

## Determination of Host Resistance to Beetle Transmission of Plant Viruses

R. C. Gergerich, H. A. Scott, and S. L. Wickizer

Department of Plant Pathology, University of Arkansas, Fayetteville 72701.

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## ABSTRACT

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Two comoviruses, cowpea mosaic virus (CPMV) and cowpea severe mosaic virus (CPSMV), were easily mechanically transmissible to *Vigna unguiculata* subsp. *unguiculata* 'Monarch' cowpea and *Phaseolus vulgaris* L. 'Black Valentine' bean. Leaf-feeding beetles, however, rarely transmitted either virus to bean, while they transmitted both viruses more frequently to cowpea. When the gross-wound inoculation technique, which mimics beetle feeding, was used to inoculate bean and cowpea with purified CPMV and CPSMV mixed with beetle regurgitant or pancreatic ribonuclease,

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the results were similar to those observed in beetle transmission tests. Monarch cowpea was more susceptible to mechanical inoculation with diluted infectious sap from CPMV- or CPSMV-infected plants than was Black Valentine bean. Four cultivars of cowpea with different susceptibilities under field conditions to the cowpea strain of southern bean mosaic virus showed a similar pattern of susceptibility when inoculated with the gross-wounding technique.

Inoculation of seedlings by mechanical transmission techniques is traditionally used for detecting resistance to plant viruses. Such inoculation introduces viruses in an unnatural manner and may not detect plant resistance to virus infection by natural vectors. The literature suggests that there are marked differences in plant susceptibility when beetle transmission results are compared with mechanical transmission tests. Jansen and Staples (8) showed that there was a difference in susceptibility of soybean and cowpea to cowpea severe mosaic virus (CPSMV) in beetle transmission trials, although both hosts were susceptible to mechanical transmission. Natural infection of soybean by CPSMV has been reported (1,2,11,13), but the incidence in soybeans growing near heavily infected cowpea fields was quite low (14), even though the beetle vector was present and fed on soybean.

The use of beetle transmission trials or field tests to evaluate resistance to virus infection by vectors would obviously be a cumbersome procedure. An inoculation technique (gross wounding) that mimics beetle feeding damage (4) may be useful for identifying resistance to vector transmission. When viruses are mixed with ribonuclease (RNase), the active inhibitory component in beetle regurgitant (3,5), and inoculated by the gross-wounding technique, only those viruses naturally transmitted by beetles infect plants (4,12), even though all of the hosts used in these studies were susceptible to mechanical inoculation by infectious sap.

Originally, it was thought that the inhibition of infection by regurgitant or RNase in the gross-wound inoculum was restricted to those viruses not naturally transmitted by leaf-feeding beetles. During experiments to validate the gross-wound inoculation technique, however, it became apparent that there were certain hosts that could be infected by mechanical inoculation but not by gross wounding. Further tests revealed that these hosts also were more resistant to beetle transmission under laboratory conditions. These results suggested that a screening procedure utilizing the gross-wounding technique might identify heretofore undetected resistance to vector transmission by leaf-feeding beetles.

The objectives of this research were to evaluate the usefulness of the gross-wounding technique in identifying resistance to infection by beetle vectors and to determine the basis for this type of resistance. A preliminary report has been published (6).

## MATERIALS AND METHODS

**Maintenance of beetles and regurgitant collection.** Mexican bean beetles, *Epilachna varivestis* Muls., were reared on *Phaseolus vulgaris* L. 'Pinto' in the greenhouse. Bean leaf beetles, *Cerotoma trifurcata* (Forst.), were collected from the field and fed on Pinto bean in the laboratory for at least 5 days before use. Beetles were induced to regurgitate by holding the beetle between thumb and forefinger and teasing its mouthparts with a capillary glass tube that then was used to collect the emitted regurgitant. Regurgitant was stored at  $-20^{\circ}\text{C}$  in closed glass capillary tubes.

