Inheritance of Resistance to Virus-Induced Lethal Wilt in Arrowleaf Clover

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Joint contribution from Texas Agricultural Experiment Station 25861, Texas A&M University, Agricultural Research and Extension Center, Overton 75684, and USDA-ARS, Mississippi State 39762. Contribution to regional project S-228, Forage Legume Viruses.
Accepted for publication 30 April 1991 (submitted for electronic processing).

ABSTRACT

Pemberton, I. J., McLaughlin, M. R., and Smith, G. R. 1991. Inheritance of resistance to virus-induced lethal wilt in arrowleaf clover. Phytopathology 81:1001-1005.

Inheritance of the lethal wilt (LW) reaction in arrowleaf clover inoculated with a pea mosaic-type virus (PMV) isolate, 204-1, of the bean yellow mosaic virus (BYMV) subgroup was investigated. The expression of LW was found to be simply inherited and conditioned by a single dominant gene (*L*). Resistance to LW was conferred by the homozygous recessive form of the gene. Interaction of the *L* gene with five other BYMV subgroup isolates was also studied. Germ plasm tested included LW-resistant (family 3-97; *ll*) and -susceptible (Yuchi; ~20% *LL* or *Ll*) populations of arrowleaf clover. The LW reaction in arrowleaf clover is virus strain-specific, and only one other isolate, TX-22, induced LW in susceptible arrowleaf clover. Lethal wilt did not occur when susceptible arrowleaf clover was inoculated with BYMV-Scott, BYMV-RC, TX-3, and clover yellow vein virus (CYVV-Pratt) isolates. Plants in the family 3-97 did not express LW when inoculated with any of the six isolates. Plants resistant to the LW syndrome were susceptible to infection with all the virus isolates tested and developed symptoms consisting of varying degrees of nonlethal wilting and systemic mosaic.

The bean yellow mosaic virus subgroup (BYMV subgroup) of potyviruses consists of four viruses: bean yellow mosaic virus (BYMV), clover yellow vein virus (CYVV), pea mosaic virus (PMV), and sweet pea mosaic virus (SPMV). This subgrouping was based on serology, including enzyme-linked immunosorbent assay (ELISA) tests, molecular cDNA hybridization experiments, and coat protein amino acid composition (3,13,19). Together, the members of this subgroup can infect nearly 200 legume species (9). Virus diseases of arrowleaf clover (*Trifolium vesiculosum* Savi) can limit forage production and reduce plant survival, either alone or in combination with root diseases or other viruses (10,18).

A lethal necrotic reaction to BYMV is controlled by the dominant *N* factor in red clover (*T. pratense* L.), and an unnamed gene in subterranean clover (*T. subterraneum* L.) (6,12). We have also reported a lethal wilt (LW) reaction in arrowleaf clover inoculated with a PMV-type isolate, 204-1 (synonymous with BYMV-Ky-204-1 in previous literature) (17).

Our objectives were to determine the inheritance of LW in arrowleaf clover inoculated with 204-1, and to determine the reaction of LW-resistant and -susceptible germ plasm to inoculation with other BYMV-subgroup isolates.

MATERIALS AND METHODS

Origin of LW germ plasm. Twenty greenhouse-grown arrowleaf clover (*T. vesiculosum* cv. Yuchi) plants were removed from pots, and two shoots with partial root systems were excised from each plant. Cuttings were rooted in fresh media and allowed to grow until the cuts callused and new shoot growth appeared. The new plants were then mechanically inoculated with the 204-1 isolate of PMV. Growth media and inoculation procedures have been previously described (17). Within 10 days after inoculation, all plants exhibited systemic mosaic symptoms. In addition, the youngest growth on two pairs of rooted cuttings began wilting. The wilt rapidly progressed basipetally to all growth above the inoculated leaf, and 8 days after the first wilt symptom appearance, the cuttings shriveled and died. The corresponding healthy mother plants, designated LW5 and LW7, were grown to maturity and handcrossed to produce seed. Two healthy mother plants (NL6 and NL13), from which cuttings showed only systemic mosaic symptoms, were included in the set of crosses. All seeds were harvested in September 1988 and stored at −9 C.

