Vascular Puncture of Maize Kernels for the Mechanical Transmission of Maize White Line Mosaic Virus and Other Viruses of Maize

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ABSTRACT

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Maize white line mosaic virus (MWLMV) was efficiently transmitted (>90%) by a vascular puncture method with insect pins to maize kernels without causing teratogenic or lethal injuries to the developing seedlings. The pins, soldered to a 10-gauge copper wire, were used to puncture vascular tissues of maize kernels. The copper wire and pins were mounted in an engraving tool for machine-assisted inoculation. Effects of preinoculation soaking of kernels in water; postinoculation moisture regimes; the size, number, and configuration of pins; and the site and frequency of inoculation on rates of transmission were evaluated. Transmission rates of MWLMV were highest (average 32%) when the preinoculation soaking of kernels was done at 4 C. In contrast, transmission rates were significantly reduced when the preinoculation soaking of kernels was done at 30 C. The average rates of transmission to inoculated kernels incubated at 30 C on paper towels moistened with either 50 or 150 ml of water in a 2-L Pyrex dish for 24 h or directly planted into soil were 42, 20, and 11% ($P > 0.003$; LSD = 14.5%), respectively. Inoculations near the side of the embryo averaged 58% transmission whereas inoculations near the tip of the plumule averaged 33% transmission ($P > 0.0001$). Subjecting kernels to one, two, or three preinoculation soak (24-h) and dry (20- to 24-h) cycles or a 4- or 20-h preinoculation soak period resulted in transmission rates of 95, 90, 87, 80, and 14% ($P > 0.0001$), respectively. The optimum conditions for MWLMV transmission were 1) the use of a machine-assisted inoculator with minuten pins positioned like the tines of a fork, 2) preinoculation soaking of the kernels at 21 C for 4 h, 3) inoculation of kernels near the side of an embryo, and 4) incubation of kernels at 30 C postinoculation for 24 h on paper towels moistened with 50 ml of water. The ranges of transmission rates with this protocol for the following maize viruses were maize chlorotic dwarf waikavirus, 1–34%; maize dwarf mosaic potyvirus (strain A), 41–82%; maize mosaic rhabdovirus, 1–19%; maize rayado fino marafivirus, 1–25%; maize rough dwarf fijivirus (maize Rio Cuarto disease), 1%; maize streak geminivirus, 1–5%; maize subtle mosaic virus, 12–48%; and wheat streak mosaic potyvirus, 3–55%.

The best characterized plant viruses are those that are amenable to mechanical transmissions (25). Hence, any new method that allows the transmission of previously nonmechanically transmitted viruses is of great interest. Early attempts at mechanical transmission of plant viruses followed methods used in the medical profession or mimicked the probing of insects. Undoubtedly, rub inoculation is presently the most widely used method for virus transmission. It probably was first used by Reddick and Stewart (19) and was greatly improved by the addition of silicon carbide to the inoculum (18). Subsequently, improved methods of mechanical transmission involved more sophisticated equipment such as air brushes (6,7), solid-stream inoculators (10,13), electro-endosmosis (17), microinoculation (5), and particle bombardment (20). Recently, an embryo-wounding technique involving two longitudinal cuts in a maize (*Zea mays* L.) embryo with a scalpel successfully transmitted maize white line mosaic virus (MWLMV) (26).

In this study, a vascular puncture method with insect pins was developed to improve rates of transmission of MWLMV and to do it without causing teratogenic or lethal injuries to maize seedlings (26). Effects of preinoculation soaking of kernels in water; postinoculation moisture regimes; inoculation variables including size, number, and configuration of pins; use of a hand-held or machine-assisted inoculator; and site and frequency of inoculation on rate of transmission were evaluated. Symptom development in plants that were inoculated by either rub inoculation or the vascular puncture method also were evaluated. The vascular puncture method also was tested for transmission of maize chlorotic dwarf waikavirus (MCDV), maize dwarf mosaic potyvirus (strain A) (MDMV-A), maize mosaic rhabdovirus (MMV), maize rayado fino marafivirus (MRFV), maize rough dwarf fijivirus, also known as maize Rio Cuarto disease (MRDV [MRCD]), maize streak geminivirus (MSV), maize subtle mosaic virus (MSMV), and wheat streak mosaic potyvirus (WSMV). A preliminary account of this study has been published (8).

