Factors Influencing Virus Titer in Maize Dwarf Mosaic Virus-Infected Sorghum

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ABSTRACT

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Concentrations of maize dwarf mosaic virus (MDMV) strains A and B were measured by the enzyme-linked immunosorbent assay (ELISA) in various tissues of grain sorghum (*Sorghum bicolor*) grown under different environmental conditions. Virus titer was higher in plants at 15 C than at 25 C and much higher than at 35 C. Differences in light quality and intensity did not affect virus titer in either expanding or fully developed leaves. Plants grown in the controlled-environment chamber had more virus than those grown in the greenhouse. Under controlled environmental conditions, the youngest (top) leaf when one-third expanded contained much less virus than older plant parts. Virus titer increased slowly and reached a maximum in the fourth or fifth leaf from the top where it remained stable and did not decline sharply as has been observed for these viruses in maize. During grain filling there were moderate to high levels of virus in all parts of the plant including the head, but the virus disappeared from the grain and declined, particularly in the older leaves, as the plant approached physiological maturity. The sorghum hybrid, Bugoff, contained more virus than susceptible *Zea mays* 'Golden Cross Bantam' and 'NE 28.'

Additional key words: host-virus relationship, quantitative ELISA.

Strains of maize dwarf mosaic virus (MDMV) and the closely related sugar cane mosaic virus (SCMV) occur worldwide in graminaceous hosts. These viruses cause significant economic loss in several crops including grain and forage sorghum, maize, millet, and sugar cane. Host plant resistance has partially reduced the losses caused by this virus disease but epidemiology, physiology, symptom expression, and some aspects of resistance are not fully understood. One measure of the relationship of the virus to the host is the titer of virus in the plant tissue.

Tu and Ford (9–11) and Shukla and Joshi (7) measured the virus concentration in maize under several conditions. In their system, sap infectivity was greatest if plants infected with MDMV strain A (MDMV-A) were given modest excesses of N, P, or K, and held at warm temperatures (26.5 C). Cooler temperatures reduced the rate of virus accumulation but stabilized the titer over time. Given optimum conditions, the virus titer in maize reached a maximum shortly after the leaves became fully expanded and then declined very rapidly. As the plant grew, each successive leaf had less virus than the preceding leaf. Similar observations on MDMV were made by Foster (3) with MDMV and maize chlorotic mottle virus, singly or in mixed infections. Factors affecting virus titer in sorghum are unknown.

We have occasionally found sorghum sap inocula to have low infectivity and have assumed, without evidence, that this was due to one or more of the factors described by the previous workers on maize. However, sorghum differs from maize in many aspects of its reaction to MDMV, especially the temperature sensitivity of some symptoms and a variety of necrotic reactions. Also, sorghum is grown in environments which are often hotter and dryer than those where maize is grown. In addition, grain sorghum, although bred to grow like an annual, is basically a perennial while maize is a true annual. For these reasons, we measured the influence of environment and leaf position on virus titer.

Clark and Adams (1) described the enzyme-linked immunosorbent assay (ELISA) which, with the proper modifications, can be used for quantitative assay of virus titer. Reeves et al (6) and Sum et al (8) have reported ELISA methods to work well for the assay of MDMV.

The purpose of the research reported in this paper was to describe, by using quantitative ELISA, the effects of environmental factors on virus titer and to discuss the implications of these findings.