Testing the F1 generation. Ten F1 families resulted from crosses among the four arrowleaf clover mother plants. All possible combinations of crosses were included, except LW5 × NL6 and the reciprocal, due to lack of flowers. Half of the seed (n = 2–23) from each cross was germinated and planted in Cone-tainers (Ray Leach Cone-tainers, Stuewe and Sons, Inc., Corvallis, OR) in February 1989. All plants were mechanically inoculated with the 204-1 isolate on days 37 and 38. Dates of first symptom appearance and LW expression were recorded. Chi-square values were calculated for groups of crosses, classified according to predicted parental genotypes.

Virus isolates and antisera. Virus isolates BYMV-Scott, PMV (BYMV-204-1) and CYVV-Pratt were from cultures maintained at Mississippi State and obtained originally from O. W. Barnett, Clemson University, Clemson, SC (3). Antisera to these isolates were also provided by O. W. Barnett. Virus isolate BYMV-RC and its antiserum were provided by S. A. Ghabrial, University of Kentucky, Lexington (24). These isolates were inoculated and maintained on *Pisum sativum* L. 'Dwarf Gray Sugar' (DGS pea) in the greenhouse. Twenty-three field grown arrowleaf clover plants exhibiting BYMV-like symptoms at Overton, TX, were indexed by direct double antibody sandwich ELISA in May 1989. Samples were shipped overnight to Mississippi State, MS, where they were processed as described (15) and tested against antisera to BYMV, PMV, and CYVV type virus isolates (Scott, 204-1, and Pratt, respectively). Nineteen of the 23 samples reacted positively with antiserum to 204-1. None reacted positively with antiserum to CYVV-Pratt or BYMV-Scott, although some samples showed a slight cross-reaction to CYVV-Pratt, which is normal for PMV-type isolates (1). Sap saved from the ELISA preparations was used to inoculate 10-day-old DGS pea seedlings. All seedlings developed systemic yellow mosaic symptoms typical of BYMV subgroup isolates. The Texas (TX) isolates were also maintained in DGS pea in the greenhouse.

Host range test. In fall of 1989, a limited host range test was conducted at Mississippi State by using the BYMV subgroup isolates Scott, RC, 204-1, CYVV-Pratt, and seven of the Texas isolates selected on the basis of relatively high, intermediate, and low absorbance (A_{405nm}) values in ELISA with 204-1 antiserum. Systemically infected leaves of DGS pea were ground with mortar

and pestle in 0.03 M $NaPO_4$ buffer (pH 7.35) containing 0.02 M 2-mercaptoethanol and rub-inoculated in a suspension with 600-mesh Carborundum onto primary leaves of *Glycine max* L. 'Davis', *Phaseolus vulgaris* L. 'Top Crop', and DGS pea. Host range plants were grown from seed sown 14 days earlier in the greenhouse. Eight to 10 plants of each host were inoculated. Symptoms were recorded on 1 November 1989, and infections in all plants were confirmed by ELISA. Two isolates, designated TX-3 and TX-22, were selected for inclusion in further tests as representative of the range of diversity among Texas isolates observed in the host range tests.

