Susceptibility to Clover Yellow Vein Potyvirus in the United States Germ Plasm Collection of Subterranean Clover

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ABSTRACT

McLaughlin, M. R., and Fairbrother, T. E. 1994. Susceptibility to clover yellow vein potyvirus in the United States germ plasm collection of subterranean clover. Plant Dis. 78:665-667.

In spring 1991, natural infections of clover yellow vein virus (CYVV) caused severe disease in 60% of experimental accessions of subterranean clover (*Trifolium subterraneum*) grown in field trials at Mississippi State, Mississippi. A search for resistance to CYVV was initiated within the United States collection of subterranean clover germ plasm. Plants of each of 261 plant introduction (PI) lines and three cultivars were grown in the greenhouse during the winter and spring of 1991–1992. Seedlings were mechanically inoculated with CYVV-Pratt and evaluated 21–28 days later. Initial DAS-ELISA confirmed an absolute correlation of symptoms with infection. Symptomless plants were reinoculated and reevaluated up to five times. Fewer than 2% of all tested plants were selected as resistant and held as first-generation parents (P_1). Selected P_1 plants (45 from 21 PI lines) were identified as var. *yanninicum* (one line), var. *oxaloides* (seven lines), and var. *flagelliforme* (13 lines) and allowed to produce first-generation self-pollinated (S_1) seed. The S_1 seed were grown and the S_1 plants tested in the summer of 1992, following the same procedures used in the selection of the P_1 plants. Six S_1 plants from four PI lines were selected for second-generation (S_2) seed production. The S_2 seed were grown and the S_2 plants tested in 1993, following procedures similar to those used for P_1 and S_1 tests. All S_2 plants were susceptible and showed uniformly severe reactions consisting of rapid systemic wilt and plant death. We conclude that no heritable resistance to CYVV exists in the major portion of the germ plasm collection tested. The severe susceptible (hypersensitive) reaction identified in some lines may be a useful management tool in limiting spread of CYVV in the field.

Subterranean clover (*Trifolium subterraneum* L.) is a legume that is grown as an overseeded winter annual in the southeastern United States (3,9). It is a predominant forage legume in Australia and is widely grown in other parts of Australasia, the Iberian Peninsula, South Africa, temperate South America, and the northwestern United States in northern California and Oregon. The prostrate stems, production of numerous flowers, and geocarpic peduncles of subterranean clover make it well suited to natural reseeding even under close and heavy grazing (11). In the U.S. Southeast, subterranean clover is used for forage and also as a winter cover crop in conservation tillage production systems (5).

Subterranean clover is considered to be predominantly self-pollinated (14). However, genetic variability and visitation of flowers by pollinating insects have been observed in nature (7), and outcrossing rates of 0.15–0.22% have been reported (4,10). These reports agree that cross-pollination could and does occur and that as a source of genetic diversity it plays a significant role in the evolution of this species. Breeding and evaluation programs have been established in Texas and Mississippi.

Subterranean clover is affected by a variety of diseases, including those caused by viruses (6). Among the viruses, clover yellow vein potyvirus (CYVV) has caused severe mosaic disease problems in subterranean clover at several locations in the southeastern United States. In a 3-yr survey (1978–1980) of six virus diseases in seven forage legumes in the Southeast, CYVV was one of the most frequently identified viruses infecting subterranean clover (13) and a virus of major concern to forage legume researchers (1,2). In 1985, CYVV was identified and shown to be widespread in subterranean clover evaluations at Mississippi State, Mississippi, and Angleton, Texas (M. R. McLaughlin, *unpublished*). During the winter of 1990–1991, subterranean clover plant introduction (PI) lines planted in field tests at Mississippi State were observed to have severe symptoms of a virus disease. Subsequent tests by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) were positive for CYVV. By the spring of 1991, 60% of 75 accessions had one or more infected plants. One year later, in a fall 1991 planting evaluated in spring 1992, 50% of 150 accessions had one or more plants with CYVV disease symptoms.

These associations of CYVV with subterranean clover suggest that virus disease caused by CYVV is an important factor in the adaptation and utilization of subterranean clover in this region. Either resistance to CYVV or a management strategy for dealing with CYVV infections will be essential if this clover species is to be successfully grown and fully utilized in the region. The available United States germ plasm has never been evaluated for reaction to CYVV. Johnstone and McLean (6) earlier called for such an evaluation of Australian germ plasm. The objectives of the present research were to classify accessions of the United States germ plasm collection of subterranean clover according to their CYVV disease reactions and to identify promising lines for future breeding work.

