Evaluation of Red Clover for Resistance to Bean Yellow Mosaic Virus

S. T. SIM, Graduate Research Assistant, and K. T. LEATH, Research Plant Pathologist, USDA-ARS, U.S Regional Pasture Research Laboratory, and C. P. ROMAINE, Associate Professor, Department of Plant Pathology, Pennsylvania State University, University Park 16802

ABSTRACT

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A mixed cultivar population of symptomless red clover (*Trifolium pratense*) plants from 2-, 3-, and 4-yr-old fields and a population of Kenstar red clover plants in the greenhouse were evaluated for resistance to bean yellow mosaic virus (BYMV) strain 204-1 after a series of mechanical inoculations, aphid transmission, and exposure to natural field conditions. Plants were evaluated for resistance by visual inspection for symptom development and by enzyme-linked immunosorbent assay for detection of viral antigen in the leaves. Initial populations of more than 500 plants each were reduced by culling virus-infected plants and general attrition to eight in the field population and seven in the seedling population. These 15 plants were not infected despite repeated mechanical inoculations with standard and concentrated inoculum; exposure for one growing season in a field of aphid-infested, BYMV-infected red clover; and a transmission feeding by viruliferous aphids. These plants were considered resistant to strain 204-1 of BYMV.

Additional key word: ELISA

Bean yellow mosaic virus (BYMV) is one of the most prevalent viruses of red clover (1,5,8,9,11,17,19). In Pennsylvania, BYMV was found in 80% of the fields surveyed and accounted for 73% of the virus-infected plants (10). Infection with BYMV can reduce hay yield by 26–56% (14) and seed yield by as much as 89% (6). BYMV probably interacts with *Fusarium* spp. to increase root rot and cause premature stand decline, as observed in red clover infected with red clover vein mosaic virus and *Fusarium* spp. (22) or in white clover infected with clover yellow mosaic virus and *Fusarium* spp. (3).

BYMV-resistant red clover clones have been identified in the United States and Europe (4,5,7,16), but only two cultivars, Kenstar and Arlington, are reported to have moderate resistance (15,20). Resistant plants were identified by visual observation, which does not eliminate symptomless, infected plants from the breeding population, and host indexing, a cumbersome practice for large numbers of plants.

Our objectives were to compare field and greenhouse populations for BYMV-resistant plants and to develop and test a system to evaluate BYMV resistance in red clover that would apply intense selection pressure, keep escapes to a minimum, and be applicable to large numbers of plants.

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MATERIALS AND METHODS

Plant populations. Five hundred eighty-eight symptomless red clover plants from several cultivars, including Arlington, Florie, Kenstar, Pennscott, Redland, and unidentified cultivars, were collected in the fall of 1980 from 2-, 3-, and 4-yr-old red clover fields in Centre and Lancaster counties of Pennsylvania. Stems, with a small piece of crown tisue attached, were cut from selected field plants and planted in rooting flats in the greenhouse. Rooted cuttings were transplanted into 500-ml clay pots containing commercial peat-vermiculite potting mix. Each plant was drenched with 15 ml of benomyl (8 g a.i./L of water) immediately after transplanting and again 8 mo later to control root rot. Plants were fertilized with soluble 20-20-20 NPK, and foliage was trimmed as needed.

Five hundred two plants of Kenstar red clover were started from seed in a greenhouse in early 1981; they were fertilized and trimmed as needed, and benomyl was applied at about 165 days.

Virus and antiserum. BYMV strain 204-1 originally purified from red clover (8), also known as pea mosaic virus strain 204-1 (12), and antiserum to 204-1 were provided by O. W. Barnett, Clemson University. The virus was maintained in alsike clover (*T. hybridum* L.) by mechanical transmission every 2–3 mo. Alsike clover was used as initial inoculum because it exhibited disease more quickly and dependably than did red clover.

