

Resistance to Viruses in *Trifolium* Interspecific Hybrids Related to White Clover

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ABSTRACT

Pederson, G. A., and McLaughlin, M. R. 1989. Resistance to viruses in *Trifolium* interspecific hybrids related to white clover. *Plant Disease* 73:997-999.

Six *Trifolium* interspecific hybrids, 24 F₂ plants from *T. ambiguum* × *T. repens* hybrid 435, and 48 *T. ambiguum* plant introductions and populations were evaluated for resistance to peanut stunt virus (PSV), clover yellow vein virus (CYVV), and alfalfa mosaic virus (AMV). One *T. repens* × *T. occidentale*, three *T. ambiguum* × *T. hybridum*, and two *T. ambiguum* × *T. occidentale* hybrids were resistant to PSV. Of these, only the *T. repens* × *T. occidentale* hybrid was fertile. Fifteen F₂ plants from *T. ambiguum* × *T. repens* hybrid 435 were resistant to PSV, CYVV, and AMV. The *T. ambiguum* plants had 99 and 100% resistance to PSV and CYVV, respectively. The *T. repens* × *T. occidentale* hybrid and *T. ambiguum* × *T. repens* F₂ plants may be valuable sources of virus resistance for incorporation into adapted white clover cultivars.

White clover (*Trifolium repens* L.) is the predominant legume used for grazing by cattle in permanent pastures in the southern United States. A major limitation of white clover is its lack of persistence in perennial grass-legume pastures. White clover stands often decline significantly after the second or third year of growth. Virus diseases may play an important role in the decline of white clover stands. Viruses have been shown to reduce white clover yield (9,16,24), persistence (19,23), seed production (4), crude fiber (16), and nodulation (11).

Peanut stunt virus (PSV), clover yellow vein virus (CYVV), and alfalfa mosaic virus (AMV) are the most prevalent viruses identified on white clover in the southern United States (3,18,19). PSV was found in 21% and CYVV in 14% of the white clover plants sampled from pastures in eight southern states during 1971-1972 (3). In space-plant tests in 11 southern states during 1978-1980, 23.5% of the white clover plants were infected with PSV (18). Infection by PSV caused the greatest

reduction in white clover yields (9,24). In filtered-air enclosures in the field, white clover yields were reduced 28% when infected with PSV and 14% when infected with CYVV (9).

Interspecific hybridization of white clover with other *Trifolium* species has long been suggested as a means to improve various characters of white clover, including virus resistance (3,6). White clover has been successfully hybridized with six other *Trifolium* species: *T. ambiguum* M. Bieb. (25), *T. argutum* Sol. (syn. *T. xerocephalum* Frenzl) (14), *T. isthmocarpum* Brot. (15), *T. nigrescens* Viv. (6), *T. occidentale* Coombe (syn. *T. repens* var. *biasoletii* (Steud. & Hochst.) Aschers. & Graebn.) (12), and *T. uniflorum* L. (20). Of these six species, *T. ambiguum* has been reported to be resistant to PSV, CYVV, and AMV (1,3,7), although susceptibility to CYVV was found in one plant introduction (1). Resistance to PSV and susceptibility to CYVV and AMV has been observed in *T. uniflorum* and *T. hybridum* (1,3,7). *T. occidentale*, *T. isthmocarpum*, and *T. nigrescens* are susceptible to PSV, CYVV, and AMV (3,7).

Most *Trifolium* interspecific hybrids have been evaluated only for agronomic potential, fertility, and meiotic pairing. Recently, *T. nigrescens* was identified as a source of resistance to the southern root-knot nematode (*Meloidogyne incognita* (Kofoid & White) Chitwood), and two fertile (*T. repens* × *T. nigrescens*) × *T. repens* backcross hybrids with nematode resistance were identified (22). Little is known of the virus susceptibility of *Trifolium* interspecific hybrids. Cell suspension cultures of *T. ambiguum* × *T. hybridum* did not support multiplication of CYVV, and intact hybrid plants were not systemically infected (13).

