Evaluation of Arrowleaf Clover for Tolerance to Bean Yellow Mosaic Virus

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ABSTRACT

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Seventy-eight half-sib families of arrowleaf clover (*Trifolium vesiculosum*) were evaluated in the greenhouse for tolerance to bean yellow mosaic virus (BYMV). Inoculated seedlings of five families exhibited more tolerance to BYMV than seedlings of reference cultivar Yuchi. These five and seven families with equal or less tolerance than Yuchi were selected for further comparisons. The 12 selected families and Yuchi were rated on symptom severity and dry matter production. There was a significant negative correlation between symptom severity and dry matter yield, $r = -0.92$ ($P = 0.01$). The effects of BYMV on growth and survival of plants inoculated at different ages were determined. The age of arrowleaf clover plants at inoculation significantly ($P = 0.001$) affected cumulative dry matter production compared with controls. The younger the plants were when inoculated, the greater the yield reductions. Overall, 92% of the inoculated A64 plants survived the resulting infections, while only 55% of the inoculated Yuchi plants survived. Yuchi plants inoculated at 17 wk of age were particularly sensitive to BYMV infection, with only 25% surviving. We conclude that tolerance to BYMV is present in the germ plasm of arrowleaf clover and that evaluation of inoculated seedlings based on symptom severity is a conservative, effective selection method.

Arrowleaf clover (*Trifolium vesiculosum* Savi) is an important legume species in the southeastern United States, where it is grown as a forage crop and as a winter annual cover crop in conservation tillage systems (17,21). Virus and root rot diseases can severely limit forage production, seed production, and stand persistence of this clover species (14,22,25).

Bean yellow mosaic potyvirus (BYMV) is transmitted by aphids in a nonpersistent manner and infects plants in 20 families, including 157 species in 30 genera of the Leguminosae (12). It has been reported from 29 *Trifolium* species, including several economically important ones: arrowleaf clover in Mississippi and Texas (21; M. R. McLaughlin, *unpublished*), red clover (*T. pratense* L.) in New York and Kentucky (1,19,20), and alsike clover (*T. hybridum* L.) in New York (1). Despite its prevalence, only a few attempts have been made to screen clovers for potential sources of resistance. Several cultivars of red clover have been released possessing resistance to BYMV (28,30). Diachun and Henson (9–11) determined that three genes control resistance to BYMV in red clover. Taylor et al (31) report on the inheritance of red clover BYMV resistance in further detail. Other investigators have reported low percentages of BYMV resistance in alsike clover, red clover, and subterranean clover (*T. subterraneum* L.) cultivars (2,15,18, respectively).

The objectives of this study were to identify arrowleaf clover germ plasm tolerant to BYMV and to compare growth and survival rates of selected families to those of a standard variety.

MATERIALS AND METHODS

General culture and inoculation methods. Arrowleaf clover seed was germinated on moist paper pads in petri dishes, then sown in plastic flats or in Cone-tainers (Ray Leach Cone-tainer Nursery, Canby, OR). All experiments were conducted in the greenhouse. Growing medium was prepared by mixing peat, perlite, vermiculite, and sand (2:1:1:1; v/v) and amended with 106 g of 0-0-60 fertilizer, 208 g of 9-45-15 fertilizer, and 1767 g each of dolomitic lime and gypsum per cubic meter of media. Seedlings were inoculated with type 'O' Rhizobium inoculant (Nitragin Co., Milwaukee, WI) 2 days after emergence. All plants in all experiments were fertilized as needed with a soluble trace micronutrient element mix (sulphur 15%, boron 1.45%, copper 3.2%, iron 7.5%, manganese 8.15%, molybdenum 0.046%, and zinc 4.5%) formulated at the rate of 1 tsp per 20 L of water.