MATERIALS AND METHODS

Virus isolates. Strains A and B of MDMV were obtained from John Hill, Iowa State University, Ames. The strains were maintained on sorghum [*Sorghum bicolor* (L.) Moench] 'Bugoff' (Asgrow Seed Co.) which reacts differently to the two virus strains (12). Strain B was maintained in the controlled-environment chamber to avoid the cold-sensitive necrotic response (red leaf), while strain A was generally propagated in the controlled environment chamber but occasionally also in the greenhouse at 25–30 C with supplemental high-pressure sodium light to give 16 hr of illumination. The controlled-environment chamber (Rheem-Sherer model CEL 511-38-HLE) was equipped with high-intensity, low-pressure sodium lights supplemented with incandescent and fluorescent light (500 $\mu E/M^2/sec$) for 16 hr days at 27 ± 1 C. Unless identified otherwise, the test plant was cultivar Bugoff. Plants were inoculated in the two-leaf stage by rubbing carborundum-dusted plants with inoculum consisting of crude sap, diluted 1:4, from plants inoculated 2–4 wk earlier and showing mosaic symptoms. Under these conditions, symptoms were normally visible on the test plants in 5 days. When the effects of environmental conditions were being measured, the plants were held under the test conditions for 10–24 days so that the tissues being tested would have been under those conditions throughout their entire developmental period.

Antibodies. Virus for antiserum production was purified from sorghum (4). The antiserum was produced in rabbits by intramuscular injection of 1 mg of purified virus mixed with

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Freund's complete adjuvant at weekly intervals for 3 wk. One week later, the rabbits received 1 mg of virus in sterile saline intravenously. The rabbits were exsanguinated on the 6th wk from the start of immunization.

The gamma globulin was concentrated by precipitation with 40% ammonium sulfate. The resultant precipitate was resuspended in demineralized water and reprecipitated with a 33% salt concentration. After resuspension, the globulin was dialyzed exhaustively against neutral 0.01 M phosphate-buffered saline (PBS) at 4 C.

The enzyme conjugate for the double antibody sandwich method was prepared according to the method of O'Sullivan and Marks (5). The optimum concentrations of gamma globulin for coating assay plates and of conjugate were determined by a checkerboard titration of 4, 2, 1, and 0.5 μg/ml of globulin reacted with 4, 2, 1, 0.5, and 0.25 μg of enzyme conjugate per milliliter. The determination of these parameters incorporated 10 μg of pure virus per milliliter and 1:4 dilution of infected plant sap in the assays; both antigens were titrated by the checkerboard method for each preparation of conjugated gamma globulin.

After addition of the appropriate concentration of gamma globulin, the assay plates were incubated on a rotating platform for 2 hr to give maximum sensitivity and uniformity and then refrigerated overnight. The plates were washed thoroughly in PBS + 0.5% Tween-20 (PBS-T) and filled with blocking buffer (PBS + 0.2% bovine serum albumin). This blocking buffer remained in the plates until they were used in tests.

Enzyme-linked immunosorbent assay. Infected tissue was ground in a mortar and pestle in neutral 0.01 M phosphate buffer (PB) in a 1:2 (w/v) proportion. The sap was subsequently diluted with PB through a series of twofold dilutions for virus assay. One-tenth milliliter of PB was added to each well prior to the addition of 0.1 ml of each dilution of sap. When purified virus was used in tests, the virus was diluted and the antigen was applied directly to the wells in 0.2 ml. Following the addition of antigen, the plates were incubated on a rotating platform shaker for 2 hr to ensure maximum contact of the reactants and then refrigerated overnight. After a thorough wash in PBS-T, 0.2 ml of the appropriate dilution of enzyme conjugate was added to each well. The mixing was stopped and the plates were washed as before. The substrate (1.25 mg of *p*-nitrophenyl phosphate per milliliter of 10% diethanolamine buffer, pH 9.8) was added (0.2 ml per well) and the plates were incubated for 1 hr. The reaction was stopped by adding 0.05 ml of 3 M sodium hydroxide per well.

Absorbance was measured with a Titertek Multiskan ELISA microtiter plate reader. The $A_{405\ nm}$ was recorded at each of nine twofold dilutions done in duplicate and the values were plotted. These plots were compared to estimate the difference in virus titer resulting from different treatments. The difference in dilution (obtained by interpolation) that gave the same virus titer, measured as $A_{405\ nm}$, for the two treatments was taken to be proportional to the difference in virus concentration (see Fig. 1). Within an experiment, the treatment giving the lowest titer was given a relative value of one and the others were proportionally larger. Each experiment was repeated three or four times. To the best of our knowledge there is no adequate mathematical or statistical model to describe the significance of the difference between two treatments. To establish confidence intervals of a single reading, four samples were drawn from the same tissue population and duplicate samples were measured through nine dilutions and five replicated readings. The average 95% confidence interval for all dilutions was less than ±22% of a dilution.

