

## Artificial Inoculation of Maize White Line Mosaic Virus into Corn and Wheat

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### ABSTRACT

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Maize white line mosaic virus (MWLMV) was transmitted to healthy sweet corn (*Zea mays*) or wheat (*Triticum aestivum*) seeds by adding purified virus to the wound sites. The seedlings that germinated from these wounded seeds showed typical symptoms of white line mosaic disease. With this technique, MWLMV was demonstrated as the causal agent for this disease. Of the major cereal crops (corn, rice, barley, sorghum, oat, and wheat) tested with this technique, only corn and wheat were susceptible to MWLMV. Results were confirmed by using indirect ELISA and cDNA techniques. No difference in susceptibility to MWLMV

was found among 11 sweet corn cultivars with four different genes (sh2, su, se, and ae + du + wx) that encode sugary phenotypes. Young sweet corn seedlings were infected with MWLMV by hypocotyl wounding when inoculated at an early stage. Crude sap from plants infected with MWLMV and MWLMV-RNA also infected corn after embryo wounding. In addition, maize dwarf mosaic virus strains A and B, a cucumber mosaic virus strain, and maize subtle mosaic virus infected corn plants by the embryo-wounding technique. This technique can easily provide a continuous supply of diseased material for further studies.

A white line mosaic disease of sweet corn (*Zea mays* L. 'Saccharum') was first reported in central New York during the summer of 1979 (2) and was later found in Vermont (7), Wisconsin (8), and Ohio (10). The disease has also been reported in Maine, Massachusetts, Michigan, and New Hampshire (3,10).

Symptoms on sweet and Dent corn consist of small discrete chlorotic lines on leaves seen in early growth stages, followed by stunting and a severe mosaic and mottled appearance in latter stages. The discrete, short, chlorotic white lines (1–2 mm × 1–2 cm) primarily within veinal tissue are distinctive of this disease (2). No obvious symptoms or malformations have been reported on roots or flowers; however, both kernel and ear production are affected. Symptomless infection may also occur in natural conditions (11). Popcorn and ornamental Indian corn are also affected (T. A. Zitter, unpublished).

An isometric virus particle (35 nm in diameter) was recovered from disease tissue and called maize white line mosaic virus (MWLMV) (2,5). MWLMV consists of a single protein species of 32,000–37,000 MW and a single-stranded RNA of  $1.25 \times 10^6$  MW (2,5,6). Some smaller viruslike particles, about 17 nm in diameter, are often associated with MWLMV.

MWLMV is not transmissible to either corn or sorghum mechanically or by aphids or leafhoppers (2,5,10). Soil transmission was reduced by using benomyl applied as a drench, which suggests a fungal vector for this disease (10). This hypothesis is supported by the higher incidence of MWLMV on the edges of fields or in low-wet areas of a given field (2,10).

Identifying a vector has been difficult because inoculum for this disease could only come from naturally infected field plants or from the greenhouse-produced plants in naturally infested soil that contained a complex of potential soilborne vectors. Although the virus has always been associated with the disease, its pathogenic function was not demonstrated because of the inability to inoculate healthy corn plants with the virus and to produce characteristic disease symptoms. Hence, as pointed out by some researchers, MWLMV should be referred to as maize white line mosaic

associated viruslike particles (MWLMVLP) until its ability to produce maize white line mosaic disease is demonstrated (2,10).

In this paper, an embryo-wounding technique was used to demonstrate that MWLMV is the causal agent of the disease. The partial host range of MWLMV was also determined. Embryo wounding may offer a uniform method of assessing the inherent resistance or susceptibility of corn to viruses. A portion of this work has already been published (18).