Reaction of arrowleaf clover to BYMV subgroup isolates. Greenhouse-grown, 100-day-old seedlings of Yuchi and family 3-97 were inoculated (n = 36–97), as described, in Overton with one of six BYMV subgroup isolates, Scott, RC, TX-22, TX-3, 204-1, and CYVV-Pratt. Half-sib family 3-97 was derived from the third cycle of phenotypic recurrent selection for tolerance to 204-1 (23). This line, which does not express the LW trait, is more tolerant to 204-1 than Yuchi (*unpublished*). Previous work has shown that approximately 20% of Yuchi arrowleaf clover carries the *L* allele (16). Early systemic wilt symptoms observed on each plant and numbers of dead LW plants were recorded on day 22 (CYVV-Pratt) or day 36 (other isolates) post-inoculation. Early symptoms were considered to be those that appeared in the two to four leaves emerging after inoculation, but before typical systemic mosaic, chlorosis, rugosity, and stunting symptoms developed in the newest growth. Inoculated plants expressed some degree of wilting and necrosis during this early symptom phase, which ceased when full-blown symptoms appeared. The only exceptions to this were plants expressing the LW trait which, of course, died. For our purposes, wilting may be described as the permanent loss of turgidity in leaflets, leaves, and/or petioles due to virus infection. The wilting was irreversible and always followed by death of affected plant parts. Early systemic wilt symptoms were classified as: 1) Lethal wilt, plants exhibited total, rapid systemic wilting and tissue collapse beginning with youngest growth and resulting in plant death; 2) nonlethal complete wilt, plants exhibited rapid systemic wilting and tissue collapse that ended on appearance of new growth with systemic mosaic symptoms; 3) nonlethal partial wilt, necrotic local lesions and/or one to very few wilted leaves or leaflets only, not all pre-mosaic growth is affected; 4) no wilt, no local lesions or pre-mosaic symptoms expressed, plants appeared unaffected until systemic mosaic symptoms developed on emerging growth.

Plants were harvested twice, on days 43 and 93 post-inoculation, for dry matter yield. Healthy control plants were included for comparison. Data were subjected to General Linear Models analysis by using PC-SAS (20).

RESULTS

Lethal wilt F1 generation. All clover plants had developed systemic mosaic symptoms by the eleventh day after inoculation with the 204-1 isolate. The first symptoms of LW appeared 8–13 days after inoculation. All LW plants developed systemic mosaic symptoms as well, although these were difficult to observe on some due to wilting. Typically, the second new leaf emerging above the inoculated leaves developed wilt symptoms first. Leaflets became flaccid and shriveled, and as wilt symptoms progressed over the course of several days, all new growth above the inoculated leaves wilted, collapsed, and the whole plant died. Because chi-square values for individual families showed acceptable fits to hypothesized ratios, crosses were combined into groups according to their predicted parental genotypes, and chi-square values were calculated for each group (Table 1). Chi-square values for all F1 crosses showed an acceptable fit to hypothesized ratios. Progeny from LW × LW crosses segregated to fit a 3:1 ratio of LW to nonlethal (NL) plants ($P > 0.995$). Progeny from LW × NL crosses segregated to fit a 1:1 ratio of LW to NL plants ($P > 0.995$). No progeny from NL × NL crosses expressed the LW.

TABLE 1. Expected and actual numbers of arrowleaf clover plants exhibiting lethal wilt (LW) after inoculation with bean yellow mosaic virus subgroup isolate 204-1

Cross[a]	Predicted parental genotypes	n	Expected ratio LW:NL	Actual ratio LW:NL	Chi-square	P
LW × LW	*Ll* × *Ll*	40	3:1	3.4:1	0.133	> 0.995
LW × NL	*Ll* × *ll*	51	1:1	0.8:1	0.490	> 0.995
NL × NL	*ll* × *ll*	10	0:1	0:1	...	...

[a] LW and NL designations represent a parent plant identified as a carrier of the lethal wild gene (*Ll*) and a noncarrier (*ll*), respectively. NL = nonlethal.

Host range test. Host plant reactions to the four BYMV subgroup isolates and two selected Texas isolates, TX-3 and TX-22, are presented in Table 2. Five other Texas isolates (data not shown) induced symptoms similar to those induced by isolate TX-3. Both 204-1 and TX-22 induced necrotic local lesions in Davis soybean. No symptoms were observed in Davis inoculated with the other isolates. Infection by CYVV-Pratt caused particularly severe symptoms in Top Crop bean and DGS pea. Only local symptoms were observed on Top Crop inoculated with TX-3 and Scott. Both TX-22 and 204-1 caused systemic mosaic and chlorotic lesions in Top Crop. All isolates induced some degree of systemic mosaic in DGS pea.

