

# Laboratory Transmission of Maize Chlorotic Mottle Virus by Three Species of Corn Rootworms

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## ABSTRACT

Jensen, S. G. 1985. Laboratory transmission of maize chlorotic mottle virus by three species of corn rootworms. *Plant Disease* 69:864-868.

Greenhouse and laboratory tests were conducted on the transmission of maize chlorotic mottle virus (MCMV) by three species of *Diabrotica*. No latent period was detected. Virus was recovered from the gut and in trace amounts from the hemolymph. There was no correlation between the presence of recoverable virus in or on the insect and its ability to transmit. No transovarial passage of the virus was detected. Larvae transmitted the virus from fresh material but not from dead plant residue. Infectious virus was not recovered from dead plant material held over the winter. The epidemiology and overwintering of MCMV cannot be readily explained in terms of the beetle-virus interaction.

Corn lethal necrosis (CLN) is a serious disease of corn caused by infection with two viruses, maize chlorotic mottle virus (MCMV) and either maize dwarf mosaic virus (MDMV) or wheat streak mosaic virus (WSMV) (5,6). Loss estimates for some fields have been as high as 75%. The destructiveness of the disease has impressed farmers to the point that field tests have been limited to fields where the disease already occurs. This has made it difficult or impossible to do field experiments that include disease-free control treatments.

To date, CLN has been confined to the Republican and Big Blue river valleys in Nebraska and Kansas. In fields with a history of mixed cropping, a sharp demarcation has been seen between infected and uninfected areas, depending on the previous years' crops. CLN occurred in areas of a field that were in corn the previous year, whereas areas that had been planted to a crop other than corn the previous year remained substantially free of the disease (8,14). All of the viruses involved have aerial vectors: MCMV is transmitted by beetles (4), MDMV by aphids (3), and WSMV by eriophyid mites. Because both MDMV and WSMV and their vectors are widespread in Nebraska and Kansas, the geographic and epidemiological limits of CLN are apparently due to restraints on

the spread of MCMV.

This paper reports the results of laboratory and greenhouse experiments on beetle transmission of MCMV designed to determine if transmission of this virus differs from that of other beetle-transmitted plant viruses in ways that may explain the unusual epidemiology of MCMV. The vector transmission properties do not readily explain the unusual features of CLN epidemiology, but they do suggest possible avenues for research with this potentially devastating disease.

## MATERIALS AND METHODS

Laboratory colonies of the southern corn rootworm (*Diabrotica undecimpunctata howardi* Barber) and the western corn rootworm (*D. virgifera virgifera* LeConte) were used to study vector-virus relationships. Insects included the standard colony of southern corn rootworms maintained at the Northern Grain Insect Research Laboratory, Brookings, SD, and two colonies of western corn rootworm: a typical wild type that has been in laboratory culture for several years and a nondiapausing strain of the same type. No differences in vector-virus interactions were found between the two strains of western corn rootworms, and either strain was used depending on availability. Transmission properties of either strain were considered representative of the species, and data were combined. Northern corn rootworms (*D. barberi* Smith & Lawrence) were collected in the field in Brookings County, SD.

The virus used in these studies was collected near Republican City, NE, and was identified as MCMV by its reaction on Blue Boy wheat and SDP2 corn, its sedimentation rate in rate-zonal sucrose density-gradient centrifugation, particle morphology, serological reaction of antiserum to this virus with authentic

MCMV, and transmission by chrysomelid beetles (4,7). SDP2 corn, an inbred line developed at South Dakota State University, Brookings, served as a test and indicator plant throughout the studies because it was highly susceptible to infection and gave pronounced symptoms.

Unless otherwise indicated, a 2-day acquisition access period for corn rootworm beetles was provided in a controlled-environment chamber ( $25 \pm 2$  C with a 16-hr photoperiod) in small, wire-screen cages in which bundles of severed young corn plants inoculated 3-4 wk earlier by insect feeding were the virus source. Larvae acquired virus for 1 day in large, plastic petri dishes lined with moistened filter paper. Virus source plants for larvae were similar to those described for beetles but with the older, outer leaves removed and the tender, young tissue exposed by longitudinal shredding of the plant. Washed roots of corn seedlings were also an effective virus source but were more difficult to obtain and supported a much smaller population of larvae.