MATERIALS AND METHODS

Selection of PI lines. Seed of 261 subterranean clover PI lines was obtained from the Regional Plant Introduction Station at Griffin, Georgia. In addition, seed of three cultivars (Koala, Meteora, and Mt. Barker) was obtained commercially, bringing the total number of accessions to 264. Ten seedling plants from each accession were grown in Jiffy Mix potting medium (CASSCO, Montgomery, AL) in 2.5 × 15 cm Cone-Tainers (Stuewe and Sons, Corvallis, OR). Three plantings, in groups of approximately 87 PI lines each, were made in the greenhouse in the fall of 1991. From 40 to 50 Mt. Barker plants were included in each planting as a control susceptible to CYVV. All plants were maintained in the greenhouse for 4–6 wk, then inoculated with virus. Group 1 plants (seeded 14 October) were inoculated with CYVV on 13 November and 7 December 1991 and on 6 January and 11 February 1992. Group 2 plants (seeded 8 November) were inoculated on 11 December 1991 and on 16 January, 11 February, 12 March, and 17 April 1992. Group 3 plants (seeded 9 December) were inoculated on 16 January, 11 February, 12 March, and 17 April 1992.

Three or four leaves of each plant were mechanically inoculated by a pestle dipped in inoculum. Viral inoculum was pre-

Journal Article No. J-8376 of the Mississippi Agricultural and Forestry Experiment Station.

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Accepted for publication 29 March 1994.

pared from 1- to 4-wk-old symptomatic leaves of alsike clover (*T. hybridum* L.) systemically infected with CYVV-Pratt. Leaves were triturated 1:10 (w/v) in 0.03 M sodium phosphate buffer, pH 7.3, containing 0.02 M 2-mercaptoethanol, and 600-mesh silicon carbide was added. Plants were evaluated visually 3–4 wk after inoculation, and symptomless plants were reinoculated (on new leaves). Initial tests of 10 entries by DAS-ELISA, following published methods (12), showed an absolute positive correlation of symptoms with positive ELISA results. Subsequent evaluations were based on symptoms, with occasional ELISA confirmation.

This inoculation and evaluation process was repeated five times in the winter of 1991–1992. Symptomless plants selected through this process were designated as first-generation parents (P_1) and allowed to flower and produce first-generation self-pollinated (S_1) seed in the greenhouse during the spring of 1992. Infraspecific taxa identities of the selected P_1 plants were determined according to the classification of Zohary and Heller (15).

Selection of S_1 and S_2 plants. The S_1 seed were planted in the greenhouse as described above, and S_1 plants were evaluated for resistance in 1992. The S_1 seed were planted 3 August and the S_1 plants were inoculated on 21 August, 11 September, and 16 October, following the same procedures used in selecting the P_1 plants. Resistant S_1 plants were selected as described for the P_1 plants and allowed to mature and produce second-generation self-pollinated (S_2) seed in the greenhouse. The S_2 seed were similarly grown and the S_2 plants were tested in 1993. The S_2 seed were planted 1 September and the S_2 plants were inoculated on 7 October and 3 November.

Table 1. Infections among susceptible cv. Mt. Barker control plants included with test plants in parental (P_1) and first (S_1) and second (S_2) self-pollinated progeny generations of subterranean clover plants inoculated with clover yellow vein potyvirus

Test	No. of plants	No. of plants infected per no. of inoculations				
		1	2	3	4	5
P_1 test						
Group 1	40	38	0	2	...	...
Group 2	51	44	3	4	...	...
Group 3	50	35	1	13	1	...
Total	141	117	4	19	1	...
Cumulative percent		83	86	99	100	
S_1 test						
Total	30	30	...	...	...	...
Cumulative percent		100				
S_2 test						
Total	12	2	10	...	...	...
Cumulative percent		17	100			
All tests						
Total	183	149	14	19	1	...
Cumulative percent		81	89	99	100	

Table 2. Taxonomic classification of *Trifolium subterraneum* lines tested for resistance to clover yellow vein virus in parental (P_1) and first (S_1) self-pollinated progeny generations