MATERIALS AND METHODS

Test plants. Test plants were cultivar Seneca Chief sweet corn (*Z. mays* L. var. *saccharata* (Sturtev.) L. H. Bailey). In the standard germination procedure, large, flat kernels (about 100 kernels in 200 ml of tap water) were soaked for 4 h at 21 C before inoculation treatments. Kernels were incubated postinoculation at 30 C for 24 h on eight layers of paper towel moistened with 50 ml of water in a 2-L Pyrex dish (28 × 18 × 4 cm) (Corning Glass

Works, Corning, NY) covered with clear plastic wrap. After incubation, five kernels were planted in each of 20 10-cm pots containing autoclaved greenhouse soil; the planting usually resulted in a 90% stand for each treatment. Noninoculated kernels similarly planted also resulted in 90% stands. Each pot of soil was supplemented with about 3 g of 14-14-14 (N-P-K) slow-release fertilizer. A systemic insecticide (*O,O*-diethyl s-2-ethylthioethyl phosphorodithioate, about 1.5 g per pot) also was incorporated into the soil for kernels inoculated with MSV or MRDV (MRCD). Plants were grown in a greenhouse maintained at 26–32 C and supplemented with 15 h of light (750 μE s^{-1} m^{-2} at 30 cm above bench level) from metal halide fixtures. MRDV (MRCD)-inoculated plants were transplanted from pots after 13–34 days into soil beds and then examined for symptoms.

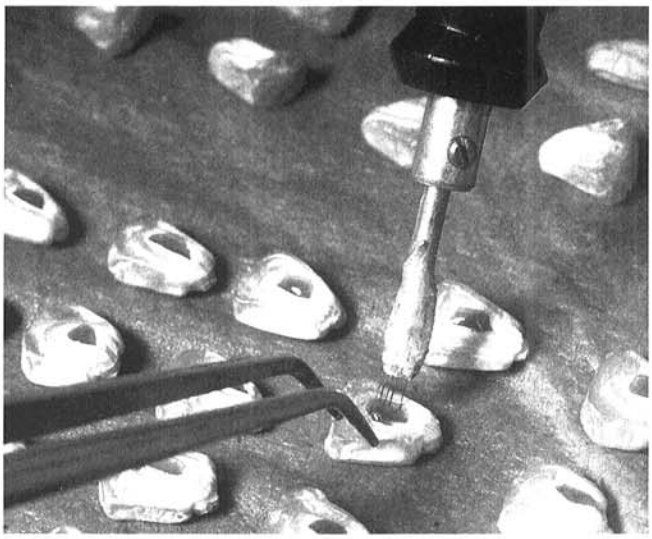

Fig. 1. Minuten pins positioned like the tines of a fork mounted in a machine-assisted inoculator. The inoculating pins were held at a 45° angle from the surface of the kernel to puncture the vascular tissues without injuring the embryo.

Inoculum preparation. Fresh inocula of MCDV, MDMV-A, MMV, MRFV, MRDV (MRCD), MSMV, MWLMV, and WSMV were prepared from infected leaves or roots (11- to 61-day-old plants) that were ground with a pestle in a mortar containing 0.01 M potassium phosphate buffer (1:5, w/v) at pH 7.0. The extracts were then squeezed through two layers of cheesecloth before use. Purified virions of MWLMV and MCDV were prepared as previously reported (3,9). A 2- to 6-μl drop of inoculum was placed on the inoculation site. MSV inoculum originally was prepared from infected 3-yr-old lyophilized leaves, but subsequent fresh inocula were prepared from infected leaves of 13- to 70-day-old plants.