The objectives of this study were to determine the responses of six *Trifolium* interspecific hybrids to PSV inoculation; to determine the responses of F₂ plants of *T. ambiguum* × *T. repens* to separate inoculations of PSV, CYVV, and AMV; and to evaluate 48 *T. ambiguum* plant introductions and populations for their responses to separate inoculations of PSV and CYVV.

MATERIALS AND METHODS

Plant material. Cuttings of six *Trifolium* interspecific hybrids were obtained from E. A. Rupert, Clemson University, Clemson, SC (8). Seed from the self-fertile *T. ambiguum* × *T. repens* hybrid 435 (25) was obtained from N. L. Taylor, University of Kentucky, Lexington. Seed of 28 *T. ambiguum* plant introductions was obtained from the Regional Plant Introduction Station at Geneva, NY. Seed of 10 additional plant introductions and nine populations of *T. ambiguum* was obtained from C. E. Townsend, USDA-ARS, Fort Collins, CO. Seed of *T. ambiguum* 'Treeline' was obtained from Australia by W. E. Knight, USDA-ARS, Mississippi State, MS.

Plants of the *Trifolium* interspecific hybrids were vegetatively cloned by stolon tip cuttings or by dividing the parent plant. Vigor of the *T. ambiguum* × *T. hybridum* and the *T. ambiguum* × *T. occidentale* hybrids was poor, and few cuttings could be made from these hybrids. All plants were grown in a greenhouse in 10-cm-diameter plastic pots containing a commercial growth medium of peat moss and vermiculite (1:1, v/v). The greenhouse was maintained at a temperature of 18 ± 3 C in the winter and 28 ± 4 C in the summer.

Virus isolates and inoculation procedure. The isolates of PSV, CYVV-Pratt, and AMV used in this study were maintained in white clover or *T. hybridum* plants in the greenhouse. All isolates were originally obtained from O. W. Barnett, Clemson University (3). All plants were mechanically inoculated in the greenhouse with sap prepared from infected *Pisum sativum* L. 'Dwarf Gray Sugar' (*T. ambiguum* test), *T. hybridum* (last two CYVV inoculations of *T. ambiguum* test), or white clover (other tests). The sap was prepared by grinding freshly collected leaves with mortars and pestles in 0.03 M sodium phosphate buffer, pH 7.35, containing 0.02 M 2-mercaptoethanol. Carborundum (600-

Contribution of the U.S. Department of Agriculture, Agricultural Research Service, in cooperation with the Mississippi Agricultural and Forestry Experiment Station, Mississippi State, MS 39762. Journal article No. 7192.

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Accepted for publication 4 July 1989.

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mesh) was mixed with the sap, and the inoculum was rubbed over three or four leaves of the test plants with a pestle. *Trifolium* interspecific hybrids were inoculated twice with PSV. Plants of *T. ambiguum* × *T. repens* F₂ were inoculated three times with AMV and two times with PSV and CYVV using a separate set of clones for each virus. Two to eight *T. ambiguum* plants from each of the 48 plant introductions or populations, for a total of 331 plants, were inoculated three times with PSV and four times with CYVV.

Tests for virus infections. The double antibody sandwich ELISA was used to test all plants inoculated with CYVV and AMV (17). ELISA was used to test *T. ambiguum* plants inoculated with PSV in addition to inoculation of the indicator host, *Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'California Blackeye,' which was used for detection of PSV in all hybrids tested. Antisera to PSV, CYVV, and AMV were laboratory stocks prepared as previously described (17). Reactions of test samples in ELISA were compared with control samples of appropriately infected and healthy plant leaf tissue. Optical density measurements at 405 nm were made on a Bio-Tek Model EL307 or EL309 microplate reader (Bio-Tek Instruments, Inc., Burlington, VT). Optical density values were judged positive when they exceeded the mean of the healthy control plus two standard deviations. All *Trifolium* test plants except *T. ambiguum* × *T. repens* F₂ hybrids were assayed for virus infections at least two times in separate tests to verify their reactions to virus inoculations. The F₂ hybrids were inoculated with CYVV and PSV and tested once each by ELISA and indicator host assay. The tests were conducted 10

and 6 wk after inoculation with CYVV and PSV, respectively.