Beetles were given inoculation access to SDP2 corn seedlings in the two- to three-leaf stage. Adult beetles were confined singly or in groups of three or five insects per plant. Unless specified otherwise, beetles were fed for 2 days and then either picked from the plants or killed by fumigation. For larval transmission, larvae were placed with roots of sprouted seedlings in large, plastic petri dishes lined with moistened filter paper, or 10 larvae were added to a 10.2-cm clay pot with three seedlings in the two- to three-leaf stage. The inoculation access period for larvae was 2-6 hr in petri dishes or 2-4 days in soil. Unless otherwise specified, all larvae were in the late second or early third instar stage, when they are the most vigorous and voracious. After inoculation access, all plants were transferred to an isolation greenhouse maintained at  $25 \pm 2$  C and a 16-hr photoperiod and examined for symptoms 2-3 wk later.

Although conditions were standardized, the amount of transmission varied from experiment to experiment but was consistent within an experiment. All experiments were conducted in an isolation greenhouse, and diseased plants were held in an insect-free isolation greenhouse. All soil was sterilized before use and all soil, plant material, and

Published as Paper 6125, Journal Series, Nebraska Agricultural Experiment Station.

Accepted for publication 12 June 1985 (submitted for electronic processing).

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insects were autoclaved after each experiment. To describe the biological interaction between the insects and the virus, the following factors were studied:

1. Factors related to transmission—beetle age and sex, temperature during inoculation access, latency and persistence, acquisition access duration, beetle numbers, transmission by wild populations, and transmission by insects correlated to virus presence in or on the insect as detected by mechanical inoculation.

2. Biological interactions between the vectors and the virus—virus presence in hemolymph, inactivation by gastric juices, gut permeability to the virus, and transovarial passage.

3. Factors that affected larval transmission—surface contamination vs. biological interaction, and transmission from dried plant material.

## RESULTS

**Factors related to transmission.** To determine if transmission efficiency varied during the season, beetles of various ages were tested and found to transmit MCMV with equal efficiency (Table 1). Likewise, both sexes transmitted MCMV with similar efficiency, with 37 of 111 males and 31 of 105 females transmitting. (These insects were later used for the genetics and transovarial

transmission tests.) Temperature, however, did affect transmission results. Beetles that had acquired the virus at 25 C consistently transmitted better at 25 C than at either 15 or 35 C (Table 2).

To test for a latent period and persistence in transmission, individual beetles were allowed a 6-hr acquisition access followed by a series of successive 6-hr inoculation access periods on healthy seedlings. There was no significant change in the transmission rate during the 54 hr after acquisition (Table 3). Statistical analysis of the probability of transmission by individual insects indicated that at these levels of transmission, the transmission by any single insect was random. An insect that had transmitted once was no more likely to transmit again than an insect that had not been a vector before.

Average transmission percentages for western corn rootworm beetles were 7.4, 11.7, and 9.0% after acquisition periods of 1, 2, and 3 days, respectively, indicating little difference in efficiency related to a prolonged acquisition access.

When there is no biological interaction between vectors on a plant, the number of plants that become infected by insect transmission should be a function of percent transmission by one insect and the number of insects per plant as described by Storey (12) and Nault et al

(3). Table 4 shows transmission as a function of number of beetles per plant, and Figure 1 is a plot of expected transmission in the absence of biological interaction. Transmission rates were well below the theoretically expected value. This is probably due to interaction between insects on a single, small plant. This interaction would not be a factor in the field on young growing plants before many beetles emerge, but beetle interaction could occur on mature plants, where populations can be high.

In a test of the transmission efficiency of the local wild populations, western and northern corn rootworm adult beetles were collected in fields in Brookings County, SD, that are free of MCMV. Sufficient field populations of southern corn rootworms were not available for similar tests. Two experiments with western corn rootworms gave 5 and 31% transmission; concurrent tests with northern corn rootworms gave 8 and 9% transmission. These tests generally agreed with tests done with laboratory-reared insects, and transmission efficiency of both groups was similar to the 15% transmission observed by insects collected in fields containing CLN in the Republican River Valley.