Alsike clover infected with BYMV strain Ky 204-1 were obtained from O. W. Barnett, Clemson University, Clemson, SC, and maintained in the laboratory for use as the inoculum source. Inoculum was prepared by grinding BYMV-infected leaves in a 0.45% (w/v) solution of diethyldithiocarbamic acid in 0.03 M Na_2HPO_4 buffer with mortar and pestle, using a ratio of one leaf to 1 ml of buffer solution. Plants were mechanically inoculated by rubbing 600-mesh Carborundum-dusted leaves with a cotton swab dipped in inoculum. The youngest and second youngest fully expanded leaves were each inoculated on consecutive days.

Origin of germ plasm tested. Three thousand arrowleaf clover plants (1,000 each of the cultivars Yuchi, Meechee, and Amclo) in spaced-plant nurseries were observed each year during five cycles of recurrent selection in the field from 1979 to 1983 under natural disease pressure. Arrowleaf clover is a self-infertile, open-pollinated crop, and no attempt to control interpollination was made. Due to the heterozygous nature of arrowleaf clover, no "pure" families were developed for this study. Seed was harvested and bulked each summer from plants lacking viral and fungal disease symptoms and exhibiting forage potential. The collected seed was then planted in the fall for the next selection cycle. Lack of consistent natural disease pressure, however, necessitated that further screening be conducted in the greenhouse. In 1983, seed from 78 selected plants was collected and stored in a freezer.

Initial screening of 78 half-sib families. Twelve to seventeen 5-wk-old seedlings of each of the 78 half-sib families of arrowleaf clover collected in 1983 and the commercial variety Yuchi were inoculated with BYMV as described. All plants were rated for symptom severity 5 wk later. Although all families exhibited some chlorosis, mosaic, leaf distortion, and stunting, not all were affected to the same degree. Plants were rated individually on a scale of 0–5 (0 = no symptoms, 1 = mild symptoms, 4 = most severe symptoms, and 5 = dead plant). The symptoms varied independently of each other. Some plants suffered minimal stunting yet were very chlorotic and exhibited severe mosaic symptoms, while others were smaller but relatively green and showed only a mild mosaic pattern. Severely stunted plants were given a rating of 4 because in all cases mosaic symptoms were moderate to severe. Plants with the mildest symptom combinations were given the lowest ratings. Families rated as most tolerant or susceptible were chosen for later reevaluation. Plants exhibiting relatively high tolerance to BYMV as determined by relatively low symptom severity ratings were hand-crossed as part of a recurrent selection for resistance to BYMV. BYMV infection was confirmed by enzyme-linked immunosorbent assay (23) in plants selected for crossing as well as the alsike clover plants used as the inoculum source.

Reevaluation of some selected families. Five of the most tolerant half-sib families and seven of the most susceptible families were chosen for further evaluation and comparison with Yuchi. From 9 to 20 plants were inoculated. Inadequate seed production prevented testing all of the most tolerant and susceptible families of the original 78 tested. All test plants were inoculated at 6 wk of age; control plants were not inoculated. Plants were rated for symptom severity 6 wk after inoculation using the scale described. Dry matter production was measured by clipping all plants to just above the growing points at 8, 12, and 16 wk after inoculation.

Effect of inoculation on plants of different ages. Half-sib family A64, identified as relatively tolerant to BYMV in initial evaluations, and Yuchi were chosen for this study. Eight to ten different plants of both family A64 and Yuchi were inoculated at five different ages. Inoculations were made at monthly intervals, commencing at 5 wk after planting. Control plants were not inoculated. Plants were transplanted from Cone-tainers to plastic pots (13× 10 cm) containing identical soil media at 7 wk. All plants were clipped to just above growing points and dry matter production measured four times at monthly intervals, beginning at 15 wk.

Experimental designs and statistical analyses. Completely randomized design with unequal numbers of replications were used in all experiments. Data were subjected to analysis of variance and treatment means were compared using Fisher's protected least significant difference (LSD) test (29).