Pin preparation. A single insect pin or three or five insect pins (no. 00, no. 0, no. 1, and 0.15-mm-diameter minutens) bundled together formed an inoculating point. The pins were placed on the flattened end of a 10-gauge copper wire (4 cm long); 5 mm of the pointed ends extended beyond the copper wire. The overlapping ends were wrapped together with 30-gauge wire and then soldered. Minuten pins also were configured on a 10-gauge copper wire as five pins laid side by side like the tines of a fork. The pins on the copper wire were mounted on either a wooden dowel for hand inoculation or in an engraving tool (catalog number 11-111, Ideal Industries Inc., Sycamore, IL) for machine-assisted inoculation (Fig. 1). The power stroke adjustment on the engraving tool was set at midpoint. To ensure that the vascular tissue would be inoculated, the location of the vascular tissue beneath maize embryos as described by Sargant and Robertson (21) was confirmed by observations of dissected kernels. For inoculation, the pins were held at a 45° angle from the surface of the kernel adjacent to the embryo and pushed through the inoculum and the pericarp covering the scutellum to a depth of 0.5–1 mm.

Experimental treatments, design, analysis, and infectivity assays. Wide variations in rates of transmission of MWLMV in early experiments appeared related to conditions used for kernel germination as well as for inoculations. Hence, the effects of water temperature and the duration of soaking the kernels before inoculation; different water regimes during incubation at postinocula-

TABLE 1. Variables tested in transmission experiments of maize white line mosaic virus

	Preinoculation variables		Inoculation variables			Postinoculation variables	
Experiment	Soak duration	Water temperature (C)	Pin size, number, and configuration	Inoculation site	Inoculator	Incubation duration and temperature	Amount of water (ml)
1	0, 4, 8, 12, 16, 20, or 24 h	4.5, 21, or 30	no. 00, five pins bundled together	Plumule end	Hand held	24 h at 30 C or direct planting	50
2	4 h	21	no. 00, five pins bundled together	Plumule end	Hand held	24 h at 30 C or direct planting	50 or 150
3	4 h	21	nos. 00, 0, 1 or minuten; one, three, or five pins bundled together	Plumule end or side of embryo	Hand held or machine assisted	24 h at 30 C	50
4	4 h	21	Minuten, five tines	One or two sides of embryo; inoculated once or twice per side	Machine assisted	24 h at 30 C	50
5	Cycle 1 = 24-h soak, 20- to 24-h dry; cycle 2 = cycle 1 repeated twice; and cycle 3 = cycle 1 repeated three times. All three cycles also included a 4-h soak before inoculation. Cycle 0 = 4-h soak only. Control = 24-h soak only.	21	Minuten, five tines	Side of embryo	Machine assisted	24 h at 30 C	50

tion; inoculation site; frequency of inoculation; size, number, and configuration of pins; and use of a hand-held or machine-assisted inoculator for transmission were tested. Five experiments, each repeated three times, were conducted to test these preinoculation, inoculation, and postinoculation variables (Table 1). In each experiment except the third, 100 kernels per treatment were inoculated. Preinoculation variables were tested in experiments 1 and 5. Inoculation variables were tested in experiments 3 and 4. Postinoculation variables were tested in experiments 1 and 2. The effectiveness of any changes in the variables was judged against the transmission rates in the earlier experiments (R. Louie, *unpublished data*) in which the protocol included a 4-h preinoculation soaking of kernels at 21 C, a hand-held inoculator with five no. 00 pins bundled together to puncture the plumule end of a kernel, and the direct planting of the kernels into soil at postinoculation.

In the first experiment, kernels were soaked in tap water for 0, 4, 8, 12, 16, 20, or 24 h at either 4.5, 21, or 30 C and then inoculated. In the second, kernels were treated with 4 h of preinoculation soaking at 21 C, inoculated, and then incubated at 30 C on paper towels moistened with either 50 or 150 ml of water in a 2-L Pyrex dish for 24 h before planting. Both experiments also included kernels that were treated with a 4-h preinoculation soaking at 21 C followed by postinoculation direct planting into soil.