To test for a relationship between the acquisition of virus and the ability to transmit, western corn rootworm beetles were allowed an 18-hr acquisition access followed by a 48-hr inoculation access and then homogenized individually. Each homogenate was mechanically inoculated onto indicator corn seedlings. Of the plants given the inoculation access feeding, 52 of 123 (43.3%) were infected. Of the plants inoculated mechanically from the homogenized insects, 20 of 129 (15.5%) were infected. MCMV was recovered both by transmission feeding and by mechanical inoculation from eight insects, which is exactly the proportion expected by random distribution. There was no correlation between the ability of the beetle to transmit and the presence of naturally acquired virus in or on its body as detected by mechanical inoculation.

**Biological interactions.** Circulative viruses are often persistent and interact closely with the vector. To determine if MCMV was circulative, attempts were made to recover virus from the hemolymph of insects after a 24-hr acquisition. Beetles were anesthetized with CO<sub>2</sub> and a rear leg was removed. The drop of hemolymph that accumulated at the wound was drawn off with a microcapil-

**Table 1.** Transmission of maize chlorotic mottle virus by corn rootworm beetles of different ages

Experiment	Age <sup>a</sup>	Species <sup>b</sup>	No. of beetles per plant	No. plants infected/ no. infested (% infected)
1	Young	WCR	3	18/68 (26.5)
	Mid	WCR	3	23/93 (24.7)
	Old	WCR	3	7/26 (26.9)
2	Young	WCR	1	16/123 (13.1)
	Old	WCR	1	51/127 (40.2)
3	Young	SCR	3	3/59 (5.1)
	Old	SCR	3	9/324 (2.6)

<sup>a</sup>WCR = western corn rootworm beetles and SCR = southern corn rootworm beetles.

<sup>b</sup>Young = 2 days old or less, mid = 3 wk old, and old = more than 4 wk old.

**Table 2.** Effect of temperature on transmission of maize chlorotic mottle virus (MCMV) by western corn rootworm

Experiment	No. plants infected/no. infested (% infected) <sup>a</sup>		
	15 C	25 C	35 C
1	4/46 (8.7)	8/46 (17.4)	4/46 (8.7)
2	10/67 (14.9)	19/54 (35.2)	5/59 (8.5)
3	13/90 (14.4)	18/88 (20.5)	4/91 (4.4)
Totals	27/203 (13.8)	45/169 (26.6) <sup>b</sup>	13/196 (6.7)

<sup>a</sup>Forty-eight hours of acquisition access on MCMV-infected corn at 25 C followed by 48 hr of inoculation access on SDP2 corn.

<sup>b</sup>\* = Significantly higher at the 95% confidence level.

**Table 3.** Transmission of maize chlorotic mottle virus by single corn rootworm beetles transferred to new corn seedlings every 6 hr after a 6-hr acquisition access

Experiment	No. plants infected/no. infested (% infected)								
	0-6 hr	6-12 hr	12-18 hr	18-24 hr	24-30 hr	30-36 hr	36-42 hr	42-48 hr	48-54 hr
1	3/60 (5.0)	...	9/60 (15.0)	6/55 (10.9)	1/54 (1.8)	...	3/53 (5.7)	2/52 (3.8)	...
2	4/60 (6.7)	3/58 (5.2)	0/56 (0.0)	1/54 (1.9)	1/53 (1.9)	0/51 (0.0)	1/47 (2.1)	2/37 (5.4)	0/26 (0.0)
3	0/60 (0.0)	1/58 (1.7)	2/56 (3.6)	0/54 (0.0)	0/53 (0.0)	1/46 (2.2)	1/43 (2.3)	1/38 (2.6)	1/35 (2.9)
Totals	7/180 (3.9)	8.5/176 (4.8)	6.5/172 (3.8)	7/163 (4.3)	2/160 (1.3)	2.5/150 (1.7)	3.5/143 (2.4)	5/127 (3.9)	1/61 (1.6)