RESULTS

In preliminary tests, we examined the reliability of the system and established parameters for subsequent tests. Three different brands of microtiter plates were compared. Under our conditions, the NUNC plates gave $A_{405\ nm}$ values about twice as high as Dynatech for the same virus concentration with either purified virus preparations or infected plant sap. Dynatech plates were about four times more efficient than Falcon plates when used with purified preparations of MDMV-A but at least 100 times more efficient with infected sap of either strain. Thus, NUNC plates were used in subsequent tests.

Cross-reactivities of MDMV-A and MDMV-B antisera to heterologous antigens were tested. The reactivity of MDMV-A antiserum with its heterologous antigen was 1/2,000 of the homologous. The heterologous reactivity of the MDMV-B antiserum was about 1/1,000 of the homologous titer. There was no reactivity with healthy plant sap.

In earlier tests, we found a good correlation between ELISA titer and infectivity of MDMV and the stability of both strains of virus in frozen sap for up to 6 mo (S. G. Jensen and M. K. Palomar, *unpublished*). Therefore, we did not test the changes in infectivity resulting from changes in all the parameters we examined for virus titer. Furthermore, if experimental design, the press of other work, or the number of samples exceeded our capacity the excess was held, usually as intact leaves, for short periods at −80 C.

Environmental factors. In the first series of tests, two groups of plants were compared by identical treatment except that, following inoculation, one lot was moved to the controlled environment chamber while the other was left in the greenhouse. After 14 days, the youngest emerging leaf and the second youngest fully expanded leaf were compared (Table 1A). With strain A, virus increased fourfold going from the youngest leaf to the fully expanded leaf,