Enzyme-linked immunosorbent assay (ELISA). The gamma globulins were purified and conjugated with alkaline phophatase essentially as described by Clark and Adams (2). The "double-antibody sandwich" version of ELISA as described by Voller et al (21) and Clark and Adams (2) was used, except leaves were sampled with a paper hole punch to cut disks of leaf tissue 6 mm in diameter (13). After sampling three disks from each of six leaves, the punch was washed with detergent and rinsed twice to remove plant sap and tissue. This procedure was adequate to prevent carryover of virus inoculum. Leaf disks were incubated (three disks per well) for 24 hr at 4 C in phosphate buffer (0.02 M phosphate, pH 7.3, 0.15 M NaCl, 0.05% Tween 20, 0.003 M KCl, 0.02 M diethyldithiocarbamate [NaDIECA], and 0.02% NaN_3 [PBDIECA]).

Polystyrene microtiter ELISA plates (Dynatech Lab. Inc., Alexandria, VA) were coated with 5 μg/ml, and later, 10 μg/ml of partially purified gamma globulin. Antibody-enzyme conjugate was used at a 1/1,000 dilution (21). The enzyme-substrate reaction was evaluated visually after incubation for 15 hr at 22 C as negative, possibly positive, positive, or strong positive. Initially, one leaf per plant (three disks per leaf) was assayed, but as the plant population decreased, two or three leaves per plant were assayed.

Inoculation procedures. Inoculations were performed sequentially in the order that they appear in Table 1. After each inoculation, all plants were indexed by ELISA when symptoms appeared on several plants (about 24 days post-inoculation). Plants that indexed positive for BYMV were discarded. Only those plants that scored negative or possibly positive were retained for the next screening procedure.

Mechanical inoculation. Inoculum was prepared by grinding young, unfolded, BYMV-infected alsike clover leaves in

0.03 M potassium phosphate buffer, pH 8.0, containing 0.02 M mercaptoethanol (PBM). Pea plants (*Pisum sativum* L. 'Dwarf Grey Sugar') at the four-leaf stage were inoculated with the alsike clover extract by dusting all four leaves with Carborundum powder (320-grit) and rubbing the extract onto them with a cotton swab. Peas were grown in potting mixture in plastic flats (12.5 × 37 cm, 12 plants per flat) with a day length of 15.5 hr at 25 C and a night temperature of 15 C. Infected pea leaf tissue was ground at 1:5 (w/v) in PBM and used to inoculate a second set of peas to increase inoculum quantity. Leaf tissue from the second set of peas was ground at 1:5 (w/v) in PBM and used to inoculate young red clover leaves. Two or three leaves on each plant were dusted with Carborundum powder and rubbed with a cotton swab dipped in pea inoculum. Inoculum was prepared frequently during inoculations to prevent possible loss of infectivity by enzymatic degradation of viral particles. With all inoculations, inoculum infectivity was checked on peas (which showed 85–100% infected plants after one inoculation), and control inoculations with buffer alone were done on control red clover plants.

Exposure to field conditions. In June 1981, the remaining 262 symptomless, ELISA-negative plants were transplanted into an established red clover field for exposure to natural inoculation. BYMV was already present in the field plants as determined by ELISA, and a natural population of aphids was present. The field was not mowed during the exposure.

In October 1981, 115 surviving plants were returned from the field to the greenhouse. Plants were indexed by ELISA, mechanically inoculated twice, and reindexed.

Controlled aphid feeding. Green peach aphids (*Myzus persicae* Sulzer) were maintained on red clover in a growth chamber with a day length of 15.5 hr at 25 C and a night temperature of 15 C; aphids were transferred to new plants every 1–2 wk. Aphids were placed in a dish for 30 min before acquisition feeding for 2–3 min on BYMV-infected alsike clover leaflets. When at least 10 aphids were feeding on a leaflet, the leaflet was placed on a test plant. Most aphids voluntarily moved onto the red clover; others were coaxed with a brush. Aphid feeding was observed with a hand lens. Aphids were left on the test plants in a growth chamber for 24 hr, then killed by spraying with malathion, and plants were returned to the greenhouse. The efficiency of aphid transmission was checked on unselected Kenstar red clover plants.