**Viruses and virus purification.** Three beetle-transmissible viruses (cowpea mosaic [CPMV], CPSMV, and the cowpea strain of southern bean mosaic [CP-SBMV]) were used in this study. The isolate of CP-SBMV was provided by Cedric Kuhn, University of Georgia. CPMV and CP-SBMV were propagated in *Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'Monarch' and 'Crimson', respectively, and harvested 10–14 days after inoculation. CPSMV was propagated in Black Valentine bean and harvested 10–14 days after inoculation. CPMV and CP-SBMV were purified by chloroform-butanol extraction followed by two or three cycles of differential centrifugation and resuspension of virus in 0.01 M phosphate buffer, pH 7.2. CPSMV was purified as described by Lin et al (10) and resuspended in 0.01 M phosphate buffer, pH 7.2.

**Virus inoculations.** For virus transmission trials with bean leaf beetles, single beetles were fed on virus-infected Black Valentine bean plants for 24 h and then transferred to young test plants for a 24-h inoculation feeding period. Test plants were grown in the greenhouse for 2 wk and then tested for virus infection by Ouchterlony gel diffusion tests.

The relative susceptibility of Monarch cowpea and Black Valentine bean to mechanical inoculation of CPSMV and CPMV was tested by inoculation of Carborundum-dusted seedlings with tenfold serial dilutions of sap (in 0.01 M sodium-potassium phosphate buffer, pH 7.2) from CPSMV- and CPMV-infected cowpea plants. Similarly, the susceptibility of four cowpea cultivars to CP-SBMV was tested by mechanical inoculation with sap from infected Monarch cowpea. Test plants were indexed for virus infection with Ouchterlony gel diffusion tests using 1% agarose gels containing 0.02% sodium azide 2–3 wk after inoculation.

Transmission tests with the gross-wound inoculation technique

(4) used infectious plant sap mixed with buffer (0.01 M phosphate, pH 7.2) or pancreatic RNase in buffer (Sigma, St. Louis, MO; final concentration of 200 µg/ml), or purified virus mixed with buffer, Mexican bean beetle regurgitant, or pancreatic RNase. These mixtures were inoculated with the gross-wound inoculation technique by punching a disk from a leaf with a glass cylinder that previously had been dipped into the inoculum. The relative susceptibility of Monarch cowpea and Black Valentine bean to gross-wound inoculation of CPSMV was tested by inoculating dilutions of purified virus (in 0.01 M phosphate buffer, pH 7.2) to seedling primary leaves.

Four cowpea cultivars with different susceptibilities to CP-SBMV under field conditions (7) were evaluated for susceptibility after gross-wound inoculation of purified virus or infectious sap mixed with buffer (0.01 M phosphate, pH 7.2) or pancreatic RNase in buffer (200 µg/ml final concentration). Seeds of the four cowpea cultivars were provided by Cedric Kuhn, University of Georgia. After 2 wk, test plants were indexed serologically by Ouchterlony gel diffusion tests as described above. The antiserum had a titer of 1:320 in gel diffusion tests and was used at a dilution of 1:10. Early Pinkeye has been shown to accumulate less virus than the other three cowpea cultivars used in these experiments (9). Therefore, plants of each cultivar that were negative by Ouchterlony gel diffusion tests also were indexed by infectivity tests on Monarch cowpea.

## RESULTS

**Effect of inoculation method on the susceptibility of bean and cowpea to CPSMV and CPMV.** Mechanical inoculation of undiluted infectious sap to Monarch cowpea and Black Valentine

TABLE 1. Transmission of cowpea mosaic virus (CPMV) and cowpea severe mosaic virus (CPSMV) by mechanical inoculation and by bean leaf beetles

Virus	Test host <sup>a</sup>	Transmission	
		Mechanical <sup>b</sup>	Beetle <sup>c</sup>
CPMV	Cowpea	25/25 <sup>d</sup>	10/52
	Bean	25/25	1/52
CPSMV	Cowpea	20/20	18/59
	Bean	20/20	3/64

<sup>a</sup> Monarch cowpea and Black Valentine bean.

<sup>b</sup> Carborundum-dusted seedlings were inoculated with infectious sap from Black Valentine bean (CPSMV) and Monarch cowpea (CPMV).

<sup>c</sup> Single beetles were given a 24-h acquisition access period on virus-infected Black Valentine bean and a 24-h inoculation access period on the test host.