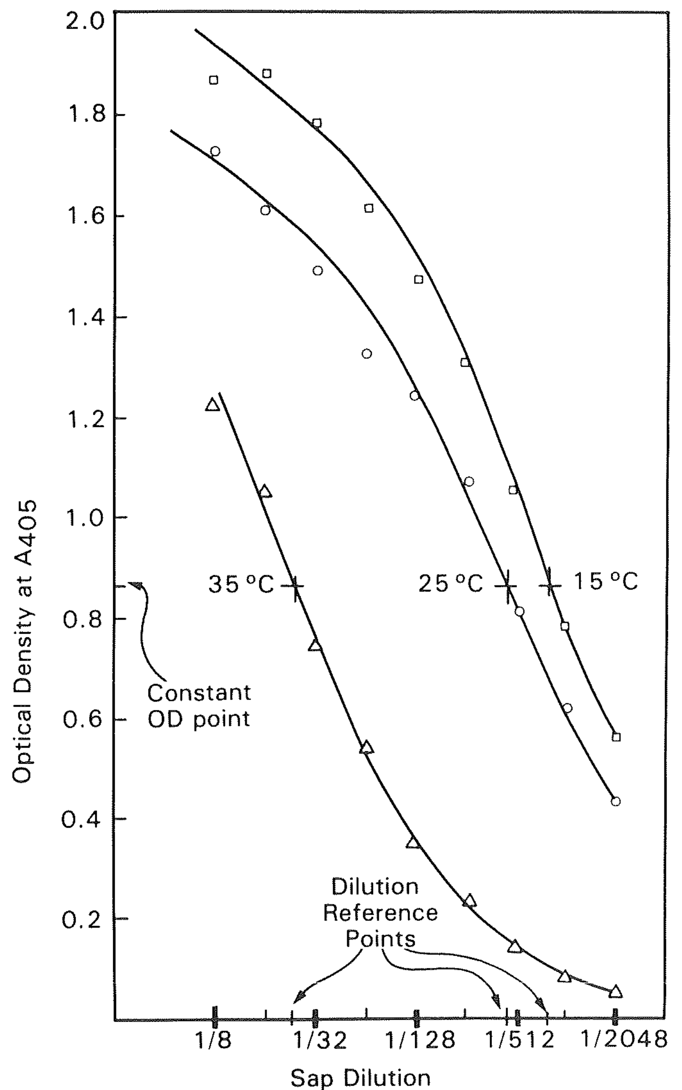

Fig. 1. A plot of the absorbance at 405 nm versus dilution of the sap of partially expanded top leaves of plants held at 15, 25, and 35 C. At a constant absorbance, the true equivalent dilution was calculated for each sample. The lowest titer was given a reference value of one, and higher titers were given proportional values based on their equivalent dilution values.

TABLE 1. Comparison of relative titers[a] of maize dwarf mosaic virus (MDMV) strains A and B in different sorghum or other plant tissues under the same conditions or in similar tissues subjected to different treatments or conditions[b]

A. Greenhouse versus chamber-grown plants:

	Expanding leaves[c]		Mature leaves	
	Greenhouse	Chamber	Greenhouse	Chamber
MDMV-A	1	13	4.6	49
MDMV-B	1	2.3	4.6	42

B. The effects of light quality and quantity:

	Expanding leaves			Mature leaves		
	Full light	40% light	40% fluor.	Full light	40% light	40% fluor.
MDMV-A	1	1	1	4.7	4.7	4.7
MDMV-B	1	1	1	6.1	7.1	7.5

C. Temperature effects:

	Expanding leaves			Mature leaves			Old leaves		
	15 C	25 C	35 C	15 C	25 C	35 C	15 C	25 C	35 C
MDMV-A	100	52	3.3	676	169	1.3	891	208	1
MDMV-B	47	8.6	<<1	231	147	<<1	304	187	1

D. Leaves in same developmental stage at different days after inoculation:

	Expanding leaves				Mature leaves			
	Days after inoculation				Days after inoculation			
	12	19	26	33	12	19	26	33
MDMV-A	1	3.2	1.3	21	10	52	23	28
MDMV-B	1	1	2.3	2.3	4.3	15	26	28

E. Top leaves of plants of various ages:

	Days after inoculation							
	4	10	16	22	28	34	40	49
MDMV-A	1.9	2.5	4.3	5.3	4.3	4.6	1.0	2.6
MDMV-B	1.0	3.3	6.2	15.8	16.9	9.7	14.7	11.2

F. Leaves at different positions on a young plant:

	Leaf position (12 is top of plant)					
	7	8	9	10	11	12
MDMV-A	5.3	6.1	3.3	4.0	2.6	1.0
MDMV-B		13.9	9.8	7.0	4.6	1.0

G. Different tissues on a fully developed plant:

	Tissues beginning at the top of the plant						
	Grain	Panicle	Flag leaf	2nd-leaf	3rd-leaf	4th-leaf	5th-leaf
MDMV-A	11	4.9	143	52	6.1	21	1.0
MDMV-B	1.9	1.0	4.8	22.6	9.2	2.8	1.4

H. Virus source plants:

	Bugoff sorghum	NE 28 corn	Golden Cross Bantam corn
MDMV-A	3.5	1.2	1.0
MDMV-B	4.3	1.0	1.9

[a] All of the values are relative to the lowest concentration found in that particular test which has been given an arbitrary value of one. *Valid comparisons can be made only horizontally, not vertically between virus strains or between experiments.* The data presented are from a representative experiment of three or four replications.

[b] See Materials and Methods for the details of the conditions or tissues involved in each test.