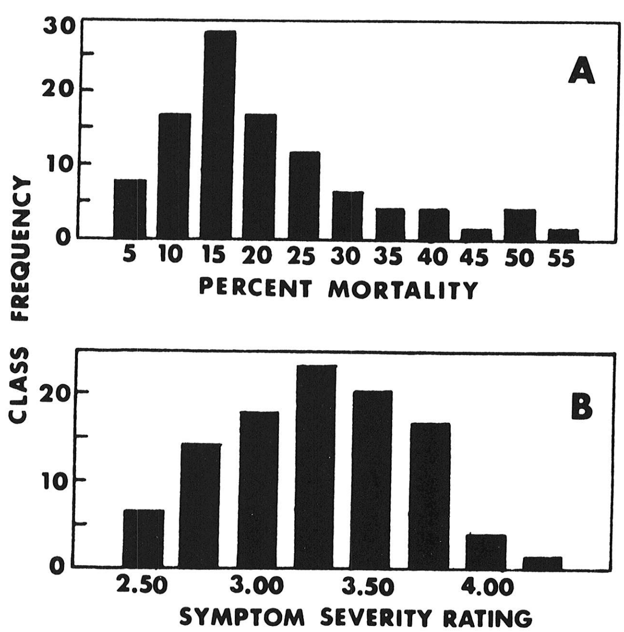

Fig. 1. Effect of bean yellow mosaic virus inoculation on class frequency within 78 arrowleaf clover half-sib families based on, **A**, percent mortality; and **B**, symptom severity rating. Symptom severity ratings in B, 1 = mildest symptoms, 4 = most severe symptoms, 5 = dead plant.

RESULTS

Initial screening. The 78 half-sib families evaluated for BYMV tolerance exhibited wide-ranging reactions to the virus, from severe systemic symptoms and death to a mild mosaic with minimal stunting (Fig. 1). Reactions within a family were varied due to each family's heterozygous nature. However, there were significant symptom rating differences between mean reactions in selected half-sib families (Table 1). Half-sib families A1, A64, A24, A21, and A71 were more tolerant to BYMV than Yuchi based on symptom ratings in initial screening experiments. Other lines did not differ significantly from Yuchi in symptom rating. Percent mortality ranged from 0 to 12% for these five families, 29% for Yuchi, and 6 to 47% for the remainder. Death of clover plants due to BYMV occurred within 5 wk of inoculation, at which time plants were 9 wk old. Dying plants first developed large necrotic areas on the leaves, followed by rapid wilting and death within 2 days.

Reevaluation of selected families. Significant differences in symptom severity ratings due to half-sib family were again observed (Table 2). Eight families were rated as significantly more tolerant to BYMV than Yuchi based on symptom severity. Of these

TABLE 1. Symptom severity ratings and percent mortality rates of 12 arrowleaf clover families and cultivar Yuchi inoculated with bean yellow mosaic virus from Initial Screening experiment

Family	Symptom severity rating	Mortality (%)	Classification[b]
A1	2.59 a[a]	6	T
A64	2.62 a	6	T
A24	2.71 a	0	T
A21	2.81 a	12	T
A71	3.12 ab	12	T
A72	3.53 bc	47	S
A30	3.76 bc	6	S
A3	3.81 cd	0	S
A17	3.81 cd	12	S
Yuchi	3.82 cd	29	S (control)
A4	3.88 cd	12	S
A36	4.00 cd	31	S
A29	4.41 d	47	S

[a] Means followed by the same letter are not significantly different according to Fisher's LSD, $P = 0.05$, $n = 12$ to 17. Symptom severity ratings: 1 = mildest symptoms, 4 = most severe symptoms, 5 = dead plant.
[b] Selected families of the 78 initially screened; these were chosen to be reevaluated. Families were classified as relatively tolerant (T) if rating was significantly different from Yuchi, otherwise given a susceptible rating (S).