Inoculation with concentrated inoculum. In a final attempt to infect remaining plants, concentrated BYMV inoculum was prepared according to the purification scheme devised by O. W. Barnett (*personal communication*). Infected pea leaves (29 g, 19 days postinoculation) were triturated in a blender at 1:1.5 (w/v) with phosphate buffer (0.5 M KPO_4, pH 7.0, 1.0 M urea, 0.5% thioglycolic acid, and 0.01 M NaDIECA). Chloroform was added at 1:1 (v/v with buffer) and blended 1 min to emulsify. The emulsion was centrifuged for 15 min at 8,000 × *g*, and the aqueous phase was poured off through glass wool, stirred for 1 hr at 4 C with 0.25 M NaCl and 4% polyethylene glycol (mol wt 8,000) (w/v), and centrifuged for 15 min at 7,700 × *g*. The pellet, resuspended in 20 ml of PBM, was used to inoculate the remaining red clover plants. The inoculum was about seven times more concentrated than the standard inoculum. Twenty-eight and 36 days after inoculation, red clover plants were indexed by ELISA. Negative plants were vegetatively propagated.

RESULTS

Field population. Of 588 symptomless plants collected from red clover fields, 249 developed symptoms of virus infection under greenhouse conditions and were discarded (Table 1). The remaining 439 plants without symptoms were indexed by ELISA and 32 indexed positive for BYMV. Over the period of 11 mo in the greenhouse and the field, 274 plants died of causes other than BYMV. Of 35 plants retrieved after 4 mo of exposure to natural field conditions, only one plant indexed positive by ELISA.

Two mechanical inoculations resulted in infection of six more plants. The remaining 25 plants were subjected to controlled aphid feeding, and 11 more plants became infected. Mechanical inoculation of the 13 remaining plants with concentrated inoculum produced no disease, although it did result in earlier and more severe symptoms in control pea plants. The eight remaining plants represented 3% of the population tested. The population tested equals the number of plants collected minus the 288 plants that died of causes other than BYMV throughout the study.

Plants collected from younger fields had lower percent BYMV infection, but more plants died from other causes than those collected from older fields ($P < 0.005$, chi-square test) (Table 2).

Greenhouse population. Of 502 Kenstar red clover plants that were seeded in the greenhouse, 21% became infected and were discarded after four mechanical inoculations (Table 1). Over the period of 10 mo in the greenhouse and the field, 285 plants died of causes other than BYMV. The remaining plants were transplanted to the field, and after 4 mo, 26 indexed positive for BYMV by ELISA and were discarded.

The remaining plants were mechanically inoculated twice. Six became

Table 1. Percentage and number of red clover plants infected with bean yellow mosaic virus (BYMV) as determined by symptom development and ELISA after each sequential inoculation during the evaluation of two populations for resistance[a]

Evaluation stage	Population[b]	
	Field plants of mixed varieties	Greenhouse-grown Kenstar
Developed virus symptoms after being brought into greenhouse	48% (281/588)	Not done
Mechanical inoculations (four times)	Not done	21% (106/502)
Field exposure (120 days)	3% (1/35)	32% (26/80)
Mechanical inoculations (two times)	17% (6/34)	11% (6/54)
Controlled aphid transmission	44% (11/25)	23% (11/48)
Mechanical inoculation (concentrated)	0% (0/13)	0% (0/13)
Uninfected plants remaining	3%[c] (8/300)[d]	4%[c] (7/178)[d]

[a] Percentages represent percentage of plants infected of those receiving that treatment. In parentheses: number of plants infected of number of plants receiving that treatment.
[b] Number of plants at the start were: field, 588; Kenstar, 502.
[c] Represents percentage of original population adjusted by subtracting the number of plants that died from causes other than BYMV.
[d] Number of plants dead from causes other than BYMV: field, 288; Kenstar, 324.