[c] Expanding leaves refer to leaves that were one-third to one-half expanded at the time of harvest while the mature leaf was the second leaf below the expanding leaf. Old leaves were fully developed under greenhouse conditions.

while with strain B the increase was from 5- to 18-fold. With either virus strain or tissue type, titers from plants in the controlled environment chamber were 2.3 to 13 times those of the greenhouse-grown plants.

Two controlled-environment chambers were used to measure the influence of light quality and quantity. Virus titers in plants grown in the Rheem-Sherer chamber (previously described in Materials and Methods) were compared with those that developed in plants grown in a smaller chamber illuminated with fluorescent and incandescent light only. Radiation at the level of the top of the plant was 200 $\mu E/M^2/sec$ in the small chamber. Metal window screening was used to reduce the radiation to 200 $\mu E/M^2/sec$ in a portion of the Rheem-Sherer chamber while the remainder received 500 $\mu E/M^2/sec$ of radiation. Ten to 14 days after the inoculated plants were placed in these conditions, samples were harvested from the youngest leaves and also from the second leaf below this young leaf. This older leaf had developed and reached full expansion under these test conditions. Results of these tests (Table 1B) show that the older leaves had more than a fourfold increase of both MDMV-A and -B over the younger leaves. This difference appeared not to be influenced by light quality or intensity.

The influence of temperature on virus accumulation was determined by using three small controlled-environment chambers, set at 15, 25, and 35 C, respectively. Sorghum cultivar Wheatland was used for most of these tests because leaves of the hybrid Bugoff became necrotic if infected with MDMV-B and held at the cool temperature. Plants were inoculated and held in the greenhouse for 1 wk until the systemic symptoms developed fully, then they were placed in the chambers. Between 10 and 24 days after the plants were placed in these temperature regimes, tissue samples were harvested from young leaves (one-third expanded), from fully expanded leaves, and from leaves that were fully expanded under greenhouse conditions. At the lowest temperature, 24 days were required to ensure sufficient growth and development while at the highest temperature, a delay in sampling of more than 10 days produced unacceptable differences in the size of the test plants. The most striking effect of temperature (Table 1C) is the great reduction in virus accumulation at 35 C; virus titer was very low in all tissues. In the expanding or mature leaves of plants infected with MDMV-B the virus was barely detectable with the ELISA procedure at a sap dilution of 1/8. It was only in the old leaves where the titer had been relatively high during the greenhouse development that sufficient virus remained to be measured. Although early experiments with MDMV-A in the hybrid Bugoff suggested that the virus titer was highest at 25 C, subsequent experiments with that combination and later experiments with cultivar Wheatland showed that if the tissues developed to full equivalent size there was consistently more virus at 15 C. Plants grew slowly at 15 C but contained 2 to 5 times more virus per gram than those at 25 C. It appears that virus synthesis is not linked to growth rate since growth was by far the fastest at 35 C and the slowest at 15 C. We did not attempt studies at lower temperatures since 15 C is near the minimum for significant plant growth.

Morphological factors. Three series of experiments were conducted to determine if the capacity of tissue to produce virus depended upon the time after inoculation. In the first tests, leaves in two developmental stages, either still expanding or fully mature, were assayed from 12 to 33 days after inoculation (DAI).

The two strains accumulated virus differently at different times (Table 1D). MDMV-A titer increased in the immature tissue throughout the test period while in the older leaf the titer peaked at 19 DAI and then declined. With MDMV-B, the titer increased slowly over the entire experiment in both immature and older tissue.

The second series of experiments followed the youngest fully expanded leaf at 6-day intervals, from 4 to 40 DAI with a final reading at 49 DAI (Table 1E). The titer of strain A was already high when the first measurement was made, but it increased slowly in subsequent assays until it had slightly more than doubled by the 22nd DAI. The titer of MDMV-B was comparatively low on the first day of sampling but it increased steadily through the 10th, 16th, and 22nd DAI and reached a peak at 28 DAI. This peak was about 16 times higher than the starting point at 4 DAI. For both strains, the virus titer in young tissue declined by the end of the experiment at 49 DAI.