Initial ELISA results showed the Texas isolates to react positively with antiserum to 204-1, but not with antisera to Scott and CYVV-Pratt (data not shown). Sap from plants in the host range study inoculated with TX-3 or TX-22 did not react with antisera to isolates CYVV-Pratt and Scott, but reacted positively with antisera to isolates RC and 204-1. Although the results of the host range study indicated there were differences between the Texas isolates, TX-3 and TX-22 could not be distinguished serologically.

Reaction of arrowleaf clover to BYMV subgroup isolates. Systemic symptoms were first observed 9–16 days after inoculation. All isolates caused similar initial symptoms in arrowleaf clover consisting of vein-clearing in the youngest, otherwise normal, leaves.

By day 13, plants inoculated with CYVV-Pratt began curling and wilting around leaflet edges of the youngest growth. This was the start of the early systemic wilt phase of symptom development. Over the next 2 days, wilt symptoms became more severe as larger areas of the leaf collapsed and died. Veinal and interveinal necrosis was also observed. This initial symptom appearance suggested LW, but symptoms never evolved to the rapidly wilting-dying stage. As systemic wilting-necrosis progressed over several days, it was apparent that all plants were not equally affected. Whereas only one or a few leaves had wilted and died on some plants, all new growth had wilted on others. Other CYVV-Pratt symptoms included leaf reddening, blackening, and chlorosis. Many plants had developed one leaf with

TABLE 2. Host reactions caused by six bean yellow mosaic virus subgroup isolates[a]

Isolate	*Glycine max* Davis	*Phaseolus vulgaris* Top Crop	*Pisum sativum* Dwarf Gray Sugar
TX-3	...	CLL / ...	... / wh-yel MO
TX-22	NLL	CLL / MO, CL	... / yel MO
Scott	...	LC / ...	... / yel MO
RC	...	LC, E / m MO	... / m MO
204-1	NLL	CLL, LC / MO, CL	... / m MO
CYVV-Pratt	...	LCN / MO, E, CL S, sev leaf curl	... / sev MO

[a] Symptom designations: (local symptoms/systemic symptoms) ··· = no symptoms, NLL = necrotic local lesions, CLL = chlorotic local lesions, LC = local chlorosis, LCN = local chlorosis and necrosis, MO = mosaic, E = epinasty, CL = chlorotic lesions, VN = veinal necrosis, St = stunting, yel = yellow, wh = white, m = mild, sev = severe. Eight to 10 plants of each host were inoculated per isolate.

TABLE 3. Death rates due to lethal wilt in two arrowleaf clover lines inoculated with six bean yellow mosaic virus subgroup isolates and in noninoculated controls

Isolate	Yuchi			Family 3-97		
	Total plants	Lethal wilt (%)	Other causes (%)	Total plants	Lethal wilt (%)	Other causes (%)
204-1	49	14	0	46	0	0
TX-22	36	11	0	44	0	0
TX-3	94	0	3	46	0	0
RC	97	0	16	91	0	0
Scott	94	0	4	90	0	0
CYVV-Pratt	93	0	11	90	0	14
Noninoculated controls	92	0	0	46	0	0

an epinastic petiole, and new growth emerging after this point showed typical systemic mosaic symptoms. Systemic wilting ceased at this time, and all plants showed very severe mosaic, chlorosis, rugosity, and stunting.