Table 2. Differences among red clover populations collected from 2-, 3-, and 4-yr-old fields in number of plants collected, percentage infected with bean yellow mosaic virus (BYMV), percentage dead from causes other than BYMV, and percentage of total population uninfected at end of screening

Field age (yr)	Plants collected (no.)	BYMV-infected (%)	Dead from causes other than BYMV (%)	Uninfected (%)
2	307	36^{L}[a]	62^{H}	1.6^{NS}
3	198	53^{NS}	47^{NS}	0.5^{NS}
4	83	62^{H}	35^{L}	2.6^{NS}

[a] L = lower than, NS = not significant from, and H = higher than mean expected at $P = 0.005$ with chi-square test.

infected. The 48 surviving plants were subjected to controlled aphid feeding and 11 became infected. The 13 remaining plants were mechanically inoculated with concentrated inoculum, but no plants became infected. The seven plants represented 4% of the total population minus 324 plants that died of causes other than BYMV.

Population comparison. Field and greenhouse red clover populations had comparable ratios of resistant plants (3 vs. 4%, respectively). In the field population, controlled aphid feeding resulted in the highest percent BYMV-infection (44%) followed by mechanical inoculation (17%) and exposure to natural conditions (3%) (Table 1). In the greenhouse population, exposure to natural conditions resulted in the highest percent infection (32%) followed by controlled aphid feeding (23%) and mechanical inoculation (21%) (Table 1).

Forty-nine percent of the field plants and 64% of the greenhouse plants died during the study from causes other than BYMV. Root rot killed some of these plants.

DISCUSSION

Red clover plants resistant to BYMV strain 204-1 were identified with about equal frequency in both field and greenhouse populations after sustained and varied attempts to infect them. The term resistance is used here to describe those plants in which no BYMV antigen was detected by ELISA after repeated inoculations.

In this study, mechanical inoculation produced the lowest percent infection in the populations being screened (14% after two and 21% after four inoculations). Controlled aphid feeding was most effective in transmitting BYMV for both test populations of red clover combined (av. 38%). Stuteville and Hanson (16) found, however, that four mechanical inoculations of BYMV strain 204-1 into eight varieties and breeding lines were equally or more effective than aphid transmission. Different methods probably account for the different results. For example, in aphid transmission, Stuteville and Hanson used a 2-day acquisition time, which decreases transmission efficiency of nonpersistent viruses (18). In mechanical inoculations, they used a more concentrated inoculum (infected tissue:buffer ratio of 1:3 or 1:2) that may have increased percent infection.

Exposure to field conditions for one growing season was an effective selection procedure for the greenhouse population (32% infection) but not the field population (3% infection), perhaps indicating aphid nonpreference for the field population in the field environment. There was no noticeable discrimination against field plants during controlled aphid feeding, however. Others (17,23) have also observed aphid preferences for or against certain clones or cultivars of red clover.

The final inoculation with concentrated virus did not result in infection of any of the resistant plants selected by previous inoculations. The concentrated inoculum might have been more effective had it preceded the inoculation by aphid transmission.

About half of the plants in both populations died of causes other than BYMV. This is not unusual with red clover grown for long periods in the greenhouse. Plants often became stunted before dying and may have been infected with another virus. Root rots probably killed most of the plants in the greenhouse, whereas rodent damage killed most of the plants during field exposure.

We concluded that 1) lack of symptom expression was not a reliable indicator that the plant was not infected with BYMV, 2) the leaf-disk ELISA protocol provided a quick and reliable way to index many red clover plants for BYMV infection, 3) mechanical inoculation by rubbing was a very inefficient method of inoculation, 4) exposing greenhouse plants to field conditions resulted in a substantial level of infection, and 5) Kenstar red clover was as good a source of BYMV resistance as the mixed population of symptomless, older field plants.

Future selection for BYMV resistance in red clover might best be accomplished by seeding or transplanting unselected germ plasm into a field surrounded by an old red clover planting that would provide inoculum. The plots should be mowed two or three times during the summer to provide young regrowth. At the end of the first and/or second year, symptomless plants should be indexed for BYMV, and those that index negative should be brought into the greenhouse. Plants should then be inoculated with concentrated inoculum to reduce their numbers, and remaining plants should be subjected to controlled aphid transmission and assayed with ELISA.

The advantages of this procedure are as follows: 1) a strong selection pressure for BYMV resistance is applied, 2) exposure to additional strains of BYMV might occur, 3) only one mechanical inoculation is required, 4) the evaluation could be completed within 1 yr, and 5) plants could be evaluated in the field for agronomic characteristics.