These studies were expanded to determine the virus accumulation in tissues of different age. In the expanded tests, virus titer was assayed in each leaf of a plant at about the 12-leaf stage, just prior to boot. This corresponds to stage 4 of Vanderlip and Reeves (13). Leaves of different positions (and therefore of different ages) were harvested simultaneously. The tissue harvest pattern is illustrated in Fig. 2 and the data are shown in Table 1F. The results show that virus concentration in these tissues increases after the leaves fully expand and that it remains stable after maximum titer has been reached. Leaf 10 in this experiment would compare with the mature leaves assayed previously. It is apparent that the virus titer increases for some time after maximum leaf expansion. Throughout these tests the youngest leaves (about one-third expanded) always had the least virus. Both strains A and B responded similarly; in general, strain B required longer to reach its maximum concentration. In fact, the titer of strain B increased progressively in the more mature leaves.

The next experiments were similar in intent, but the plants were assayed in the milk stage of heading rather than in the preboot stage. The youngest tissue, namely the grain and panicle, had considerable virus, but less than the older leaves (Table 1G). The maximum virus with strain A was in the flag leaf but with strain B the maximum was in the first leaf below the flag leaf. This supports the concept that strain A builds up in the tissue faster than strain B. An additional observation in this experiment was the apparent rapid decline in virus titer as the older leaves senesced. Subsequent studies showed that by blackening of the abscission layer of the grain, which signals physiological maturity, virus was no longer detectable in the grain.

Host comparisons. Results of the preceding tests show the influence of environment on changes in virus titer at different stages of growth and development of sorghum. They suggest that

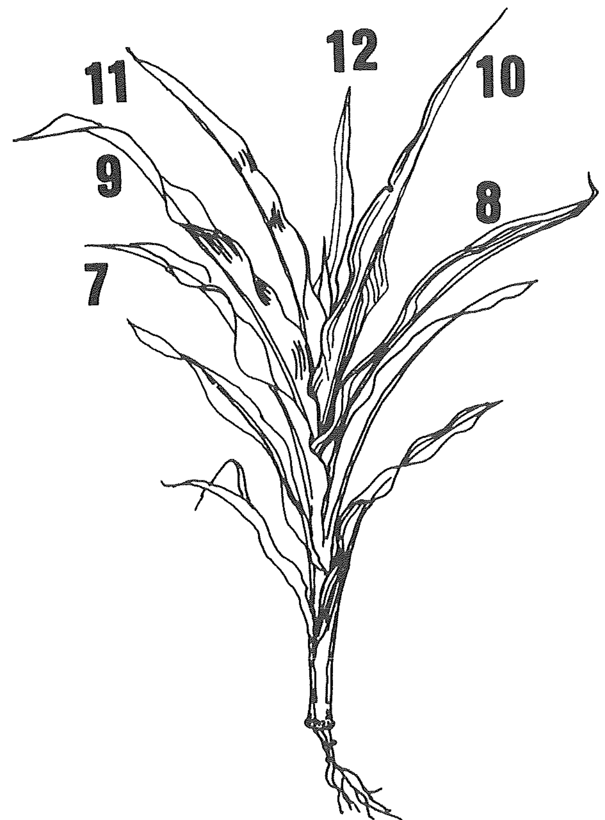

Fig. 2. Drawing of a plant in the preboot stage identifying and characterizing the tissues that were sampled.

sorghum may have some advantages over maize as a host for virus purification. Virus is commonly purified from Golden Cross Bantam sweetcorn and the inbred line NE 28. These cultivars were grown and inoculated the same as sorghum and when strong mosaic symptoms were apparent, about 2 wk after inoculation, the virus titers in the second leaves down from the top were compared with those from sorghum. With strain A, both corn cultivars contained about the same amount of virus which was between one-half and one-quarter the amount in the sorghum. With strain B, the results with Golden Cross Bantam were again only one-half or less of the amount in sorghum, but NE 28 was an even poorer host, having only one-half the virus titer of Golden Cross Bantam.