A factorial experiment (experiment 3, Table 1) was conducted to test the effect of pin sizes with various combinations of inoculators and inoculation sites for virus transmission. There were 50 kernels for each treatment of either one, three, or five pins of the four different sizes and bundled together to inoculate either near the tip of the plumule or near the side and midway along the embryo with either the hand-held or machine-assisted inoculator. Pin size was the main block. The pin size and hand-held or machine-assisted inoculator (method); the pin size, method, and number of pins; and the pin size, method, number of pins, and inoculation site were split, split-split, and split-split-split blocks, respectively.

Because minuten pins mounted singly instead of bundled together appeared to cause less injury to the kernel and because five minuten pins positioned like tines of a fork were more efficient than a single pin, an experiment (experiment 4, Table 1) on the effect of the frequency of inoculation on transmission was conducted with the machine-assisted inoculator and minuten pins positioned like tines of a fork. Treatments consisted of one or two inoculation punctures on one side or one inoculation puncture on both sides midway along the embryo.

In the fifth experiment, kernels were subjected to one, two, or three cycles of preinoculation soaking (24 h) and drying (20–24 h) at 21 C followed by a 4-h soak before inoculation; or kernels were soaked for only a 4-h period (i.e., 0 cycle) or soaked for only a 20-h period before inoculation. They were then inoculated in the midregion parallel to the embryo with five minuten pins in a fork configuration.

Controls were kernels mock inoculated with buffer with the hand-held inoculator or inoculated with virus with the hand-held or machine-assisted inoculator. After inoculation, the kernels in the control treatments were directly planted (i.e., without incubation at 30 C) into soil. Symptom evaluations began 4–5 days after inoculation and continued for another 17–23 days, but evaluations for MRDV (MRCD) symptoms continued for 8–11 wk.

Confirmations of virus transmission were based on enzyme-linked immunosorbent assays (ELISA), bioassays, *Graminella nigrifrons* (Forbes) transmission of MCDV, and symptomatology. ELISA tests (2,9) of plants infected with each of the nine viruses were performed, and a sample was judged positive when its absorbance was greater than the mean of the healthy control plus three times its standard deviation. Transmission of MCDV from infected plants to inbred Oh28 by *G. nigrifrons* resulting in diagnostic veinbanding symptoms constituted a positive recovery of virus (14). Rub inoculation with sap from plants inoculated by vascular puncture with MDMV-A, MSMV, and WSMV of inbred Oh28 maize, Monon wheat (*Triticum aestivum* L.), and Atlas sorghum (*Sorghum bicolor* (L.) Moench) resulting in symptoms typical of the respective virus on the differential host also constituted successful virus recovery (15,24).

When the data (percentages based on the number of germinated kernels) were clustered at the extremes, they were first transformed by the arcsine square root transformation and then analyzed by an analysis of variance. The means were separated by the least significant difference (LSD) at $P = 0.05$. The results reported are nontransformed, and only in analyses with nontransformed data are LSDs presented.

RESULTS

Transmission of MWLMV by vascular puncture. In the first experiment, transmission of MWLMV was significantly greater in plants from kernels treated with a 12- to 24-h preinoculation soak at 4 C than in those treated at either 21 or 30 C (Fig. 2). Transmission rates of MWLMV from kernels soaked at 4 C were generally unaffected throughout the test period, but transmission rates of MWLMV from kernels soaked at 21 and 30 C were significantly reduced after the 12- and 8-h treatments, respectively. Regardless of the temperature treatment, rates of transmission were most variable when kernels were soaked for 4 h, inoculated, and then directly planted (i.e., without an incubation period at 30 C) into soil.