PI no.	P_1 plants		S_1 plants		Infraspecific taxa
	No. tested	No. resistant	No. tested	No. resistant	
493151	10	8	71	1	*oxaloides*
493152	10	1	10	0	*oxaloides*
493155	10	1	10	0	*oxaloides*
493158	10	1	10	0	*oxaloides*
493159	10	1	10	0	*flagelliforme*
493160	10	1	10	0	*flagelliforme*
493161	10	3	30	0	*flagelliforme*
493162	10	2	20	0	*flagelliforme*
493166	10	1	10	0	*flagelliforme*
493194	12	6	59	3	*flagelliforme*
493195	13	6	59	1	*flagelliforme*
493219	11	1	12	0	*flagelliforme*
493227	10	1	12	0	*flagelliforme*
493270	12	1	10	0	*flagelliforme*
493304	10	1	10	0	*oxaloides*
517162	10	1	6	0	*flagelliforme*
517165	11	2	15	0	*flagelliforme*
517166	10	4	40	0	*flagelliforme*
535705	13	1	10	0	*oxaloides*
535718	12	1	10	0	*oxaloides*
535765	10	1	10	1	*yanninicum*
Totals	224	45	434	6	

RESULTS AND DISCUSSION

We tested 261 PI lines. Although the U.S. Germplasm Resource Information Network (GRIN) lists 314 lines in the United States collection, seed was not available for some lines. In all, 2,870 plants were evaluated in selecting P_1 plants. Of these, 2,825 (98.5%) were infected by CYVV, including all 141 of the Mt. Barker plants used as controls. In addition, PIs 189396, 277438, and 279012 were also Mt. Barker and were all infected (30 plants total) after two inoculations. Of the Mt. Barker control plants tested alongside the P_1 plants, 83% were infected after one inoculation, 99% after three inoculations, and 100% after four inoculations (Table 1). Symptoms exhibited by the infected plants generally appeared first as systemic vein-clearing and mosaics on new leaves 11–21 days after inoculation. Subsequent new growth exhibited mild to severe mosaics and mild to severe reductions in leaf size and petiole length. Some accessions exhibited necrotic local lesions followed by systemic necrosis and rapid plant death.

Lines from which all plants were susceptible were as follows (10 plants per PI unless listed otherwise in brackets following the number): 99476, 134772, 158387, 168638, 184962, 189396, 190558, 190561, 190564, 190567, 190568, 190572, 190577, 206389 [8], 209924, 209926, 209927, 209928, 209930, 233866, 233867, 233868, 233869, 233870, 233871, 233872, 233873, 233874, 239901, 239902, 239904, 239906, 239907, 239908 [13], 239910 [7], 241461, 249847, 249848, 268067, 268132, 277431, 277433, 277434, 277435, 277436, 277437, 277438, 277439, 279012, 287998, 291871, 291880, 291892, 291894, 292494, 302977, 311497, 318933, 319140, 319141, 319142, 319143, 319145, 319146, 324287, 353434, 378136, 401556 [9], 401571, 419334, 419413, 432334, 449327, 458015, 493149, 493150, 493153, 493154, 493156, 493157, 493163 [9], 493164, 493165, 493167, 493168, 493169, 493170, 493171, 493172 [12], 493173, 493174, 493175, 493176, 493177, 493178, 493179 [9], 493180, 493181 [11], 493182, 493183, 493184 [11], 493185, 493186 [11], 493187, 493188, 493189 [12], 493190 [12], 493191 [12], 493192 [13], 493193 [14], 493196 [12], 493197 [12], 493198 [12], 493199 [12], 493200 [11], 493201 [11], 493202 [12], 493203 [11], 493204, 493205 [13], 493206, 493207, 493208 [11], 493209 [11], 493210, 493211, 493212, 493213, 493214, 493215, 493216 [18], 493217 [12], 493218, 493220, 493221, 493222, 493223 [13], 493224, 493225, 493226, 493228, 493229, 493230, 493231, 493232, 493233, 493234,

493235, 493236, 493237, 493238, 493239, 493240, 493241, 493242, 493243, 493244, 493245, 493246, 493247, 493248, 493249, 493250, 493251, 493253, 493254, 493255 [8], 493256, 493257, 493258, 493259, 493260, 493261, 493262, 493263, 493264, 493265, 493266, 493267, 493268 [9], 493269 [11], 493271 [11], 493272, 493273, 493274, 493275, 493276, 493277, 493278, 493279 [11], 493280 [12], 493281, 493282 [9], 493283, 493284, 493285, 493286, 493287, 493301, 493302, 493303 [8], 517157, 517158, 517159, 517160 [13], 517161, 517168 [12], 517171, 517173 [11], 517174 [13], 517178, 535699 [11], 535702, 535703 [12], 535704 [12], 535706 [11], 535707 [12], 535708 [14], 535709 [12], 535710 [11], 535711 [13], 535712 [13], 535713 [12], 535716, 535717 [12], 535719 [12], 535720, 535721 [13], 535722, 535723, 535724, 535726 [11], 535727, 535728, 535732, 535733, 535734, 535761, 535762, and 535764. A summary of these results was submitted for inclusion in the GRIN database.