lary tube and diluted 1/10, 1/100, and 1/1,000 with distilled H<sub>2</sub>O to deter potential inhibitors. A second undiluted sample and the three dilutions were rubbed onto leaves of Carborundum-

dusted corn seedlings. As a control, leaves of virus source plants were ground with an equal volume of H<sub>2</sub>O and the sap was diluted by the capillary tube method used for the hemolymph and rubbed onto

leaves. Infection of corn seedlings was obtained with the undiluted hemolymph from two of 33 insects but not with diluted hemolymph. Plants inoculated with sap diluted at 1/200 were consistently infected, and those inoculated with sap diluted at 1/2,000 were occasionally infected. Inhibitors were apparently not a factor. In two other experiments with 20 insects each, no transmission was obtained with undiluted hemolymph. Slack and Scott (10), with good evidence of southern bean mosaic virus in beetle hemolymph, had much higher levels of virus recovery.

In an effort to determine if gastric juices inactivated the virus after ingestion by the corn rootworm, the head with attached midgut was removed from adult insects that had completed the acquisition feeding period. The midgut tissue along with the ingested plant material was ground in a drop of distilled water and inoculated onto corn seedlings. Gastric juices did not inactivate the virus. In two experiments, a total of 33 of 39 plants inoculated with homogenized beetle midgut became infected. In a similar experiment, southern corn rootworm larvae were allowed acquisition access and eight larvae, which had green plant material in their gut, were dissected and the midgut and contents were mechanically inoculated to corn seedlings. Mechanical transmission from larval midgut occurred in all eight attempts. Larvae from the same population, most of which had eaten green plant material as determined by visual inspection, transmitted MCMV by feeding to only 12 of 101 plants.

Storey, in his classic work (12), discovered that gut permeability determined transmission, so his experiments were emulated and 78 western corn rootworm beetles were pierced with a fine insect mounting pin through the ventral side posterior to the metathoracic segment midway through a 40-hr acquisition access period. This allowed the gut contents to mingle with hemolymph. Only one of 78 beetles given the gut puncture treatment transmitted MCMV, whereas 15 of 59 beetles from the same population, which were allowed 48 hr of undisturbed acquisition access, transmitted MCMV. Impermeability of the gut was not a factor in transmission. The beetles with gut punctures appeared distressed and did not eat as often or as much as the controls, but as indicated by leaf damage, they did feed. This reduced feeding may account for the reduced transmission, but certainly gut puncture did not increase efficiency.