Postinoculation moisture regime also significantly affected the rate of MWLMV transmission (experiment 2, Table 1). Average rates of transmission were 42, 20, and 11% for kernels incubated at 30 C on paper towel moistened with either 50 or 150 ml of water or directly planted in the soil, respectively ($P > 0.003$; LSD = 14.5%).

In the factorial experiment (experiment 3, Table 1), transmission rates of MWLMV for a particular site of inoculation were significantly affected by whether the inoculator tool was hand held or machine assisted. Machine-assisted inoculation was significantly more efficient. The average rates of MWLMV transmission, for all pin combinations, were 33 and 58% for the tip of the plumule and side of the embryo, respectively, when inoculation was machine assisted ($P > 0.0001$) (Fig. 3). On the other hand, when the hand-held inoculator was used, the average transmission rates for a particular inoculation site were not significantly different: 17 and 13%, respectively. In addition, the effects of pin size and the number of pins bundled together to form the inoculating

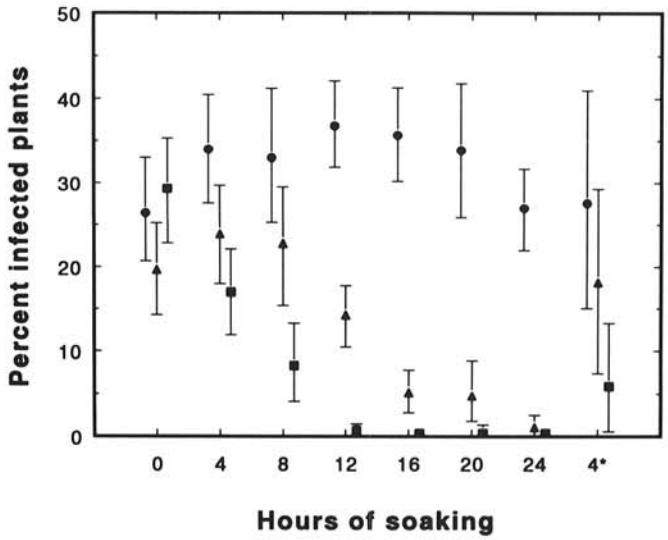

Fig. 2. Effect of preinoculation water temperature and soaking duration on transmission of maize white line mosaic virus. Water temperatures for the preinoculation soak were 4.5 C (●), 21 C (▲), and 30 C (■). Inoculated kernels planted into soil without a 24-h incubation period at 30 C are indicated by an asterisk. Pooled means (the vertical bars represent ±1 least significant difference) from three experiments are shown.

point for both the inoculator and inoculation site combinations also were not significantly different. The average transmission rates for one, three, and five pins, respectively, were for pin size no. 00, 32, 29, and 23%; no. 0, 32, 34, and 29%; no. 1, 34, 33, and 26%; and minutens, 32, 30, and 26%.

The effect of frequency of inoculation on MWLMV transmission was tested by using five minuten pins positioned like the tines of a fork in a machine-assisted inoculator (experiment 4, Table 1). Results of treatments consisting of inoculation twice on one side and once on both sides were significantly different (73 versus 94%; $P > 0.04$). However, results of inoculation either once on one side of the embryo or once on both sides of the embryo were not significantly different (85 versus 94%). This was also true for inoculations done once on one side or twice on one side (85 versus 73%).

Subjecting kernels, prior to machine-assisted inoculation, to one, two, three, or zero preinoculation soak and dry cycles or a 20-h preinoculation soak period resulted in 95, 90, 87, 80, and 14% transmission, respectively (experiment 5, Table 1). All treatments were significantly different from the 20-h soak treatment ($P > 0.0001$). Although the percent transmission from one cycle of soaking and drying was significantly different from the percentages in the three and zero cycles and the percent transmission from zero cycle was significantly different from the percentages in the one and two cycles, the differences were marginal. Differences between the two and three cycles were not significant.