<sup>d</sup> Number of plants infected/number of plants tested.

TABLE 2. Transmission of cowpea mosaic virus (CPMV)<sup>a</sup> and cowpea severe mosaic virus (CPSMV)<sup>b</sup> to Black Valentine bean or Monarch cowpea by gross-wound inoculation with purified virus in the presence of regurgitant, ribonuclease (RNase), or buffer, or with infectious sap

Virus	Test host	Inoculum <sup>c</sup>			
		Purified virus			Infectious sap
		Regurgitant <sup>d</sup>	RNase <sup>e</sup>	Buffer <sup>f</sup>	
CPMV	Cowpea	49/60 <sup>g</sup>	51/60	60/60	40/40
	Bean	0/60	0/60	3/60	0/40
CPSMV	Cowpea	12/26	27/28	28/28	23/25
	Bean	3/25	6/25	21/25	3/25

<sup>a</sup> Concentration of purified virus was 16–21 mg/ml.

<sup>b</sup> Concentration of purified virus was 14–19 mg/ml.

<sup>c</sup> Inoculum consisted of equal parts of purified virus mixed with regurgitant, RNase, or buffer, or infectious sap from Black Valentine bean (CPSMV) and Monarch cowpea (CPMV).

<sup>d</sup> Mexican bean beetle regurgitant.

<sup>e</sup> Pancreatic RNase at 200 µg/ml, final concentration.

<sup>f</sup> 0.01 M phosphate buffer, pH 7.2.

<sup>g</sup> Number of plants infected/number of plants tested.

bean resulted in 100% infection with CPSMV and CPMV. Beetle transmission trials, however, indicated that cowpea was more susceptible than bean to the two viruses (Table 1). Similarly, when infectious sap was inoculated to bean and cowpea by the gross-wound inoculation technique, cowpea was more susceptible than bean (Table 2). The addition of beetle regurgitant or pancreatic RNase to the gross-wound inoculum of purified virus caused fewer plants to become infected for all virus/host combinations, and no plants were infected when purified CPMV was inoculated to bean in the presence of regurgitant or RNase (Table 2). When undiluted infectious sap containing CPSMV or CPMV was used as inoculum for gross wounding, bean was much less susceptible than cowpea (Table 2).

**Inoculum concentration and relative susceptibility of bean and cowpea to CPSMV and CPMV by mechanical inoculation.** The above results suggested that bean and cowpea differ in their susceptibility to CPMV and CPSMV. Traditional mechanical inoculation tests were conducted to compare the susceptibility of these two hosts to CPMV and CPSMV using a tenfold dilution series of infectious sap. At dilutions of 1:1,000 or less, all bean and cowpea plants that were inoculated with CPMV and CPSMV became infected. Fewer bean plants than cowpea plants became infected with CPMV or CPSMV at higher dilutions of infectious sap (Fig. 1).

**Inoculum concentration and relative susceptibility of bean and cowpea to CPSMV by gross-wound inoculation.** Gross-wound inoculation of bean and cowpea with CPSMV at virus concen-