TABLE 2. Symptom severity ratings and percent mortality of 12 arrowleaf clover families and cultivar Yuchi inoculated with bean yellow mosaic virus from experiment reevaluating selected families

Family	Symptom severity rating	Mortality (%)	n
A24	1.78 a[a]	0	9
A4	1.83 a	0	12
A64	1.93 a	0	14
A21	1.94 a	0	18
A71	2.00 a	7	14
A1	2.00 a	8	13
A3	2.08 a	8	12
A29	2.47 ab	0	15
A17	2.64 abc	7	14
A30	3.06 bc	0	16
A36	3.15 bc	5	20
Yuchi	3.26 cd	30	19
A72	3.93 d	50	14

[a] Symptom severity ratings: 1 = mildest symptoms, 4 = most severe symptoms, 5 = dead plant. Means of n plants. Means followed by the same letter are not significantly different according to Fisher's LSD, $P = 0.05$.

eight families, three had been classified as susceptible in the initial screening. The three families may be more variable than others tested, indicating a possible need for more replications in future studies. The two lines with highest (most severe) symptom ratings (Yuchi and Family A72) averaged 30 and 50% mortality, respectively, while the remaining 11 families averaged less than 8% mortality. Cumulative dry matter production per plant ranged from 0.22 to 0.86 g (Table 3). Families A4 and A1 produced significantly more dry matter than Yuchi. Only Family A72 produced significantly less dry matter than Yuchi. There was a significant negative correlation between symptom severity rating and dry matter yield, $r = -0.918$ ($P = 0.01$). Families with low ratings (mildest symptoms) produced the greatest amount of dry matter. Figure 2 compares dry weights of uninoculated control plants and inoculated plants. Dry weights of control plants ranged from 0.92 (Family A36) to 1.72 g (Yuchi). There were no deaths among the uninoculated control plants. Family A24 averaged a 29% yield reduction, while Yuchi averaged 69%. Family A72, the most severely affected by the virus, averaged an 84% yield reduction.

Effect of inoculation on plants of different ages. The age of arrowleaf clover plants at the time of inoculation with BYMV significantly affected cumulative dry matter production ($P = 0.001$), when compared to controls. The earlier the plants were inoculated, the greater the resulting reductions (Table 4). Late inoculation (21 wk) did not significantly reduce production but might have if the study had run longer.

TABLE 3. Cumulative dry matter production of 12 arrowleaf clover families and cultivar Yuchi inoculated with BYMV or uninoculated from reevaluation experiment

	Inoculated with BYMV		Uninoculated		
Family	Mean dry matter production (g/plant)	n	Mean dry matter production (g/plant)	n	Yield reduction due to BYMV (%)
A4	0.86 a[a]	12	1.59 ab	3	46
A1	0.85 a	13	1.48 ab	4	43
A24	0.75 ab	9	1.06 ab	3	29
A71	0.75 ab	14	1.67 ab	4	55
A3	0.74 ab	12	1.51 ab	3	51
A21	0.71 ab	18	1.50 ab	5	53
A64	0.70 ab	14	1.18 ab	3	41
A29	0.66 abc	15	1.36 ab	4	51
A30	0.64 abc	16	1.63 ab	4	61
A17	0.62 abc	14	0.85 a	4	37
Yuchi	0.53 bc	19	1.72 b	5	69
A36	0.44 cd	20	0.92 a	4	52
A72	0.22 d	14	1.37 ab	3	84

[a] Means in each column followed by same letter are not significantly different according to Fisher's LSD, $P = 0.05$.

Percent yield reduction was greater for Yuchi than for family A64 at each inoculation date (Table 4). Although percent yield reduction decreased with progressively later inoculation dates, percent survival rates were not similarly affected. While family A64 averaged a 92% survival rate for all inoculation dates, only 55% of the Yuchi plants survived. Yuchi plants inoculated at 17 wk were particularly sensitive to virus infection; only 25% survived until the end of the study.

Although 45% of the Yuchi plants inoculated with BYMV died compared with only 8% of Family A64 plants, there were no significant differences in total dry matter production between the two entries (Table 4). Although not virus tolerant, Yuchi is a vigorous, high-yielding cultivar, and thus the surviving plants produced more than the experimental family.

All plants that died as a result of BYMV infection did so between 14 and 24 wk of age, with majority dying between 20 and 24 wk of age. Large necrotic areas appeared on the leaves, and the plants wilted. The still green foliage then dried up and the plant died, all within a few days. Approximately one-third of the Yuchi population and one-tenth of the A64 population died in this manner.