### MATERIALS AND METHODS

**Preparation of virus inoculum.** Infected leaf tissue with typical maize white line mosaic symptoms was obtained from a local sweet corn field. Virus (ATCC PV489) was purified according to the procedure of de Zoeten et al (5). The virus yield was determined by using a spectrophotometer set at 254 nm. The purified virus was diluted to 5 mg/ml with 0.02 M sodium phosphate buffer, pH 7.0, before it was used as inoculum. Crude sap inoculum was prepared by grinding fresh leaf tissue with a (1:5, w/v) dilution of 0.02 M sodium phosphate buffer, pH 7.0. The sap was filtered through two layers of cheesecloth and used as inoculum. MWLMV-RNA was extracted by adding an equal volume of two times the virus extraction buffer A (200 mM Tris-Cl, pH 8.5, 1 M NaCl, 1% sodium dodecyl sulfate [SDS], 2 mM ethylene-diaminetetraacetic acid [EDTA]) to the purified virus preparation followed by two times extraction with phenol/chloroform (1:1), one time chloroform extraction (equal volume), and overnight ethanol precipitation at  $-20$  C. The MWLMV-RNA was pelleted by centrifugation at 10,000 rpm for 20 min at 4 C in a Beckman JA 14.1 rotor (Beckman Instruments, Inc., Fullerton, CA). The pellet was washed with 70% ethanol (cold), dried, and resuspended in deionized, distilled water. The concentration of the MWLMV-RNA preparation was determined and adjusted to 100  $\mu$ g/ml and then used as inoculum.

**Antiserum preparation.** Antiserum against MWLMV was prepared by injecting a New Zealand white rabbit intradermally and intramuscularly. The first injection was with 1 ml of purified virus (1 mg/ml) emulsified in Freund's complete adjuvant. Subsequent injections (1 mg of the purified virus mixed with

Freund's incomplete adjuvant) were made 10 times at 1 wk intervals. Serum titers were determined by bleeding the rabbit 2 wk after the last injection and using the ring-interface precipitin test with 1 g of purified virus in each tube.

**Procedure for virus detection.** Leaf and root tissue of inoculated plants were tested for infectivity by both indirect enzyme-linked immunosorbent assay (ELISA) and by a dot-blot complementary DNA (cDNA) hybridization technique. The indirect ELISA procedure described by Voller et al was followed (17). Tissues (0.5 g) were ground in a mortar with a pestle in 2 ml of sodium-carbonate buffer, pH 9.8, usually with the addition of liquid nitrogen. The juice was filtered through cheesecloth and dispensed into duplicate wells (200  $\mu$ l/well) of round-bottom microliter ELISA plates (Dynatech Laboratories, Inc., Alexandria, VA). Wells with healthy and infected tissue and coating buffer served as controls. The immunoglobulin G (IgG) concentration used was 1  $\mu$ g/ml and the conjugate concentration was a 1:1,000 dilution of the anti-rabbit IgG alkaline-phosphatase conjugate (Sigma Chemical Co., St. Louis, MO). The substrate was allowed to react for 20 min and then 50  $\mu$ l of 3 M NaOH was added to each well to stop the reaction. Absorbance values were measured at  $A_{405\text{nm}}$  with a Dynatech Microelisa AutoReader. Samples were considered positive if the absorbance was five times the mean value of the uninfected control.

The dot-blot hybridization procedure described by Palukaitis et al was followed (14). Plant tissue (0.1–0.2 g) for each sample was ground in AMESS extraction buffer (0.5 M sodium acetate [pH 6.0], 10 mM  $\text{MgCl}_2$ , 20% [v/v] ethanol, 3% [w/v] SDS, 1.0 M NaCl, and 0.05% [w/v] bromophenol blue) in 1.5-ml Eppendorf tubes and 20  $\mu$ l of extract was added to the wells of a "Minifold" filtration apparatus (Schleicher and Schuell, Keene, NH) with aspiration through a nitrocellulose membrane. Radiolabeled cDNA probes made from MWLMV-RNA were prepared by a procedure described by Palukaitis (13). About  $0.5 \times 10^6$  cpm of the  $^{32}\text{P}$ -MWLMV-cDNA probe was used for each dot-blot hybridization. After hybridization, the membranes were washed and autoradiographed as described by Palukaitis et al (15). Uninoculated corn plant tissue served as negative controls.

**Embryo-wounding technique.** Fifty Sunbeam or Silver Queen sweet corn seeds were soaked in 40 ml of tap water for 4 hr at room temperature and were dried on paper towels for the standard inoculation test. One drop (about 2  $\mu$ l) of the virus preparation was placed on the embryo side of each seed. Two longitudinal cuts, about 1 mm deep and directed to the mid-region of the embryo were made with a scalpel through the drop of virus preparation so that the virus entered directly through the wound. The inoculated seeds were planted in autoclaved greenhouse soil, with five seeds per 15-cm clay pot; they were kept in the greenhouse under natural light. Seeds that were embryo-wounded and inoculated with 0.02 M sodium-phosphate buffer (pH 7.0) served as controls. Seedlings were observed for symptom appearance over a 1-mo period before being discarded, although some seedlings (root and leaf tissue) were tested for virus recovery before this time.