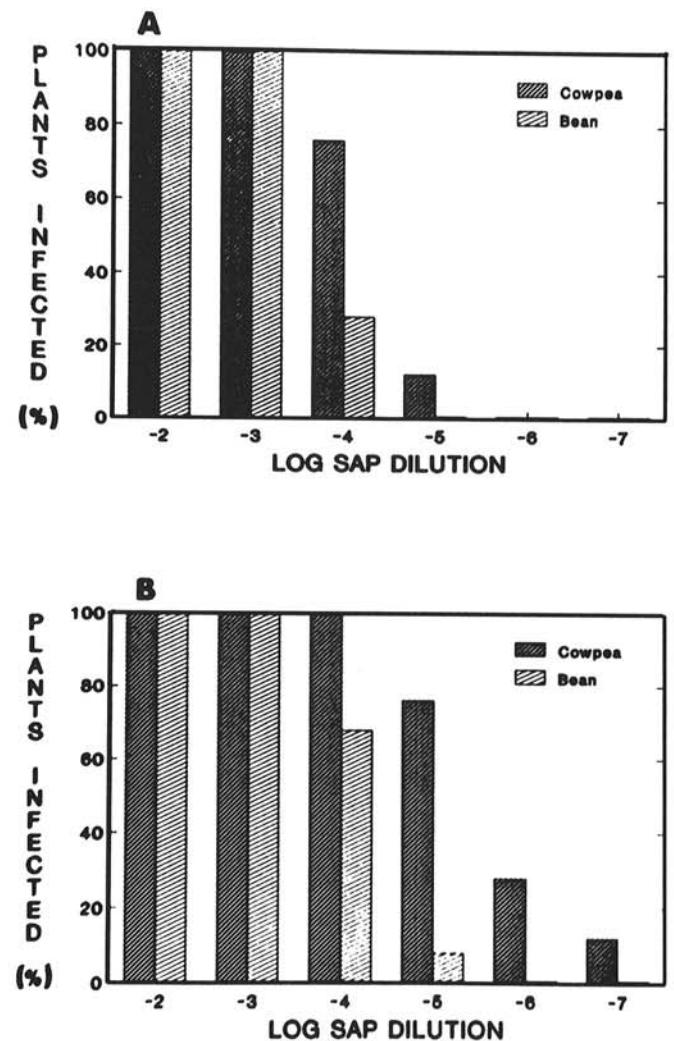


Fig. 1. Susceptibility of Monarch cowpea and Black Valentine bean to mechanical inoculation of A, cowpea severe mosaic virus, and B, cowpea mosaic virus, with dilutions of infectious sap from Monarch cowpea (25 plants per treatment).

trations of 10 mg/ml resulted in infection of almost all of the plants (Fig. 2). However, when the concentration of virus in the inoculum more closely resembled that naturally occurring in plant tissue, fewer bean than cowpea plants became infected. At inoculum concentrations of 0.01 mg/ml of purified CPSMV, no bean plants were infected while 45% of the cowpea plants were infected. Similarly, the use of undiluted infectious sap for gross-wound inoculation resulted in 92% infection of cowpea and only 12% infection of bean (Table 2).

**Effect of inoculation method and type of inoculum on the susceptibility of four cowpea cultivars to CP-SBMV.** Four cowpea cultivars that differ in susceptibility to CP-SBMV under field conditions (7) were inoculated with CP-SBMV by gross wounding to test their relative susceptibility to this virus compared to their susceptibility to beetle transmission in the field. Mechanical inoculation of infectious sap (diluted 1:2 with 0.01 M phosphate buffer, pH 7.2) resulted in 100% infection of the plants of all cultivars (Table 3). Inoculation by gross wounding resulted in a low percentage of infection of Early Pinkeye cowpea regardless of the type of inoculum used. The other three cultivars were all susceptible to gross-wound inoculation when inoculum consisted of purified virus or purified virus mixed with pancreatic RNase. When infectious sap mixed with RNase was used for gross-wound inoculation, however, Coronet was less susceptible than California Blackeye cowpea to CP-SBMV. This inoculum more closely resembles the virus concentration and RNase content of beetle regurgitant and resulted in infection levels in Coronet that more

closely resembled those found under natural transmission conditions in the field.

Because the concentration of CP-SBMV in Early Pinkeye cowpea is much lower than that of the other three cowpea cultivars used in this experiment (9), the plants that were negative for virus in Ouchterlony double-diffusion tests also were indexed for virus by back-inoculation to Monarch cowpea. In some experiments at 2 wk postinoculation, a few Early Pinkeye plants that indexed negative by Ouchterlony tests were evaluated as positive in back-inoculation tests. However, this also happened occasionally when plants of the other three cultivars that were negative by Ouchterlony tests were evaluated by back-inoculation tests.

## DISCUSSION

Susceptibility to beetle transmission of plant viruses appears to be controlled by host factors that are not apparent when these viruses are mechanically transmitted in infectious sap. In the course of experiments that were designed to verify that the gross-wound inoculation technique mimics the virus transmission process of leaf-feeding beetles, it became apparent that some susceptible hosts are resistant to inoculation by gross wounding (12). Subsequent beetle transmission trials with these virus/host combinations revealed only low levels of virus transmission compared with other hosts (Table 1). These findings suggested that hosts that are susceptible to mechanical transmission of a virus may be resistant to transmission by natural beetle feeding, and that the gross-wound inoculation technique would be a useful tool for detecting this type of resistance.