In contrast to the severe symptoms caused by CYVV-Pratt, plants inoculated with PMV or BYMV isolates generally exhibited mild to moderate mosaic, chlorosis, rugosity, and stunting symptoms. Despite the milder symptoms, 204-1 and TX-22 induced LW in 14 and 11% of inoculated Yuchi plants, respectively, and these plants died within 2 wk of first wilt symptom appearance (Table 3). No plants from family 3-97 expressed LW in response to any of the six isolates tested. Infections by isolates incapable of causing LW eventually killed 3–16% of inoculated Yuchi plants, but deaths were not characterized by the rapid, sudden wilting associated with LW, and occurred as late as 75 or more days after inoculation. Except for CYVV-Pratt-infected plants (14%

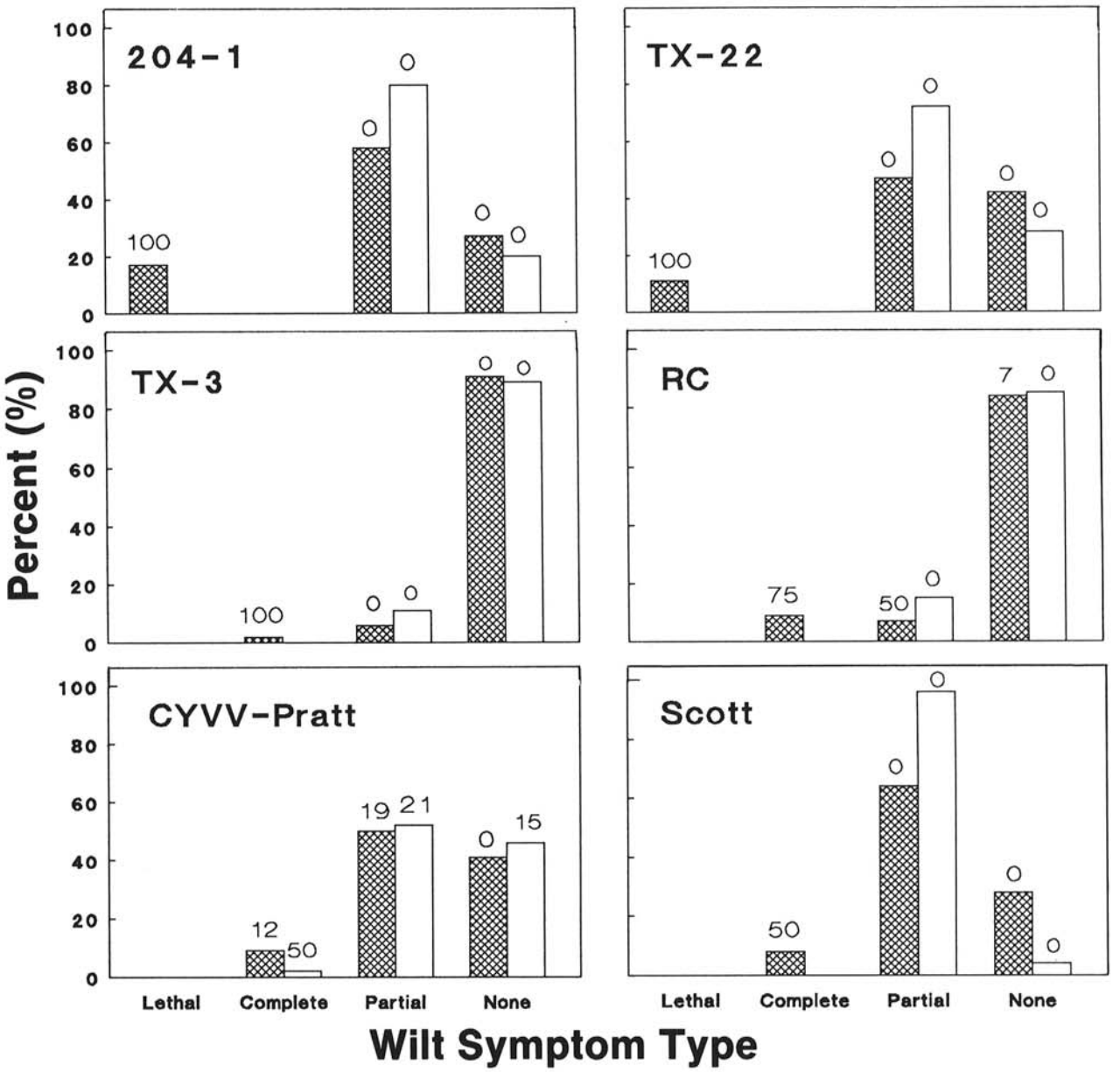

Fig. 1. Early wilt symptom distribution and death rates for arrowleaf clover inoculated with six bean yellow mosaic virus subgroup isolates. Wilt symptom type designations: LETHAL, lethal wilt = plants exhibited total, rapid systemic wilting and tissue collapse beginning with youngest growth and resulting in plant death; COMPLETE, nonlethal complete wilt = plants exhibited rapid systemic wilting and tissue collapse that ceased upon appearance of systemic mosaic symptoms; PARTIAL, nonlethal partial wilt = necrotic local lesions and/or one to very few wilted leaflets/leaves; NONE, no wilt = no local symptoms before systemic mosaic symptom development. Hatched bars represent cultivar Yuchi; open bars represent virus-tolerant family 3-97. Numbers above bars indicate percentage of dead plants of all plants in that symptom category. All plants eventually exhibited systemic disease symptoms (i.e., mosaic, chlorosis, stunting, etc.), including those in NONE category.

mortality), all inoculated plants in family 3-97 survived until the end of the study.

Early wilt symptom distributions are presented in Figure 1. In general, wilt symptoms were milder for family 3-97 than Yuchi; 404 of 407 total 3-97 plants tested (all isolates) were classified with nonlethal partial wilt symptoms or no symptoms, only three plants with nonlethal complete wilt symptoms, and none that expressed LW. In contrast, 39 of 555 Yuchi plants exhibited LW or nonlethal complete wilt symptoms in response to inoculation with BYMV-subgroup isolates. Distribution patterns were similar for two pairs of isolates: RC and TX-3 (most inoculated plants exhibited no wilt symptoms) and TX-22 and 204-1 (only these two isolates induced LW, but not nonlethal complete wilt). Higher death rates appeared to be associated with more severe wilt symptoms.