ELISA and infectivity assays. The presence of each of the nine viruses in infected plants grown from inoculated kernels was confirmed by ELISA. MCDV was also confirmed by using *G. nigrifrons* to acquire and transmit the virus from infected plants resulting from inoculation of maize kernels. Bioassays of plants from kernels inoculated with MDMV-A, MSMV, and WSMV resulting in typical viral symptoms of each respective virus on differential hosts confirmed transmission by the vascular puncture method. No contamination was detected in any of the control plants from mock-inoculated kernels.

Transmission of other maize viruses. The ranges of transmission rates (percentage followed by number of successful trials, each based on inoculations of 100 kernels) obtained with the vascular puncture method for the following viruses were MCDV, 1–34%, 48; MDMV-A, 41–82%, 20; MMV, 1–19%, 13; MRFV, 1–25%, 14; MRDV (MRCD), 1%, 2; MSMV, 12–48%, 5; MSV, 1–5%, 7; and WSMV, 3–55%, 11.

DISCUSSION

MWLMV was transmitted consistently (>90%) by the vascular puncture method. This efficiency was achieved by using a machine-assisted inoculator with pins positioned like the tines of a fork,

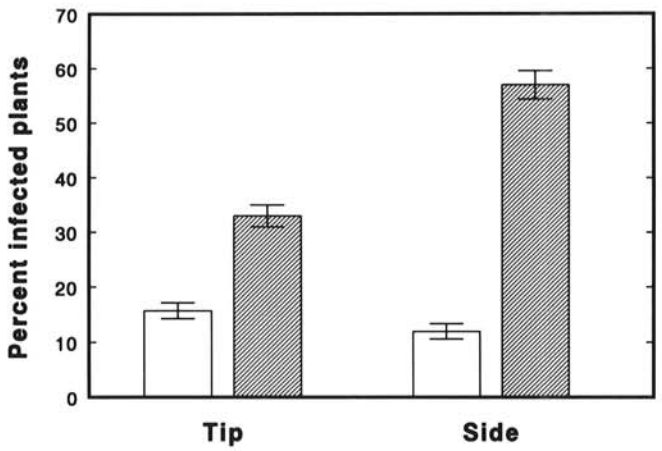

Fig. 3. Effects of using a hand-held (open bar) or machine-assisted (hatched bar) inoculator and of inoculating either the area at the apex of the plumule (Tip) or the side of the midregion of the embryo (Side) on transmission of maize white line mosaic virus. Pooled means (the vertical bars represent ±1 standard error) from three experiments are shown.

kernels that were first given a preinoculation soaking at 21 C for 4 h, and a postinoculation incubation period at 30 C for 24 h on paper towels moistened with 50 ml of water. During inoculation, the pins were held at a 45° angle from the surface of the kernel adjacent to the embryo (in the midregion) and pushed through (once or twice) the inoculum and the pericarp. The presumed site of inoculation is the vascular tissue because of where the pins were directed during inoculation and because attempts to transmit MWLMV by similarly puncturing the endosperm, scutellum, or embryo resulted in little or no transmission of MWLMV (26; R. Louie, *unpublished data*). The vascular puncture method had high rates of transmission but did not cause teratogenic or lethal injuries to the seedling. Apparently, puncturing the vascular tissues in the kernel does not injure the embryo as does the cutting of the embryo with a scalpel in the embryo-wounding technique (26).