The effect of inoculation procedure on susceptibility was most pronounced for CPMV in bean. Gross-wound inoculation was very inefficient for transmission of purified CPMV to bean, and no infection occurred when virus was inoculated to bean in the presence of beetle regurgitant or RNase (Table 2). In contrast, inoculation of cowpea with CPMV was quite efficient regardless of the type of inoculation procedure or the presence of regurgitant or RNase in the inoculum. Gross-wound inoculation of cowpea with sap from CPMV-infected plants consistently resulted in 100% infection, whereas no bean plants became infected in parallel experiments.

The effect of the inoculation method was less pronounced with CPSMV than with CPMV in bean and cowpea. Cowpea was more susceptible than bean to gross-wound inoculation of CPSMV regardless of the inoculum used. Transmission to bean was very efficient when gross-wound inoculum contained highly concentrated, purified virus. However, when regurgitant or RNase was added to the inoculum to mimic the inoculum delivered by beetles, the efficiency of transmission to bean was greatly reduced. The use of infectious sap for gross-wound inoculation provided

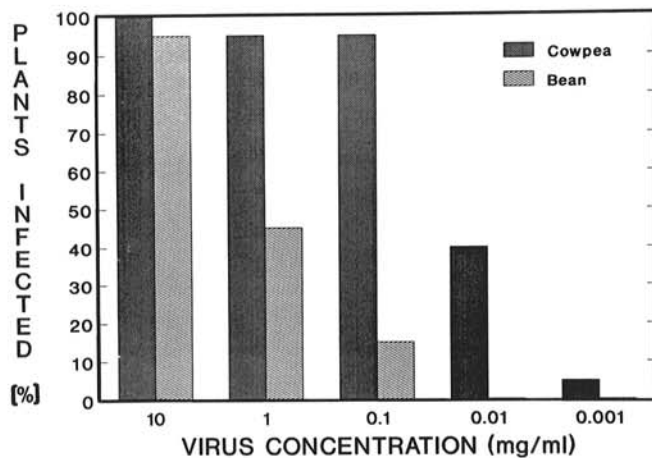


Fig. 2. Susceptibility of Monarch cowpea and Black Valentine bean to gross-wound inoculation of dilutions of purified cowpea severe mosaic virus (20 plants per treatment).

TABLE 3. Effects of different inocula and methods of inoculation on susceptibility of cowpea lines to cowpea southern bean mosaic virus

Cowpea cultivar	Host susceptibility <sup>a</sup>					
	Average field incidence <sup>b</sup> (%)	Mechanical inoculation <sup>c</sup> (%)	Gross-wound inoculation			
			Purified virus <sup>d</sup>		Infectious sap <sup>e</sup>	
			Buffer (%)	RNase (%)	Buffer (%)	RNase (%)
California Blackeye	81	100	100	83	98	75
Knuckle Purple Hull	36	100	90	90	83	63
Coronet	35	100	100	76	83	38
Early Pinkeye	2	100	11	11	13	3

<sup>a</sup> Results of two experiments with 20 or more plants per experiment. Plants were evaluated for virus infection 2 wk after inoculation using Ouchterlony gel double diffusion tests. Plants that were negative serologically for virus infection were evaluated for virus infection by back-inoculation to Monarch cowpea.

<sup>b</sup> As determined by Hobbs and Kuhn (7). Average of data for 2 yr.

<sup>c</sup> Sap from infected Monarch cowpea was diluted 1:2 with 0.01 M sodium-potassium phosphate buffer, pH 7.2, and inoculated to Carborundum-dusted primary leaves of young cowpea plants.

<sup>d</sup> Virus inoculum concentrations were 25–30 mg/ml in 0.01 M phosphate buffer, pH 7.2. Virus was mixed with equal parts of 0.01 M phosphate buffer, pH 7.2, or with a solution of pancreatic ribonuclease (400 µg/ml) in 0.01 M phosphate buffer, pH 7.2.