Figure 2 represents total dry matter harvested from plants in this study. Dry matter yields were significantly greater for family 3-97 than Yuchi ($P = 0.05$) when inoculated with any isolate except CYVV-Pratt. There were no significant differences between yields of healthy control plants.

DISCUSSION

The sudden LW response of arrowleaf clover inoculated with the 204-1 isolate is a simply inherited trait controlled by a single dominant gene, *L*. The following genetic symbols are proposed: *L* for LW susceptibility and *l* for LW resistance. The *L* allele has been eliminated by selection from arrowleaf clover family 3-97, and the *ll* gene pair confers complete LW resistance. Approximately 20% of the plants in a population of Yuchi arrowleaf clover carry the *L* allele (16). Necrosis in red clover infected with this isolate is controlled by the single factor, *N*, which is dominant to the mottling reaction, *n*. For necrosis to become systemic (i.e., lethal), the presence of an additional factor, *h*, is required (6–8). The lethal reaction in subterranean clover (*T. subterraneum*) to BYMV is also heritable and dominant in most cases (12). However, the occurrence of symptomless plants among the inoculated red and subterranean clovers suggests the influence of other yet unexamined factors. In contrast, dominance of the *L* allele for LW in arrowleaf clover appears to be complete. Systemic mosaic symptoms were observed on susceptible (*LL* or *Ll*) as well as resistant (*ll*) arrowleaf clover plants inoculated with PMV, indicating these were not mutually exclusive responses as in red clover. Additional modifying factors apparently do not play a role in the LW response of arrowleaf clover. The *L* allele, present in Yuchi but not family 3-97, conferred LW susceptibility to only two of six BYMV subgroup isolates tested. Thus, the LW response in arrowleaf clover is virus isolate-specific and may be useful in distinguishing between isolates in this subgroup.

Plant deaths not attributed to LW were not characterized by the rapid, sudden wilting associated with LW, and occurred as late as 75 or more days after inoculation. All occurred sometime after the first clipping and may have been related to harvesting stress. Typically, severely stunted plants and those expressing severe wilt symptoms became progressively weaker. Regrowth, if any, was sparse and some plants eventually died. Higher death rates were associated with more severe wilt symptoms for virus-infected Yuchi plants. All control plants survived until the end of the study.