In addition to purified virus, twenty Silver Queen seeds in one test were wound-inoculated with MWLMV-RNA at 100 ng/ $\mu$ l and two crude sap preparations. The crude sap inoculum was prepared by macerating either fresh or frozen ( $-20^\circ\text{C}$ ) leaf tissue in 0.02 M sodium-phosphate buffer, pH 7.0, (1:1, w/v) in a mortar with a pestle.

To determine which sites on a seed were susceptible to wound inoculation, 50 seeds of the cultivar Sunbeam were wound-inoculated on the scutellum, plumule, radicle, and endosperm by using purified virus (Fig. 1A). Wounds were about 1–2 mm in length. Seeds were planted as described and were observed for symptom development.

In addition to the inoculation of ungerminated seed, 2  $\mu$ l of purified MWLMV (5 mg/ml) was applied either to the hypocotyl or the primary root portions of young seedlings. Seedlings were allowed to germinate on Whatman filter paper for up to 5 days, and then were wounded with scalpel cuts similar to the embryo-wounding procedure (Fig. 1B). The inoculated seedlings were

transplanted to pots containing autoclaved greenhouse soil and were kept in the greenhouse under natural light for observation. The experiment was repeated.

Eleven sweet corn varieties with four different sugary gene types (su, sh2, se, and ae + du + wx) were selected and inoculated by embryo wounding to identify their relative susceptibility to MWLMV.

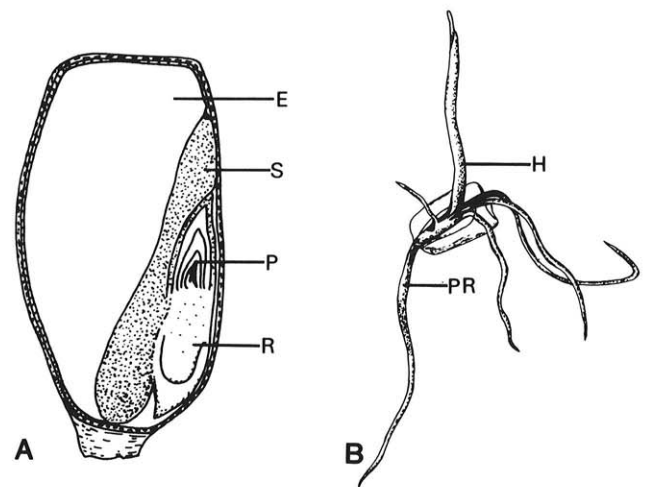
Rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), sorghum (*Sorghum bicolor* (L.) Moench), pearl millet (*Pennisetum glaucum* (L.) R. Br.), oat (*Avena sativa* L.) and barley (*Hordeum vulgare* L.) seeds were also inoculated with purified MWLMV by the embryo-wounding technique. Twenty seeds of each crop were inoculated and the experiment was repeated.

**Inoculation with other viruses.** Silver Queen seeds were inoculated with additional viruses by embryo wounding: maize subtle mosaic virus (MSMV) (12), maize dwarf mosaic virus strain A and B (MDMV-A, -B), and a muskmelon- and squash-infecting strain of cucumber mosaic virus (CMV-FNY) (1). The inocula in all cases were crude sap preparations. Infections were confirmed by indirect ELISA except with seedlings infected with CMV, which were assayed by cDNA hybridization as previously described.