Variables affecting the kernel physiology at pre- and postinoculation significantly affected the transmission rates of MWLMV. Imbibition of water is an important variable because it starts many processes in the quiescent kernel. Foremost, it initiates enzymatic activation, breakdown, translocation, and use of storage materials; it also causes leakage of solutes and changes in membrane structure (12,22). Cell damage associated with water imbibition can be extensive (1,11) and may be enough to preclude virus infection. However, the continual transmission of MWLMV to seedlings of inoculated maize kernels after as many as three soaking and drying cycles (as long as the last soaking duration was not longer than 4 h at 21 C) and after soaking for as long as 24 h at 4 C suggests otherwise. Still, treatment of kernels with prolonged preinoculation imbibition periods (e.g., for 12 and 8 h at 21 and 30 C, respectively) or with excessive water moisture at postinoculation (e.g., 150 ml of water during incubation at 30 C or direct planting into soil) reduced the rates of MWLMV transmission. As previously suggested (26), it seems likely that the processes of germination and growth beyond some point affect MWLMV transmission rates. Kernel size and shape and the amount of water imbibed were highly variable. They affected the ease of inoculation but, apparently, not the transmission rate. Transmission rates also were not obviously affected by factors affecting MWLMV titer and stability (R. Louie, *unpublished data*).

Of the variables tested by inoculation, pin number and pin size did not result in significant differences. However, the frequency of accidental injuries to the first leaf was nil with the use of minuten pins, and the small diameter of the minuten pin also gave better control to access the vascular tissues. Transmission with the machine-assisted inoculator was significantly higher than with the hand-held inoculator, and this difference could explain in part why pin inoculation was ineffective in previous studies. In contrast to the embryo-wounding technique (26), this method showed a significant increase in transmission when the inoculation site was near the side of the embryo compared with inoculation near the tip of the plumule. Most likely, this increase was related to the ease of puncturing the vascular tissue when inoculation was from the side of the embryo compared with inoculation from the plumule end.

For transmission of MWLMV, the importance of increasing the number of nonlethal wounds in the vascular tissue appeared subordinate only to the variables that affected the process of germination. As previously mentioned, the most suitable combination of variables tested to cause this type of injury was the machine-assisted inoculator with five minuten pins positioned like the tines of a fork to puncture the kernel in the midregion parallel to the embryo. This combination produced the first confirmed repeated mechanical transmissions of MCDV, MMV, MRFV, and MSV. However, this combination did not necessarily ensure that the transmission of these viruses would be as efficient as that for MWLMV. Thus, as with any other technique, transmission variables must be optimized for each virus-host combination.

Although widely used in earlier studies with plant viruses, pin inoculation for mechanical transmission of plant viruses has been mostly replaced by rub inoculation. Pin inoculation was used

for transmission of MRDV to three of 12 plants by puncturing the stem of young corn seedlings (4). S. Lenardon and A. Marinelii (*personal communication*) also confirmed transmission of MRDV (MRCD) (1%) with this vascular puncture method. However, only when minuten pins positioned like the tines of a fork and a machine-assisted inoculator directed at the vascular tissues in the kernel were used, as in this study for MWLMV, was a high level of transmission achieved.

Maize kernels are ideally suited to vascular puncture inoculation. The anatomy and morphological development of the embryo are well described (16,21,23); the location of the embryo is easily determined; and many kernels are large and flat. Furthermore, the time required for seedling emergence can be as short as 4–5 days after inoculation. Symptoms usually occur on the first emerging leaf of a plant infected with MDMV, MWLMV, or WSMV. However, with MCDV, MMV, MRFV, MSV, and MSMV, they often occur on the second or third emerging leaf. For MRDV (MRCD) infections, symptoms are observed only on leaves above the ear. Finally, virus symptoms that develop in maize after vascular puncture inoculations are typical of natural infections.

The sweet corn cultivar Seneca Chief is not susceptible to WSMV by rub inoculation, but it was susceptible to WSMV by the vascular puncture method. Systemic infection with MDMV-A does not occur in inbred Pa405 by rub inoculation, but it did occur by vascular puncture inoculation (R. Louie, *unpublished data*). In addition to the capability of transmitting previously vector-limited maize viruses that have different vector relationships, particle morphologies, and nucleic acid compositions, these observations suggest that the vascular puncture method may be used to study the mechanisms for virus resistance in different maize genotypes or resistance to systemic infection. On a broader scale, this method might be adapted to other pathogen-host systems in which the pathogen has been previously transmitted only by a vector.

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