Family 3-97 was developed from several cycles of recurrent selection for tolerance to 204-1 (23). It is significant that in the absence of LW, few or no harvest-related plant deaths, milder symptoms, and greater yields were observed for this family when inoculated with other BYMV-subgroup isolates as well. Family 3-97 suffered no plant deaths when infected with five of the six isolates. Despite the improvements made in symptomatic tolerance to BYMV subgroup isolates, family 3-97 was as susceptible to CYVV-Pratt as Yuchi, and both exhibited severe symptoms and non-LW plant deaths. Barnett and Diachun (2) have also reported the occurrence of mosaic and systemic necrosis in CYVV- and BYMV-infected arrowleaf clover.

Although the six BYMV subgroup virus isolates used in this

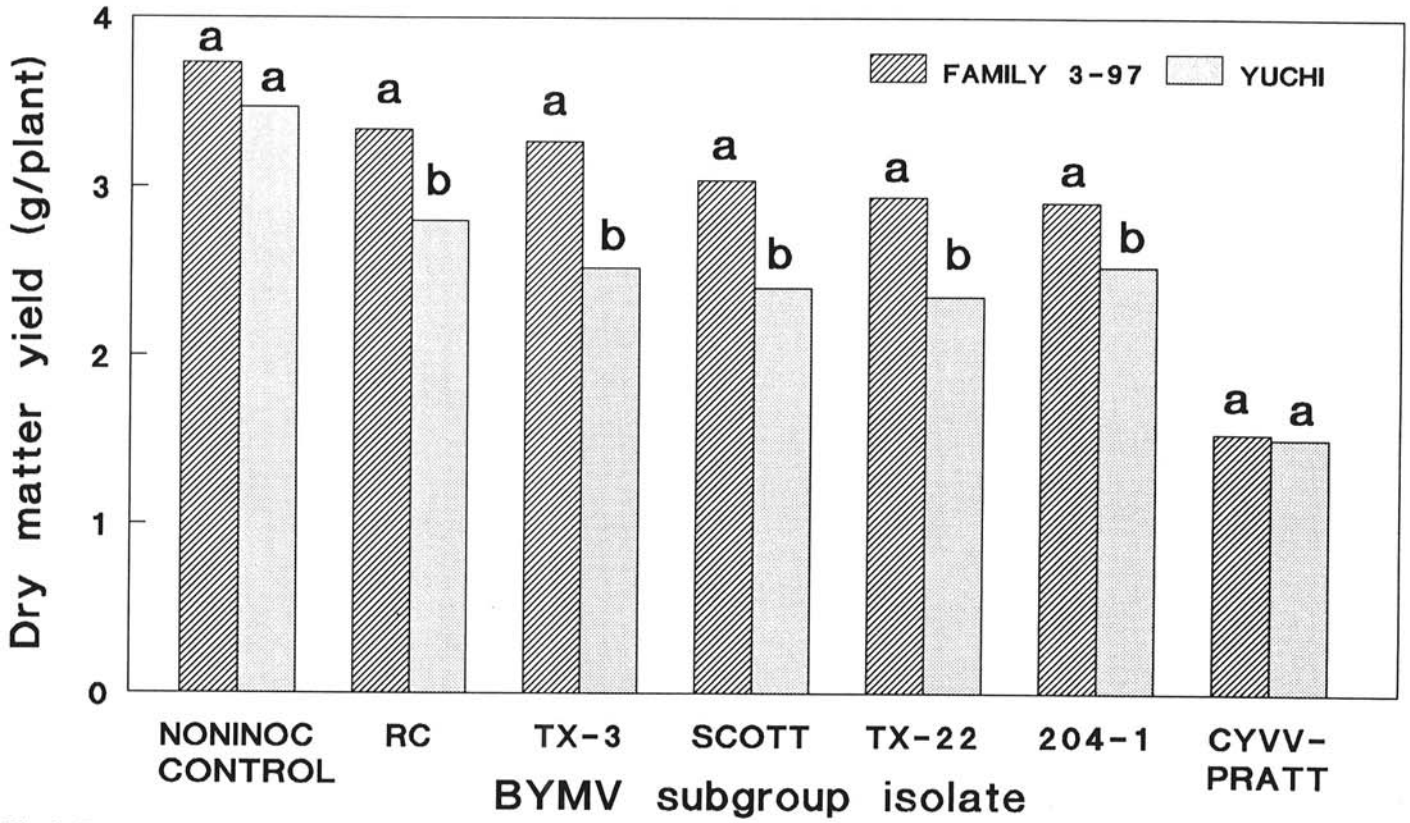

Fig. 2. Total dry matter yield for arrowleaf clover inoculated with six bean yellow mosaic virus subgroup isolates. All plants were harvested 43 and 93 days post-inoculation. Same letter above a pair of bars indicates means were not different according to Fisher's protected least significant difference test ($P = 0.05$). Each bar represents the mean of 36–97 replications.

study are related, conclusions regarding strain interrelationships can be ambiguous and dependent on the biological property examined (2–4,13). Host range studies alone are usually inadequate for isolate identification among the BYMV subgroup viruses (3). Although symptom incidence across the isolates we tested was identical (i.e., all induced mosaic, chlorosis, and wilt), symptom severity was not uniform. This in itself would not differentiate between isolates, but the occurrence of a genetically identified, reproducible response, such as LW, suggests that host response data may be helpful in isolate identification. The ability of tobacco mosaic virus to induce a local lesion host response in *Nicotiana sylvestris* Speg. can be affected by single amino acid changes in a number of sites in the viral coat protein (5). It is not surprising that BYMV subgroup isolates, which differ in serological and host range properties, should also differ in symptom production in arrowleaf clover. ELISA results in our