DISCUSSION

Results of these experiments demonstrate that the ELISA procedure can rapidly and effectively measure virus titer and the effects of the factors that affect it. Admittedly, the method does not measure infectivity but rather only virus coat protein. Dean (2) has published an infectivity test for MDMV that is more sensitive to virus titer than ELISA, but time, space, and the number of plants involved prohibit the use of infectivity tests for routine virus titer testing.

In general, the environmental influences on virus titer described by Tu and Ford (9–11) for maize are similar for sorghum but there is some variation. Our high temperature of 35 C was considerably higher than theirs (26.5 C) and we observed a drastic drop in titer between 25 and 35 C. The sorghum plants flourished at 35 C. Several workers have experienced difficulty in purifying virus from maize grown in the summer at elevated temperatures. This is perhaps due to a very rapid peak and drastic decline in virus concentration in maize at higher temperatures. There was a similar accumulation pattern at moderate temperatures (26.5 C). The problem is to select appropriate tissue since at high temperatures only a small amount of maize tissue has maximum virus titer at any one time. In sorghum at 25 C, the increase and decline of MDMV-A and -B in each leaf are more gradual. With maize, the question has been raised (but not answered) as to whether plants at high temperatures are good virus sources for aphid transmission. Higher temperatures reduced virus titer considerably in sorghum. Strain B was reduced to barely detectable levels at 35 C while strain A, although greatly reduced, was still easily detected. This reduced concentration of MDMV-B along with the presence of overwintering, MDMV-A-susceptible Johnsongrass in warm climates may be the reason that the strain A predominates in the south while strain B is more common in the north.

The temperature that led to the maximum virus concentration in sorghum (15 C) does not correspond to the optimum temperature for virus accumulation observed for maize (26.5 C). To grow plants for virus purification, it appears best to grow and inoculate the plants at a warm temperature to ensure rapid growth and systemic spread and then to hold them at a cool temperature to let the virus accumulate.

Light quality or intensity failed to influence virus titer, but it influenced the rate of plant growth. Plants grown at high light intensities were much larger. However, even with high-intensity lights, the plants received only about one-half the intensity of full sunlight. At constant temperature, the virus titer was proportional to the growth rate since virus titer per gram fresh weight remained constant. The difference in virus titer observed in a comparison between the controlled-environment chamber and the greenhouse is more difficult to explain. The average duration of light and the average temperature were the same, but fluctuations were more frequent and pronounced in the greenhouse. The stress that this puts on the plant can be seen in the appearance of small necrotic areas on plants of greenhouse-grown cultivar Bugoff infected with strain B. This combination of host and virus strain shows the severe temperature-sensitive necrosis that kills the whole plant if the temperature drops to 10–15 C for several hours. The fact that both strains had a reduced titer in the greenhouse suggests that temperature stress or fluctuation significantly affects virus replication even if it fails to cause obvious tissue damage.

On a tissue-by-tissue basis, a comparison of virus titers in sorghum and maize shows that they differ greatly. In maize, the virus accumulated rapidly as the leaf expanded, peaked at about the time of full expansion, and then declined rapidly (11). The lower leaves were capable of producing a higher virus titer than the upper leaves (11). In sorghum, the virus buildup was slower and did not reach the maximum until many days after the leaf was fully expanded. The middle leaves or those just below the flag leaf appear to have the highest concentration of virus. There also was little tendency for the virus titer to decline after reaching a maximum concentration unless the plants were grown at high temperatures or the grain had reached maturity.

These results demonstrate that MDMV has a very different relationship with sorghum than with maize. Based on our tests, sorghum would be a preferred host for virus purification. By maintaining high virus titer through a range of conditions, sorghum has the potential to be an effective reservoir for aphid transmission at moderate-to-warm temperatures but may not be a good source in hot seasons. Some of the differences between sorghum and maize may be related to the perennial growth habit of the former.

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