## RESULTS

**Embryo-wounding and inoculation techniques.** Embryo-wounded corn seed usually emerged and showed typical MWLMV disease symptoms on leaves as soon as they expanded (approximately 7–10 days). The percentage of infection varied among experiments due to how carefully the technique was performed and the number of seeds that germinated after inoculation. Seedlings often appeared misshapen but displayed typical maize white line mosaic symptoms. ELISA and cDNA tests confirmed the recovery of MWLMV from leaf and root tissue of plants with symptoms. In no instance (in over 100 attempts) was virus detected in asymptomatic plants. This included the failure to recover virus from 48 leaf and root tissue samples taken from 3- to 4-wk-old seedlings from different trials of asymptomatic seedlings grown in the same pots as seeds successfully inoculated by embryo wounding. Positive ELISA values for leaf and root tissue ranged from 0.22–0.88 (av. 0.67) compared with a 0.93 value for diseased control tissue and 0.03 for healthy control tissue. Results of a typical dot-blot assay of root and leaf tissues are shown in Figure 2.

The effect of wounding sites on the percentage of transmission



**Fig. 1.** Diagrams of ungerminated and germinated seeds of corn, illustrating how the embryo-wounding technique was used to inoculate seed and germinated seedlings. **A**, Two longitudinal cuts were made at a single site either on the endosperm (E), scutellum (S), plumule (P), or radicle (R) of each seed. **B**, Diagrammatic drawing of young sweet corn seedlings that shows the wounding site near the meristematic region of the hypocotyl (H) or on the primary root (PR). The twin cauline "wedging" roots appear near the top of the seed.

of MWLMV is shown in Table 1. The highest infection levels occurred when the plumule and radicle were inoculated. Plants never became infected when the endosperm was inoculated.

To better define the conditions that would produce consistent transmission, the presoaking time was varied for 0, 0.5, 1, 2, 4, 8, 12, and 24 hr before inoculation of Sunbeam seed. The highest infection rate (28%) was obtained with a 4-hr presoaking interval, and this procedure was followed in all subsequent tests. Another variable tested was the length of time between inoculation of seeds and planting. Fifty Sunbeam seeds for each time interval tested (0, 1, 2, 4, 8, 16, 32, 48, and 64 days) were presoaked for 4 hr in water, embryo-wound inoculated, and held in paper bags on a laboratory bench at 21 C before planting. Our results showed that the virus could remain infectious for as long as 64 days when inoculated seeds were maintained in a dry state. The percentage of infected plants ranged from 11 to 81% (av. 41%) over the 64-day period.

In addition to using purified virus as inoculum, seeds were successfully wound-inoculated with MWLMV-RNA and with the two crude sap preparations. Infection levels were similar with the RNA and frozen crude sap inocula (27 and 22%, respectively), but were lower with freshly prepared crude sap (5.5%).

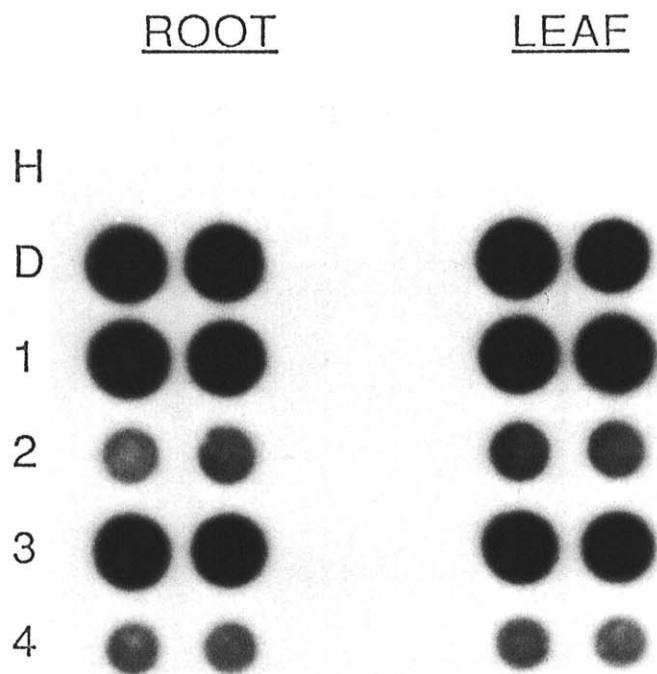


Fig. 2. Autoradiograph of cDNA hybridization assay of maize tissue. Extracts of healthy (H) and diseased (D) control tissue were loaded into the top two wells of the dot-blot apparatus. Extracts of MWLMV-diseased root and leaf tissue were loaded into the bottom four wells. Each paired spot represents a sample from a different plant infected for 3 wk.