study indicated the two Texas isolates, TX-3 and TX-22, were more closely related to RC and 204-1 than to Scott or CYVV-Pratt. Of the two, only TX-22 induced LW in arrowleaf clover and caused symptoms in Davis soybean. Distribution of wilt symptoms in arrowleaf clover was very similar for the two isolates. Although both Texas isolates reacted strongly to RC antisera, only TX-3 produced wilt symptoms similar to those of RC.

Lethal systemic necrogenesis in red clover inoculated with 204-1 has been associated with nearly a fivefold increase in peroxidase activity over healthy control plants, whereas mottling and hypersensitive necrosis reactions only doubled activity (21). The C2 peroxidase isozyme was unique to hypersensitive necrosis clone KyC13 and possibly played a role in virus localization and tissue necrogenesis (22). Enhanced peroxidase activity has also been associated with induced resistance of cucumber (*Cucumis sativus* L.) to *Colletotrichum lagenarium* (11). Healthy transgenic tobacco (*Nicotiana tabacum* L.) plants with peroxidase activity two- to 10-fold higher than wild type plants, exhibited chronic severe wilting that could not be alleviated by increased watering (14). Although wilted plants recovered turgor pressure at night, symptoms became increasingly severe after 10 days. These studies suggest that although slightly elevated peroxidase levels in the plant are advantageous during pathogen attack, many-fold increases could result in chronic wilting, systemic necrosis, and death. The LW response of arrowleaf clover inoculated with the 204-1 and TX-22 isolates may also be due to virus-triggered overproduction of peroxidase.

We conclude that the LW response of arrowleaf clover is simply inherited and controlled by the single dominant gene, *L*. This response is virus-specific. The interaction of the *L* gene with the six BYMV subgroup isolates demonstrated that only PMV-type isolates (204-1 and TX-22) induced LW in Yuchi arrowleaf clover. Resistance to LW (*ll*) and increased tolerance developed in family 3-97 against one isolate conferred varying degrees of tolerance to other BYMV subgroup isolates tested.

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