The main disadvantage of this selection procedure is that it exposes plants to possible infection by other viruses and pathogens during field exposure, which could eliminate BYMV-resistant plants.

LITERATURE CITED

1. Akita, S. 1981. Virus diseases of red clover in pasture of Japan. II. Viruses of red clover and symptoms. Bull. Nat. Grassl. Res. Inst. 20:93-102.
2. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.
3. Denis, S. J., and Elliot, E. S. 1967. Decline of red clover plants infected with red clover vein mosaic virus and *Fusarium* species. Phytopathology 57:808-809.
4. Diachun, S., and Henson, L. 1960. Clones of red clover resistant to four isolates of bean yellow mosaic virus. Phytopathology 50:323-324.
5. Fiedorow, Z., and Margowska, M. 1977. Studies on resistance of red clover to bean yellow mosaic virus. Zesz. Probl. Postepow. Nauk Roln. 195:117-126.
6. Goth, R. W., and Wilcoxson, R. D. 1962. Effect of bean yellow mosaic virus on survival and flower formation in red clover. Crop Sci. 2:426-429.
7. Henson, L., and Diachun, S. 1959. Selection for resistance to a strain of bean yellow mosaic virus in Kenland red clover. Phytopathology 49:541.
8. Jones, R. T., and Diachun, S. 1976. Identification and prevalence of viruses in red clover in central Kentucky. Plant Dis. Rep. 60:690-694.
9. Khan, H. A., and Banfield, W. M. 1971. Some factors involved in the failure of red clover in Massachusetts. Phytopathology 61:129.
10. Leath, K. T., and Barnett, O. W. 1981. Viruses infecting red clover in Pennsylvania. Plant Dis. 65:1016-1017.
11. Musil, M., Smrz, J., and Vacek, V. 1977. The occurrence of virus diseases in the assortment of red clover (*Trifolium pratense* L.). Ochr. Rost. 12(3/4):183-188.
12. Reddick, B. B., and Barnett, O. W. 1983. A comparison of three potyviruses by direct hybridization analysis. Phytopathology 73:1506-1510.
13. Romaine, C. P., Newhart, S. R., and Anzola, D. 1981. Enzyme-linked immunosorbent assay for plant viruses in intact leaf tissue disks. Phytopathology 71:308-312.
14. Smith, R. R., and Maxwell, D. P. 1971. Productivity and quality responses of red clover (*Trifolium pratense* L.) infected with bean yellow mosaic virus. Crop Sci. 11:272-274.
15. Smith, R. R., Maxwell, D. P., Hanson, E. W., and Smith, W. K. 1973. Registration of Arlington red clover (Reg. No. 16). Crop Sci. 13:771.
16. Stuteville, D. L., and Hanson, E. W. 1964. Resistance to viruses in red clover. Crop Sci. 4:631-635.
17. Stuteville, D. L., and Hanson, E. W. 1965. Viruses of red clover in Wisconsin. Crop Sci. 5:59-62.
18. Sylvester, E. S. 1969. Virus transmission by aphids—a viewpoint. Pages 159-173 in: Viruses, Vectors and Vegetation. K. Maramorsch, ed. Interscience, New York. 666 pp.
19. Tapio, E. 1970. Virus diseases of legumes in Finland and in the Scandinavian countries. Ann. Agric. Fenn. 9:1-97.
20. Taylor, N. L., and Smith, R. R. 1978. Breeding for pest resistance in red clover. South. Pasture Forage Improv. Conf. Proc. 35:125-137.
21. Voller, A., Bartlett, A., Bidwell, D. E., Clark, M. F., and Adams, A. N. 1976. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). J. Gen. Virol. 33:165-167.
22. Watson, R. D., and Guthrie, J. W. 1964. Virus-fungus interrelationships in a root rot complex in red clover. Plant Dis. Rep. 48:723-727.
23. Wilcoxson, R. D., and Peterson, A. G. 1960. Resistance of Dollard red clover to the pea aphid, *Macrosiphum pisi*. J. Econ. Entomol. 53:863-865.