In general, less time elapsed between inoculation and plant death for later inoculation dates compared with earlier inoculation. There was a significant negative correlation between the age of a plant at inoculation with BYMV and the time of that plant's death postinoculation ($r = -0.763$, $P = 0.01$). Yuchi plants inoculated on the same date usually died at the same time or within a short time of each other. The virus stunted plant growth soon after inoculation, and sharp decreases in dry matter production were observed for inoculations at 17 and 21 wk (dates 4 and 5, Figure 3). These two treatments were the only ones inoculated after clippings commenced (clippings 1 and 2, respectively). Yield reductions due to the virus were evident in comparison with control plants. Yields of plants inoculated at 5 wk (date 1) and 9 wk (date 2) were significantly reduced for all clippings, as were yields of plants inoculated at 13 wk (date 3), excluding the first clipping.

DISCUSSION

We conclude from results of this study that tolerance to BYMV is present in arrowleaf clover germ plasm and that a seedling assay

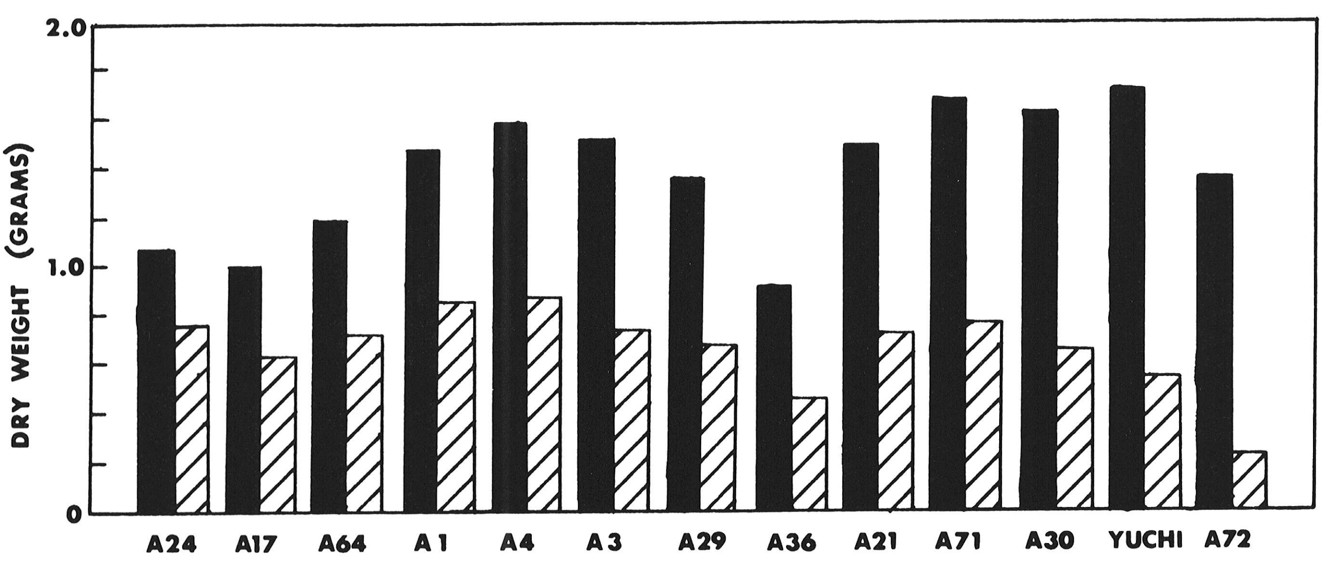

Fig. 2. Cumulative dry matter production per plant from three harvests of 13 arrowleaf clover entries inoculated with bean yellow mosaic virus and uninoculated controls. Filled bars represent control plants, hatched bars represent inoculated plants.

