaining in plots of the two replications used in analysis.

[c] First number represents additional plants that were not tested after 4 mo of field exposure. Number in parentheses indicates number of individual plants that tested negative for PSV infection after 4 mo of field exposure and were retested after 11 mo.

[d] Number in parentheses is used to differentiate genotypes having the same pedigree.

DISCUSSION

The prospects of obtaining genes for resistance to PSV from kura clover in the present hybrid populations are doubtful, inasmuch as H-435 and H-262 are susceptible. The genotypes of the Turkish kura clover that were female parents of the two hybrids were not available for evaluation in these investigations, and it is not known if those genotypes were susceptible to PSV. A recent study has shown a low level of susceptibility to PSV in the kura clover germ plasm (16). It is not known whether the resistance observed in HBC-E was of kura clover origin, via H-435, or was transmitted from its SC 83-10 white clover parent. If one assumes this resistance to be of kura clover origin, it implies that expression of resistance may be modified by genetic background. This may be the case, since in a screening of a larger H-435 F_2 population, 21 of 24 plants were free from infection after mechanical inoculation with PSV (16).

Second backcross and backcross intercross progenies may show tolerance to virus infection. Even though ELISA indicated PSV infection in all of these entries, they showed few or no symptoms. The incidence of infection in backcross intercross and second backcross progenies relative to the incidence in other entries may have been inflated, since they were propagated from the 1987 field nursery. Although such plants showed no viruslike symptoms before transplantation to the 1988 nursery, they were subjected to an additional 13 mo of the field environment.

Several obstacles exist to a rapid introgression of genes. The first is that *T. ambiguum* × *T. repens* hybridization is difficult because of the need for embryo rescue, which itself is often unsuccessful (22). Second, we have little knowledge of the inheritance of traits being selected for (e.g., persistence, rooting habit, virus resistance). This latter drawback is less limiting, however, inasmuch as breeders have selected successfully for many traits without knowledge of their inheritance. That PSV resistance appears to be conditioned by both general and specific combining ability (4) suggests a multigene inheritance. Even if resistance to single strains of the virus were provided by single dominant genes, several such genes would need to be incorporated rapidly into breeding material to protect the forage against the inevitable occurrence of new virus strains. Inheritance information would be useful, however, for developing materials resistant to several viruses and for developing plants that express the rhizomatous or stoloniferous habit. To identify more persistent genotypes from the second backcross population, a more comprehensive evaluation with a larger population will probably be needed. After 1 yr in the field, the present small

backcross populations were morphologically indistinguishable from white clover. Third, the 4*x* kura clover germ plasm is poorly adapted to cultivation inasmuch as the plants flower only once a year, are extremely dormant in winter, and do not regrow rapidly after harvest. Hexaploid kura clover suffers from some of these same problems but is more vigorous. Incorporation of genes from the more agronomically adapted 6*x* kura clover at the earliest possible stage in the development of future hybrid populations would, therefore, be desirable.

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