RESULTS

Responses of *Trifolium* interspecific hybrids to PSV inoculation. A wide range of reactions to PSV inoculation occurred among the six *Trifolium* interspecific hybrids tested (Table 1). Three cuttings from two plants of *T. ambiguum* × *T. occidentale* and five cuttings from two other plants of *T. ambiguum* × *T. hybridum* not shown in Table 1 were uniformly negative in PSV assays. The *T. isthmocarpum* × *T. repens* hybrids were uniformly positive for PSV. Comparing the response of plants within hybrids, we found that the *T. repens* × *T. occidentale* hybrids showed the greatest variation. *T. repens* × *T. occidentale* (plant 5-4-1) had no cuttings with positive PSV reactions, whereas *T. repens* × *T. occidentale* (plant 6-2-1) was uniformly positive for PSV.

Of six hybrids having no positive PSV reactions, only *T. repens* × *T. occidentale* (plant 5-4-1) was fertile (22). The *T. ambiguum* × *T. hybridum* and *T. ambiguum* × *T. occidentale* hybrids were sterile. Of the hybrids with few positive reactions, only (*T. repens* × *T. nigrescens*) × *T. repens* (plants 2-3-1 and 2-3-2), *T. repens* × *T. occidentale* (plant 6-2-2), and *T. repens* × *T. uniflorum* (plant 2-3-4) were fertile (22). Only one of 10 cuttings of (*T. repens* × *T. nigrescens*) × *T. repens* (plant 2-3-2) tested positive for PSV in one of two indicator host assays. The cutting was inadvertently discarded before a third host assay was conducted.

Responses of *T. ambiguum* × *T. repens* F₂ plants to PSV, CYVV, and AMV inoculation. The F₂ plants from the *T. ambiguum* × *T. repens* hybrid 435

showed some susceptibility to PSV, CYVV, and AMV. Of 24 F₂ plants tested, three were susceptible to PSV, three to CYVV, and three to AMV. Although no plant was infected by more than one virus, 37.5% were infected by one of the viruses.

Responses of *T. ambiguum* to PSV and CYVV inoculation. Of 331 *T. ambiguum* plants inoculated, none was infected by CYVV and only three (0.9%) were infected by PSV. These plants were from PI 238154, PI 405935, and No. 222 (University of Reading, England). One of the five plants tested from PI 405935, described as the cultivar Treeline by the Plant Introduction Station, Geneva, NY, was found to be infected with PSV. No PSV-infected plants were detected among 10 grown from Treeline seed obtained directly from Australia. No PSV-infected plants were detected for PIs 108699, 206482, 206483, 225827, 225828, 225829, 228370, 229624, 229625, 231890, 231891, 258787, 258788, 277535, 277865, 291770, 297979, 297980, 314484, 314552, 314553, 314554, 325484, 325486, 325487, 325488, 325489, 325490, 369329, 405119, 405120, 405121, 405122, 405123, 405124, and 405934 (cv. Summit), and FC 33109, FC 36132, Canada PI 205, Canada PI R1-17, Treeline, and Nos. 220 and 221 (University of Reading, England).

DISCUSSION

T. ambiguum was generally resistant to infection by mechanical inoculation with PSV and CYVV. These results corroborate and extend previous reports of resistance to AMV, bean yellow mosaic virus (BYMV), CYVV, PSV, red clover vein mosaic virus (RCVMV), and white clover mosaic virus (WCMV) in *T. ambiguum* (1,3). Although susceptibility to CYVV, WCMV, clover yellow mosaic virus, and RCVMV has been reported for *T. ambiguum* (1,3) and susceptibility to PSV is reported for the first time in this study, in all cases the majority of plants tested were resistant to these viruses. The number of PSV-susceptible plants of *T. ambiguum* was so few that the species should be considered predominantly resistant. Resistance to virus infections in *T. ambiguum* was also found in all interspecific hybrids involving *T. ambiguum*. Resistance to PSV, CYVV, and AMV was maintained in most plants of the F₂ generation of the *T. ambiguum* × *T. repens* hybrid 435. Although all *T. ambiguum* hybrids except *T. ambiguum* × *T. repens* were sterile, further hybridization with *T. ambiguum* appears to be a promising means of incorporating virus resistance into other *Trifolium* species. Recent success in improving the fertility of *T. ambiguum* × *T. repens* hybrid 435 via colchicine doubling of the chromosomes (2) offers further promise for backcrossing with this hybrid.