TABLE 1. Effect of inoculating different wound sites on seeds of Sunbeam sweet corn with maize white line mosaic virus<sup>a</sup>

Site of wounding	Germination		Diseased	
	Number	%	Number	%
Scutellum	40	80	1	2.5
Plumule	39	78	11	28.2
Radicle	44	88	12	27.0
Endosperm	43	86	0	0

<sup>a</sup>Seeds were inoculated by making two longitudinal cuts (about 1–2 mm) through a 2  $\mu$ l drop of purified virus. A total of 50 seeds was used for each site of wounding.

Transmission occurred when the hypocotyl region of 1- and 3-day-old seedlings (attaining a length of 2 mm and 5 cm, respectively) were wound-inoculated. The percentage of infection decreased from 42% at 0 time, to 11% at day 1, and 3% at day 3. No infection occurred when 4-day-old seedlings were wound-inoculated (10 cm length). In all cases, wound-inoculation of primary roots of young seedlings with MWLMV produced no infections as confirmed by ELISA and dot-blot tests.

**Inoculation of sweet corn cultivars and other cereal crops.** Eleven cultivars of sweet corn reacted positively to the embryo-wounding technique, showing typical maize white line mosaic disease symptoms (Table 2). Sunbeam and Silver Queen (both su sugary types) produced the largest number of infected seedlings. The percentage of seedlings infected varied from 5 to 27% among the other sugary types tested. Among the wounded cereal crop seeds tested, only corn and wheat were infected by MWLMV. The symptoms on wheat infected with MWLMV showed a mild mosaic, but no necrotic lesions appeared as they did on corn.

**Inoculation with other viruses.** Corn seeds were successfully infected with MSMV, MDMV-A, and -B, and CMV-FNY by the embryo-wounding technique. The efficiency of infection varied between two trials conducted with MDMV-A and -B (av. 17% infection).

## DISCUSSION

Our work showed that MWLMV can be transmitted by wounding and inoculating the seed embryo of its host plants. By this technique, we have demonstrated that MWLMV can induce typical white line mosaic symptoms in sweet corn plants and hence is responsible for maize white line mosaic disease. By means of this technique, we established that wheat is also susceptible, confirming an earlier report of natural field infection of wheat by MWLMV (8). No other cereal crops tested by the embryo-wounding technique were infected.

A clearer understanding of corn seed anatomy and germination provided in a 1905 study by Sargent and Robertson (16) can help to explain the successful infection of corn by wounding of the plumule, radicle, and, to a lesser extent, the scutellum regions. They reported that the coleoptile or stem-sheath encloses the plumule and is stiffened with a vascular hurdle on either side, while the root-sheath or coleorhiza covers the radicle until the primary root emerges. Wounding in each of these areas would allow early systemic virus movement through the interconnecting

TABLE 2. Effect of embryo-wounding of 11 sweet corn cultivars with varying sugary phenotypes on infection with maize white line mosaic virus<sup>a</sup>

Cultivar	Sugary gene type <sup>b</sup>	Germination		Infected	
		Number	%	Number	%
Silver Queen	su	46	92	16	35
Sweet Sue	su	41	82	5	12
Sunbeam	su	36	72	14	39
Sprite	su	30	60	8	27
Zenith	sh2	43	86	2	5
Hawaiian Super Sweet #9	sh2	30	60	3	10
Landmark	sh2	32	64	5	16
Seneca Sentry	se	34	68	7	21
Snowbelle	su + se	23	46	3	13
Spring Calico	su + se	36	72	8	22
Pennfresh ADX	ae + du + wx	24	48	2	8
Sunbeam (uninoculated)	su	37	74	0	0

<sup>a</sup>Fifty seeds of each cultivar were presoaked for 4 hr in 40 ml of tap water before inoculation. Cultivars were tested once and were inoculated on the same date.