Table 4. Survival, yield reduction, and cumulative dry matter production for arrowleaf clover Family A64 and cultivar Yuchi inoculated with BYMV at five different ages

Inoculation date	Plant age at inoculation (wk)	Survival[a] (%)		Yield reduction (%)		Cumulative dry matter production (g/plant)
		Yuchi	Family A64	Yuchi	Family A64	
1	5	62	87	84	63	3.48 a[b]
2	9	50	100	74	73	3.64 a
3	13	62	87	61	36	6.86 b
4	17	25	100	36	27	9.42 c
5	21	80	87	15	5	12.36 d
$\bar{x}$		55	92	54	41	
Controls	···	100	100	···	···	13.82 d

[a] $n = 8$ to 10.
[b] Means followed by the same letter are not significantly different according to Fisher's LSD, $P = 0.001$, $n = 16$ to 18. Means are averaged over the two arrowleaf clover lines, A64 and Yuchi. Differences in dry matter production due to Line, or Line × Inoculation Date were not significant and are not reported.

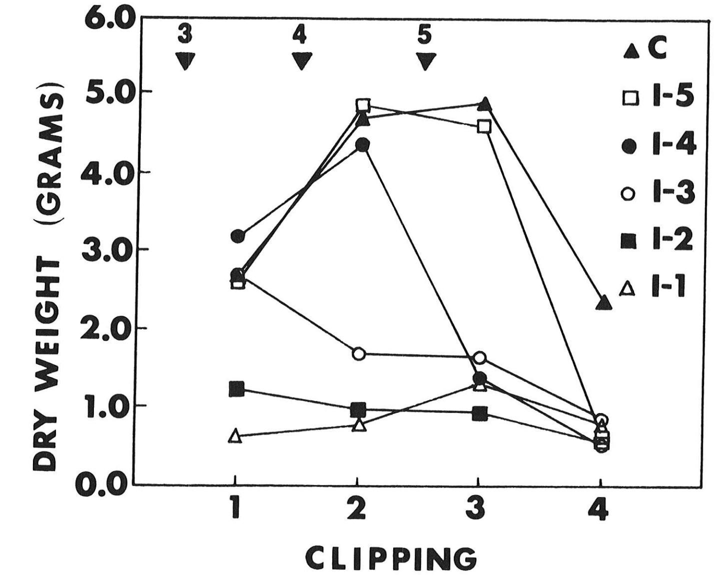

Fig. 3. Dry matter yield of arrowleaf clover inoculated with bean yellow mosaic virus at five different ages and clipped four times. Means are averaged over half-sib family A64 and cultivar Yuchi. Arrows at top of graph indicate date of inoculations 3, 4, and 5. Inoculations 1 and 2 occurred 10 and 6 wk before clipping 1, respectively, and are not indicated on graph.

based on symptom severity is a useful selection method. We identified families with significantly reduced disease severity, greater dry matter production, and increased survival compared with the commercial cultivar, Yuchi.

Clipping imposed additional stress on arrowleaf clover already infected with BYMV. The survival of Yuchi plants inoculated on date 4 (at 17 wk) was only 25%—the lowest of all inoculation treatments. Date 4 plants were inoculated midway between the first and second clippings, during a time when growth rate was the highest. Other inoculation date treatments fell before or after their respective rapid growth phases. Based on the apparent slowdown in dry matter production between the second and third clippings for uninoculated controls, we conclude that vegetative growth peaked between clippings 1 and 2, as plants entered a reproductive growth phase. This physiological condition may result in increased sensitivity to virus infection and may account for the high mortality rate in plants inoculated at that age. Gibson et al (14) conducted a similar study with arrowleaf clover but without periodic clippings. They found Yuchi plants inoculated with BYMV at 18 wk had a 100% survival rate and, in fact, exhibited slightly higher dry matter production at harvest than controls (14). Regular defoliation during the growing season is representative of rotational grazing or hay production. An early BYMV infection in the field may cause greatest yield reductions, but later infections may be just as devastating if they occur during a sensitive growth stage and stands are severely diminished due to plant mortality.

Although Family A64 was identified as more tolerant to BYMV than Yuchi based on survival rate and yield reduction, both were stunted by virus infection, and yield reductions were greatest the earlier the plants were inoculated. In another study, three inoculation dates spread over a 7-wk period also demonstrated that early infections reduced yields of Yuchi more than late infections (14). In our study, inoculation treatments covered a 4-mo period imposed with regular clippings.