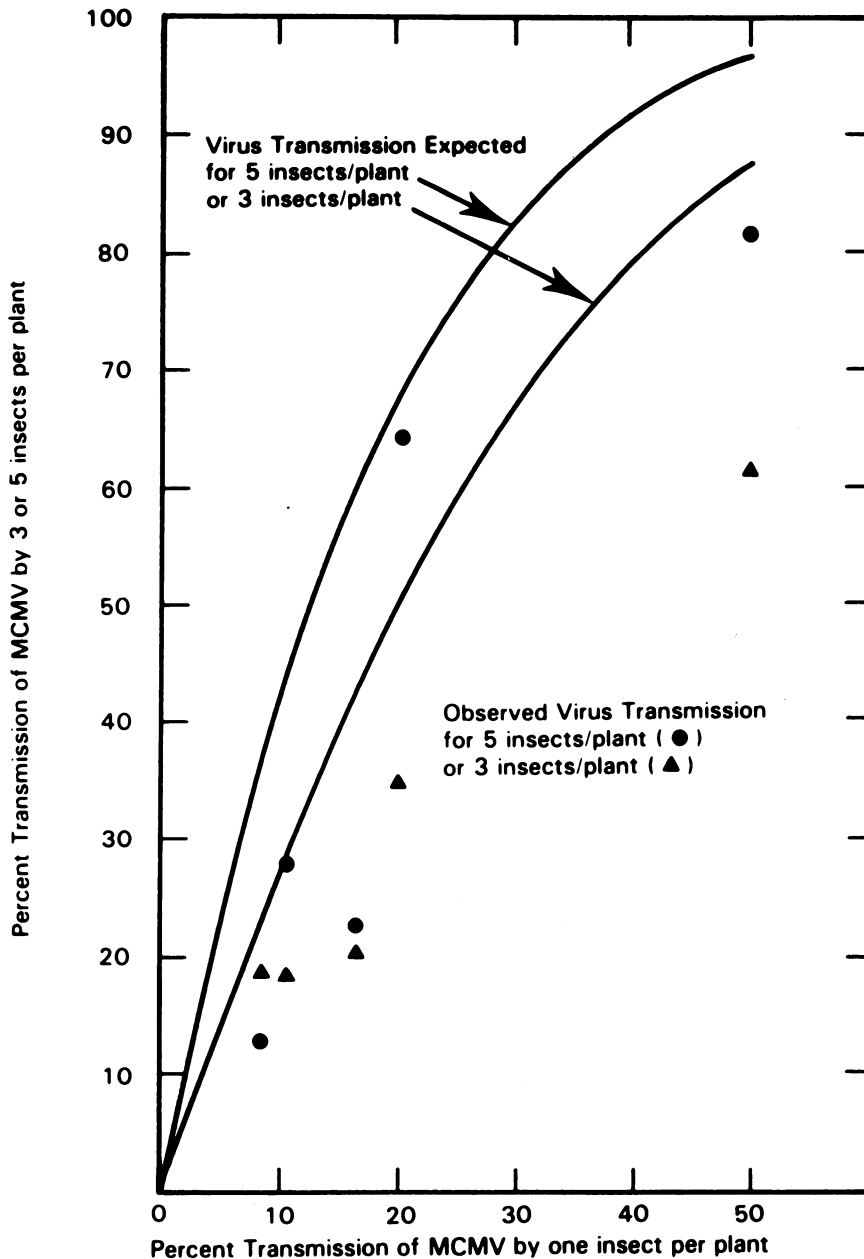
Storey (11) was the first to show that transmission efficiency could be controlled genetically. In an attempt to select an efficiently transmitting strain and also to test for the possibility of transovarial transmission, virgin male and female corn rootworms were segregated into separate populations immediately after

**Table 4.** Influence of corn rootworm beetle numbers on the transmission of maize chlorotic mottle virus<sup>a</sup>

Experiment	Species <sup>b</sup>	No. plants infected/no. plants infested (% infected) with indicated number of beetles per plant		
		1	3	5
1	SCR	81/161 (50.0)	32/53 (61.5)	14/17 (82.0)
2	WCR	16/151 (10.6)	6/32 (18.8)	5/18 (27.8)
3	WCR	16/80 (20.0)	16/46 (34.8)	9/14 (64.3)
4	WCR	21/128 (16.4)	8/39 (20.5)	5/22 (22.7)
5	WCR	13/158 (8.2)	9/47 (19.0)	3/23 (13.0)
Totals	WCR	66/517 (12.8)	39/164 (23.9)	22/77 (28.6)

<sup>a</sup> After 2 days of acquisition access in a controlled-environment chamber and 2 days of inoculation access in a greenhouse.

<sup>b</sup> SCR = southern corn rootworm beetles and WCR = western corn rootworm beetles.



**Fig. 1.** Transmission of maize chlorotic mottle virus (MCMV) by one beetle per plant vs. transmission by three or five beetles per plant.  $N = 1 - (1 - Y)^n$ , where  $N$  = percent transmission by  $n$  insects.  $Y$  = percent transmission by one insect and  $n$  = number of insects.

emergence, and only those individuals that transmitted MCMV were included in a breeding population. None of the 914 larvae from eggs from these matings tested transmitted the virus, so no transovarial passage of the virus was seen. The transmission of 148 F<sub>1</sub> adults that matured from these larvae was 12% compared with 28% for the parental population. Genetic selections did not effectively increase transmission.