Forty-five P_1 plants representing 21 PI lines were selected as resistant and were classified taxonomically (Table 2). Taxonomists recognize several taxa of subterranean clovers. In 1965, Katznelson and Morley (8) grouped these into three subspecies: *subterraneum* L., *yanninicum* Katzn. & Morley, and *brachycalycinum* Katzn. & Morley. In 1974, Katznelson (7) elevated these to species. In the most recent taxonomic treatment of the subterranean clovers (the one used in the present study), Zohary and Heller (15) considered all of these as members of a single species, *T. subterraneum*. They recognized two subspecies, *subterraneum* and *brachycalycinum*, each consisting of a single variety, *subterraneum* and *brachycalycinum*, respectively. They also recognized six varieties of *T. subterraneum* (which they did not consider subspecies): *yanninicum, brachycladum, majurculum, graecum, oxaloides*, and *flagelliforme*. In accordance with this classification, we identified the selected P_1 plants taxonomically as *Trifolium subterraneum* var. *yanninicum* (one PI line), var. *oxaloides* (seven PI lines), and var. *flagelliforme* (13 PI lines).

None of the cultivars tested showed any resistance to CYVV. Most of these cultivars are classified as "true" subterranean clovers (*T. subterraneum* subsp. *subterraneum* var. *subterraneum*), which comprise the majority of commercially grown cultivars. The "true" subterranean clover cultivars found susceptible to CYVV were Daliak (PI 99476), Dwallganup (PI 277434), Bacchus Marsh (PI 134772), Howard (PI 277436), Mt. Barker (PIs 189396, 277438, and 279012), Nangeela (PI 190568), Tallarook (PI 277439), and Woogenellup (PI 268067). Two of the three other susceptible cultivars, Clare (PIs 277433 and 449327) and Koala, are classified as *T. subterraneum* subsp. *brachycalycinum* var. *brachycalycinum*, and the third, Meteora, is classified as *T. subterraneum* var. *yanninicum*. These are the cultivars of subterranean clover presently available in the United States.

After two inoculations of S_1 plants, all Mt. Barker control plants included with the S_1 plants were infected, and the S_1 plants were evaluated. Of 434 S_1 plants tested, 139 (32%) remained healthy after two inoculations. After three inoculations, only six S_1 plants, representing four original PI lines, remained healthy (Table 2). These were PI 493151 (one plant of var. *oxaloides*), PI 493194 (three plants of var. *flagelliforme*), PI 493195 (one plant of var. *flagelliforme*), and PI 535765 (one plant of var. *yanninicum*). These six resistant S_1 plants were maintained in the greenhouse for S_2 seed production in the spring and summer of 1993, but only S_1 plants of two of the lines (PIs 493194 and 493195) produced seed. Twelve S_2 plants of each of these lines were grown for evaluations in the fall of 1993. After two inoculations of these S_2 plants, all of 12 Mt. Barker control plants were infected; 11 had typical mosaic and stunting and one died from severe systemic wilt. All of the inoculated S_2 test plants were also infected and showed severe systemic wilt reactions 2–3 wk after inoculation. Wilted plants did not recover and, beginning with the smallest plants, began to die 3–4 wk after inoculation, manifesting a supersusceptible systemic hypersensitivity to CYVV.

We conclude that no CYVV-resistant cultivars are commercially available in the United States and, furthermore, that no heritable resistance to CYVV exists in the U.S. germ plasm collection tested. We propose that if resistance to CYVV cannot be found and incorporated into U.S. cultivars, either through conventional breeding or genetic transformation, management strategies for limiting spread of CYVV must be developed to precede widespread commercial use of subterranean clover in the Southeast. The inheritance of the severe systemic hypersensitive reaction and its potential use in limiting within-field spread of CYVV are being investigated.

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