Table 1. Reaction of five *Trifolium* interspecific hybrids to peanut stunt virus (PSV)

Hybrid	Plant number	Number of cuttings inoculated	Number of PSV-positive cuttings ^a
<i>T. ambiguum</i> × <i>T. hybridum</i>	1-3-1	10	0
<i>T. isthmocarpum</i> × <i>T. repens</i>	5-2-6	10	10
	5-4-5	10	10
	(<i>T. repens</i> × <i>T. nigrescens</i>) × <i>T. repens</i>	2-3-1	10
<i>T. repens</i> × <i>T. occidentale</i>	2-3-2	10	1 ^b
	2-3-3	10	4
	5-2-2	10	5
	5-2-3	10	7
	5-4-1	10	0
<i>T. repens</i> × <i>T. uniflorum</i>	6-2-1	10	10
	6-2-2	10	1
	6-2-3	10	2
	6-2-6	10	1
	2-3-5	10	2 ^b
	2-3-6	10	10
	3-4-1	9	6
	3-4-2	10	10
3-4-3	10	8	
3-4-4	10	4	

^aPositive reaction to PSV on *Vigna unguiculata* subsp. *unguiculata* 'California Blackeye' in at least two of three host assays.

^bPlant reported as PSV-positive gave a positive reaction on only one of two host assays.

The reaction of the *T. repens* × *T. occidentale* hybrids, especially plant 5-4-1, was unexpected. Both parent species have been reported to be susceptible to PSV, although some variability in reaction among plants within both species was noted (3). Evaluation of a greater number of *T. occidentale* genotypes than used previously (3) would provide a better indication of the PSV susceptibility of this species. Backcrosses of *T. repens* × *T. occidentale* (plant 5-4-1) to white clover will be evaluated to determine if this virus resistance can be incorporated into white clover. Additional testing will be required to ensure that susceptibility to pea mosaic virus (formerly BYMV-204-1) reported for *T. occidentale* (3) is not also incorporated into white clover.

A number of the *Trifolium* inter-specific hybrids had only one or two of 10 cuttings giving a positive reaction to PSV (Table 1). Because all cuttings from a particular hybrid were genetically identical, it would be expected that if one or two cuttings were found to be PSV-infected, then all cuttings should be susceptible. Although the reason that all susceptible plants were not infected is unknown, others (3,5) have had difficulty in achieving 100% infected plants by mechanically inoculating susceptible clover. In this study, the virus titer and condition in the inoculum and the procedures used were sufficient for uniform PSV infection of a number of susceptible hybrids and of all indicator hosts inoculated after each clover inoculation. This suggests that the limiting factor in mechanical inoculations is the host genotype. Possibly, resistance to mechanical inoculation or to systemic movement of PSV may occur in some hybrids or infections may not have been detected because of low virus titers in the infected plants.

The recent release of a multiple-virus-resistant germ plasm of white clover (SRVR) gives researchers the first opportunity to incorporate virus resistance into adapted white clover cultivars (10). Although the clones composing

SRVR were selected for PSV, CYVV, and AMV resistance at numerous locations throughout the southern United States, in evaluations of SRVR at Mississippi State, the germ plasm was relatively susceptible to AMV (21). Additional sources of virus resistance will be needed to obtain multiple virus resistance in white clover cultivars for the southern United States. Further evaluation of (*T. repens* × *T. nigrescens*) × *T. repens* (plant 2-3-2) is needed to determine if it may be an additional fertile source of PSV resistance. The PSV resistance of *T. repens* × *T. occidentale* (plant 5-4-1) and the multiple virus resistance of the *T. ambiguum* × *T. repens* F₂ plants may be valuable sources of resistance if they can be incorporated into adapted white clover cultivars.

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