**Studies on larval transmission.** One possible method of transmission is simple surface contamination. To test this possibility, larvae were treated in three ways: 1) Larvae were fed on infected tissue for an acquisition access of 24 hr, then transferred directly to sprouted corn seedlings. 2) Larvae, which had been given acquisition access on diseased tissue, were washed carefully by repeated dipping and swirling in successive distilled water baths, then transferred to sprouted seedlings. 3) Larvae were fed for 24 hr on healthy corn tissue, then washed extensively in a homogenate of diseased corn seedlings diluted in distilled H<sub>2</sub>O (1:5, w/v).

Larvae given an acquisition access (treatment 1) transmitted 17.4% of the time. Similar insects washed to remove surface contamination (treatment 2) still transmitted 16.5% of the time. Conversely, larvae washed in infectious plant sap (treatment 3) did not transmit. These experiments suggest that surface contamination alone is not sufficient for transmission.

To determine if larvae could transmit the MCMV from dead plant tissue, infected plant material was dried naturally in the greenhouse. When this material was shredded or powdered, larvae would not feed on it even if it was moistened. If it was mixed with a standard agar base diet used for insect propagation, the insects ate it readily. However, larvae did not transmit virus from dead plant tissue in two studies involving a total of 150 larvae. The virus was easily recovered by mechanical transmission from this mixture of powdered leaf tissue and agar diet. Control larvae fed fresh infected tissue readily transmitted the virus.

Infectivity was lost rapidly from this dried leaf tissue when tested by mechanical inoculation. During the first 2 mo after drying, while the tissue was being used for the larval transmission studies, infectious virus was recovered readily by mechanical transmission from material being stored at room temperature; however, after 3 mo, only a trace of infectivity remained, and after 4 mo, the virus could no longer be recovered. Naturally infected corn plants from the Republican River Valley were held in an unheated isolation greenhouse and allowed to mature and dry naturally. MCMV was recovered from this material from September through December but not thereafter.

## DISCUSSION

The results suggest that the mechanism of transmission of MCMV is similar to that of other beetle-transmitted plant viruses. No latent period was detected and infectivity was found only in trace amounts in the hemolymph. Transovarial passage was not detected in 914 progeny of viruliferous beetles. Furthermore, the ability to transmit MCMV was not related to age, sex, or genotype of the beetle. The virus was not inactivated by ingestion. Transmission continued for more than 2 days, but during this time, transmission of the virus by an individual insect was random, usually occurring only once or twice during a series of plant-to-plant transfers.

Temperature significantly affected transmission efficiency, but the effect could be explained by the activity and feeding behavior of the insects at different temperatures. At 35 C, the beetles congregated quietly in the coolest corner of the feeding cage; at 15 C, they sought the warmest place at the top of the cage; and at 25 C, they swarmed over the test plants and fed regularly. This response to temperature can also be seen in the daily cycle of activity of the insects in the field. Thus, the temperature effect probably does not indicate a biological interaction between vector and virus.

Jansen and Staples (2), Dale (1), and Walters and Henry (15) reported a correlation between the amount of plant material eaten and the efficiency of transmission of a virus by beetles. Such a pattern was not seen in these studies. Western corn rootworm beetles were usually voracious, whereas the northern corn rootworm beetles were fastidious on corn. Differences in transmission efficiencies between these species were not attributable to feeding habits.

These factors suggest a simple mechanical association between the virus and the insect. However, I was unable to show a correlation between the presence of the virus in or on the insect as detected by mechanical inoculation and the ability of the insect to transmit the virus during feeding. No inhibitors were found in the hemolymph or gut, so failure to detect the virus on or in the insect is probably due to the inefficiency of mechanical inoculation techniques. With only trace amounts of virus recovered from hemolymph, the circulative system is probably not the key to biological interaction.

In serial transmissions, an insect that had transmitted once was no more likely to transmit again than an insect that had never transmitted before; therefore, the probability that any individual insect will transmit during any given time seems to be random. However, even with the stress of being handled repeatedly, some insects in the longest experiments were still transmitting on the third day.