<sup>e</sup> Sap from infected Monarch cowpea was mixed with equal parts of 0.01 M phosphate buffer, pH 7.2, or with a solution of pancreatic ribonuclease (400 µg/ml) in 0.01 M phosphate buffer, pH 7.2.

an inoculum concentration that more closely resembled natural CPSMV concentrations in beetle regurgitant and resulted in much higher levels of infection in cowpea than in bean.

Four cowpea cultivars that differ in field susceptibility to CP-SBMV (7) were used to test the hypothesis that resistance to beetle transmission is detectable by gross-wound inoculation. Early Pinkeye, which is resistant to beetle transmission in the field, had consistently lower levels of infection after gross-wound inoculation, regardless of the type of inoculum, than did three susceptible cowpea lines. Although these three susceptible cowpea cultivars differed in their field incidence, this difference was not detectable with gross-wound inoculation of purified virus or purified virus mixed with pancreatic RNase. However, when infectious sap or infectious sap mixed with RNase was used in the gross-wound inoculum, the percent infection was consistently highest in California Blackeye cowpea, the cultivar most susceptible to beetle transmission in the field.

We conclude that a high level of resistance to beetle transmission of CP-SBMV, such as that seen in Early Pinkeye cowpea, can be detected by screening with gross-wound inoculation with infectious sap or purified virus. Intermediate levels of resistance to beetle transmission such as that seen in Coronet and Knuckle Purple Hull cowpea, however, are less readily apparent with gross-wound inoculation and may require careful selection of the inoculum components to achieve the desired differential infection. The addition of RNase to infectious sap in the inoculum resulted in levels of infection for the cultivars Coronet and Knuckle Purple Hull that more closely resemble those observed in the field. For CPMV, the inoculum constituents for gross wounding are not critical for detection of resistance in bean, i.e., all inocula had low infectivity in bean and high infectivity in cowpea. For CPSMV, however, purified virus was highly infectious to both bean and cowpea. Only when RNase or regurgitant was added to purified virus or when infectious sap was used as inoculum was resistance in bean apparent. These results suggest that RNase, a component of beetle regurgitant, is important in determining virus resistance in certain plant/virus combinations.

The gross-wound inoculation technique may prove to be a valuable tool for identification of host resistance to beetle transmission of plant viruses. This type of resistance is not associated with inhibition of beetle feeding or with interaction of the virus with the beetle vector but rather appears to be related to host susceptibility when virus is delivered to the plant and inoculated with the type of feeding damage produced by leaf-feeding beetles.

The lack of susceptibility to virus inoculation in certain hosts by beetle feeding or gross-wounding appears to reflect differences in the natural susceptibility of these hosts to virus infection. The host plants in this study that were resistant to beetle transmission also were resistant to mechanical inoculation. A similar difference in susceptibility was noted by Hobbs and Kuhn (7) when dilutions of purified CP-SBMV were mechanically inoculated to four cowpea cultivars differing in their field incidence of CP-SBMV. They reported that Early Pinkeye cowpea, which is resistant to CP-SBMV under field conditions, is less susceptible to mechanical inoculation of dilutions of purified virus than the three varieties that are susceptible under field conditions. These results suggest that inoculation with beetle feeding or gross wounding is very inefficient when compared with mechanical inoculation and that

plants that are less easily infected by mechanical inoculation may be resistant to beetle transmission and gross-wound inoculation.

Subtle differences in susceptibility to mechanical inoculation apparently can translate into critical differences in susceptibility to gross-wound inoculation. When plant sap was used as gross-wound inoculum, the plants that were resistant to beetle transmission were not infected (CPMV in bean) or were infected only rarely (CPSMV in bean and CP-SBMV in Early Pinkeye cowpea). This suggests that not only is the method of inoculation important in determining resistance, but that the virus concentration in the inoculum should reflect that occurring naturally in infected plants. We propose that a screening procedure using gross-wound inoculation of infectious sap can be used to detect resistance to beetle transmission of plant viruses.

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