<sup>b</sup>Genes controlling sweetness in different sweet corn types: su = sugary (homozygous, also called standard or normal); sh2 = shrunken-2 (homozygous, also called supersweet or ultra or extra sweet); se = sugary enhanced (also called modified sugary and EH, with half or full complement of se and a full complement of su); ADX has a full complement of ae (amylose extender), du (dull), and wx (waxy).



vascular tissue. The scutellum is described by Sargant and Robertson as a "sucking" organ that provides food to the embryo from supplies present in its own tissue and from the endosperm during a gradual dissolving process (16). The vascular system in the upper scutellum consists of a narrow, slender bundle with numerous branches. The bundles of the scutellum merge on the stele of the axis at the main vascular junction, providing connection with the plumule (hypocotyl), radicle (primary root), and the twin-cauline "wedging" roots (Fig. 1B). Wounding of the scutellum would account for another method of virus uptake and systemic movement, although less efficiently as indicated by our results (Table 1).

After inoculation, MWLMV remained infectious in dried seed for up to 64 days in our experiment. This implied that once the virus was placed in a suitable site within the embryo, it remained stable until germination occurred. Although these results may suggest a possible role for seed transmission, Louie et al suggested that if seed transmission does occur, it must be at an extremely low rate (less than 0.01%) (10). In our attempts to demonstrate seed transmission, we found no indication after examining several thousand seedlings derived from seed harvested from infected plants that seed transmission had occurred (T. A. Zitter, unpublished data). Even after germination, the embryo-wounding technique still resulted in a systemically infected plant, as long as the hypocotyl is inoculated and has not exceeded about 5 cm in length. No infected plants resulted from inoculation of the primary roots of young seedlings. Nor were any infections detected by ELISA or cDNA hybridization when root or leaf tissue from healthy-appearing seedlings were tested. Our results indicate that for seedlings to be artificially infected, virus must be introduced before germination or during early radicle and hypocotyl development, and that passive root infection does not occur. Studies by Louie et al demonstrated that transmission can occur when MWLMV-infected roots that originated in infested soil are placed directly on seedling roots or within 1–2 cm of the test seedlings (9). Although we were unable to demonstrate symptomless infection by embryo wounding, this phenomenon is known to occur with natural infections (11).

We have observed that sweet corn cultivars may differ greatly in their susceptibility to MWLMV under natural field infection, and that this may depend to some extent if they are early maturing cultivars or if they produce weakened seedlings (19). This was especially true among certain sugary phenotypes, including those with the mutant endosperm gene, *shrunken-2* (*sh2*). Germination of these cultivars is delayed and root growth is suppressed in cold, wet soils (4). These conditions would favor infection if a soilborne method of natural transmission applies, and if the weakened root system is more vulnerable to infection for an extended period of time (19). It was not surprising to find that there was no apparent difference in susceptibility to MWLMV among eleven sweet corn cultivars with differing genes that encode sugary phenotypes when the artificial embryo-wounding technique was used. Although we experienced wide variation in the percentage of infected seedlings obtained, this can be explained by the physical appearance of the hybrid seeds tested (Table 2). Many of the seeds, especially *shrunken-2* hybrids, are very small, wrinkled, and irregularly indented on the embryo side, making it difficult to achieve uniform wounding. These results suggested that the differences observed in the field under natural conditions of infection were due to differences in susceptibility of the cultivars to the vector of MWLMV.

Evidence to support this conclusion is provided by examining the effects of embryo wounding on two inbred Dent corn lines. In 1982, a large (8-ha) commercial Dent corn production field in central New York was surveyed for MWLMV and showed an average of 2% infection based on visual symptoms. A plot, 9.1 m × 30.1 m, was studied in detail; it included 14 pollinator rows and six seed parent rows that showed an average of 15% of visible symptoms in the seed parent plants. No symptoms appeared in the pollinator rows. ELISA tests performed on leaf and root tissue from the pollinator plants on two occasions were

negative, while high readings were obtained when similar tests were performed with parent plants. However, we found that both pollinator and seed parent plants were equally susceptible to MWLMV when inoculated by embryo wounding.

The wounding technique has allowed us to pursue other studies on MWLMV. We have demonstrated the satellite nature of the viruslike particles associated with MWLMV (SV-MWLMV). We have also established a testing system for some possible fungal vectors of MWLMV. Besides obtaining infection of corn with MWLMV by the embryo-wounding technique, we successfully transmitted other mechanically transmitted corn viruses. These results suggest that this technique would allow for transmission of viruses and strains that prove difficult to transmit by conventional means, and could be a useful method for studying other virus-host interactions.

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