Two to 3 days is a long time in the active life of a beetle, and during that time, it may visit many plants and migrate

as far as several hundred miles. These observations on persistence of MCMV in corn rootworms, which confirm the previous observations of Nault et al (4), combined with the highly active nature of the insect, raise questions about the failure of the virus to move out of the Republican River drainage basin. At this time, it remains the only region on this continent with a chronic, high incidence of the virus.

Furthermore, there are often sharp zones of demarcation across a field between "healthy" plants and plants diseased with CLN (5,14), which does not seem to be compatible with an active wide-ranging vector such as the adult corn rootworm beetle.

Experiments reported in this paper demonstrate that larvae can effectively vector MCMV from fresh, infected plant material. Although there was no evidence of a biological interaction between the larvae and the virus, transmission did not result from experimental surface contamination. It appears that for transmission to occur, the virus must be acquired through feeding. Possibly, infection occurs by regurgitation of the ingested infectious material during inoculation feeding. Unlike some soil-inhabiting insects, larvae of the corn rootworm do not emerge from the soil or travel great distances. Their movements within the soil are only a few centimeters (9,13). Considering these limited movements, it is possible that corn rootworm larvae serve as vectors that would spread MCMV to other plants immediately surrounding an infected plant.

The question then remains: How do these plants become infected in the first place? Transovarial passage could not be demonstrated in 914 larvae, but that number is small compared with field populations. Without other indications of biological interaction between the virus and the beetle, however, there is little reason to suspect this mode of overwintering for the virus. I was unable to demonstrate that MCMV remains viable in plant debris for long periods (over winter). Uyemoto (14) reported recovering infectious MCMV from corn residue in April, but that was a rare observation from many, many samples. Also, there are still 2 mo of intense biological activity in the soil that would promote virus degradation before infection appears on corn in the field in June. This diminished inoculum potential even further reduces the likelihood that young plants become infected by corn rootworm larvae feeding or even through mechanical contact of roots with viruliferous plant debris during their normal growth and development. Uyemoto (14) also reported a single larval transmission from "nonsucculent" tissue, but the details of the experiment were not given. In my controlled experiments with plant material of known infectivity potential, larvae would not transmit even

when induced to eat the dried, rehydrated viruliferous plant material. Feeding habits of the larvae are not well known, but second instar larvae can be recovered when eggs are hatched in sieved soil devoid of plants or large particles of organic material. The assumption is that they survive by feeding on soil microbes or even other eggs or larvae. Certainly, according to my observations and given the options available to larvae in the soil during the corn growing season, they would prefer corn roots to nonliving plant debris.

Therefore, the theory that the virus overwinters in plant residue, is recovered by corn rootworm larvae, and is transmitted to corn seedlings is based on three highly unlikely assumptions. The first is that the virus could survive in significant quantities in the plant residue until the rootworm eggs hatch in the spring. The second is that larvae would willingly eat this residue. The third is that they would transmit the virus from this inoculum source. The probability of an infection would be the product of the probability of each of the three low-probability events. The observed frequency of diseased plants in the field seems to far exceed this probability.

It seems safer to assume there are other factors relating to virus overwintering that we do not know. Possibly, some other host such as a perennial plant, a soil insect, a nematode, or a fungus that is indigenous may harbor the virus through

the winter and provide the inoculum for the following spring.

In the field, the first symptoms of MCMV infection appear on scattered plants in early July, suggesting that infection occurs 1-2 wk earlier. This primary infection, which appears before the corn rootworm beetles emerge in mid-July, can account for most of the disease seen in the field. By the time most beetles emerge and can transmit, the corn is so large that symptoms may not result.

Corn rootworm beetles are active and migratory in their habits. During an epidemic CLN year, the large numbers of viruliferous insects available and moving out of the Republican River Valley make it nearly certain that the virus has been spread repeatedly by these insects to other areas. That the disease has not been observed outside of the original area probably reflects our inability to detect late, symptomless infections. However, even these plants could provide virus-containing residue if that were all that is necessary for overwintering. To date, overwintering occurs only in the originally described area. Apparently, other factors are involved in the overwintering of MCMV.

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