Metabolic effects of viruses on infected plants can include changes associated with senescence (24), protein synthesis (16), translocation of sugars (32), and carbon fixation (6). The plants killed by BYMV died between 20 and 24 wk of age, regardless of their age when inoculated. This indicates a sensitive growth stage must be reached before the virus infection kills the plant. Perhaps BYMV infection triggers a senescence mechanism in arrowleaf clover resulting in necrotic lesions and plant death in severe reactions. We have also noted a significant decrease in leaf chlorophyll content in BYMV-infected arrowleaf clover plants (*unpublished*). Other host genes may independently control severity of symptoms such as stunting, mosaic, and leaf distortion in arrowleaf clover. In contrast, resistance to BYMV in pea is controlled by a single gene (27). Inheritance of a lethal necrosis response in red clover is controlled by a single pair of dominant genes (8,9). The lethal reaction is also a dominant trait in subterranean clover (18). We identified Family A64 as more tolerant to BYMV than Yuchi based on milder symptoms and a lower death rate. The gene or genes controlling a similar systemic lethal necrosis occurred at a lower frequency in Family A64.

Backcrossing, polycross progeny testing, and phenotypic recurrent selection are the most widely used breeding methods for development of virus resistance in forage legumes (13). These approaches were used to develop two red clover cultivars, Arlington and Kenstar, which possess some resistance to BYMV-204-1 (28,30). During field trials to evaluate Kenstar for virus resistance, a new strain of BYMV was discovered (RC strain) which overcame the resistance to strain 204-1 (31). The prevalence and relative importance of a forage legume virus varies with location (13), thus the occurrence of strain RC was attributed to the fact that soybean acreage had recently increased in the area (31). This new strain was similar to other previously described strains from soybean (31). Thus it would seem advantageous to screen for resistance to several strains of BYMV simultaneously, depending on location, crop of interest, and prevalence of other host crops.

We are aware, however, of some uncertainty concerning the current classification of viruses within the BYMV-subgroup of potyviruses. This subgroup includes BYMV, pea mosaic virus (PMV), sweet pea mosaic virus (SPMV), and clover yellow vein virus (CYVV) (4). Depending on the biological property examined, i.e., host range, serology, or molecular hybridization, different patterns of strain interrelationships emerged among isolates of the four viruses (3,4,7,20). In fact, after prolonged passage in a single host species and transfer to another host species, BYMV, CYVV, and PMV appeared to be in a state of flux regarding host range and symptom expression, but not serological properties (3). It is apparent why the nomenclatural confusion has arisen. Reddick and Barnett (26) suggested an evolutionary continuum relates BYMV, CYVV, and PMV to each other and concluded the viruses are different, but related, and each possesses many strains. In contrast, Jones and Diachun (20) placed all isolates into three distinct serotype groups, but referred to all as BYMV strains.

The strain used in this study, BYMV-204-1, has been reclassified by some as PMV-204-1 (4,26). Sequence homology between 204-1 and PMV-Pratt (previously BYMV-Pratt) has been found (26), but 204-1 showed no close relationships to any of 17 BYMV-subgroup isolates, including PMV-I (4). We have referred to isolate 204-1 throughout this study as BYMV-204-1 to avoid confusion when referring to previous studies. An isolate collected from arrowleaf clover in Texas (BYMV-T-1) was related to BYMV-204-1 based on host range, presence of inclusion bodies, and serological tests (5). We have also found a BYMV isolate on arrowleaf clover in Texas that exhibits a serological relationship to BYMV-204-1 (M. R. McLaughlin, *unpublished*). Ideally, arrowleaf clover should be screened for resistance employing all viruses in the BYMV-subgroup, especially those occurring in this area, because resistance to one strain of a virus may not ensure resistance to other strains, and our future work will include some of those viruses.

Identification of BYMV-tolerant families and subsequent breeding to increase virus tolerance is important to ensure the continued success of arrowleaf clover as a forage crop. Current work is under way to develop arrowleaf clover lines with a high degree of tolerance or resistance to BYMV and a high forage production potential.

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