Vector Relations

Effect of Beetle Regurgitant on Plant Virus Transmission Using the Gross Wounding Technique

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ABSTRACT

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Regurgitant from Mexican bean beetles and bean leaf beetles inhibited the transmission of zucchini yellow mosaic virus (ZYMV) not transmissible by beetles and common tobacco mosaic virus and had little or no effect on beetle-transmissible squash mosaic virus when mixed with purified virus preparations and inoculated to systemic hosts using the gross wounding technique. Transmission of the beetle-transmissible cowpea severe mosaic virus (CSMV) was unaffected by regurgitant when cowpea was used as the test plant. No transmission occurred, however, when bean was inoculated with a mixture of regurgitant and CSMV using the gross wounding technique. Two members of the bromovirus group, which are inefficiently transmitted by beetles, behaved differently when mixed with regurgitant and inoculated using the gross wounding technique. Brome mosaic virus was not inhibited, whereas cowpea chlorotic mottle virus (CCMV) showed the same pattern of inhibition as a virus not transmissible by beetles. Recovery of CCMV and ZYMV from virus:regurgitant mixtures by fractionation in CsCl gradients resulted in virus preparations that were infectious when inoculated to plants using the gross wounding technique. This demonstrates that viruses not transmissible by beetles and the inefficiently transmitted CCMV are not inactivated by beetle regurgitant.

Additional key words: *Cerotoma trifurcata, Epilachna varivestis.*

Recent studies reported the presence of a factor(s) in the regurgitant of leaf-feeding beetles that prevents infection by viruses not transmissible by beetles but has no effect on beetle-transmissible viruses (4). The selective nature of the factor(s) is evident only when a gross wounding inoculation technique, which simulates the injury done by beetles during feeding, is used. Dilution of a virus:regurgitant mixture before inoculation with the gross wounding technique resulted in recovery of infectivity of a virus not transmissible by beetles, which indicated that the selective factor(s) from beetle regurgitant does not inactivate the virus (4).

The objectives of this work were to determine whether the effect of regurgitant on virus transmission using the gross wounding technique is a general phenomenon by testing additional viruses

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and virus-host combinations, and to further elucidate the mode of action of beetle regurgitant on viruses not transmissible by beetles.

MATERIALS AND METHODS

Viruses and virus purification. Two beetle-transmissible viruses, squash mosaic (3) and cowpea severe mosaic (2), two members of the bromovirus group that have been reported to be inefficiently beetle-transmissible, brome mosaic (11) and cowpea chlorotic mottle (6), one member of the potyvirus group that is aphid-transmitted, zucchini yellow mosaic (10), and the common strain of tobacco mosaic (14) were used in this study.

Squash mosaic virus (SqMV) was propagated in squash, *Cucurbita pepo* L. 'Early Prolific Straightneck,' and purified by the method described by Lastra and Munz (8). Cowpea severe mosaic (CSMV) and cowpea chlorotic mottle (CCMV) viruses were propagated in 'Monarch' cowpea, *Vigna unguiculata* (L.) Walp subsp. *unguiculata*, and purified as described by Lin, Anjos, and Rios (9) and Bancroft (1), respectively. Brome mosaic virus (BMV) was propagated in wheat, *Triticum aestivum* L. 'Rosen' and purified by the method described by Lane (7). Tobacco mosaic virus (TMV) was purified by the method described by Gergerich et al (4) for the cowpea strain of TMV from systemically infected tobacco plants, *Nicotiana tabacum* L. 'Kentucky 16,' harvested 10–14 days after inoculation.

The method used for purification of zucchini yellow mosaic virus (ZYMV) (obtained from Dr. M. F. Ouf, University of Minia, Minia, Egypt, and identified by Dr. R. Provvidenti, Geneva, NY) was a combination of the procedures described by Purcifull and Hiebert (13) and Gonsalves and Ishii (5) for purifying watermelon mosaic virus 2 and papaya ringspot virus, respectively. Systemically infected pumpkin, *C. pepo* 'Small Sugar,' was harvested 21 days after inoculation and homogenized in 0.5 M potassium phosphate buffer, pH 7.5, containing 0.01 M EDTA and 0.25% sodium sulfite (2 ml/g tissue). The homogenate was clarified overnight by stirring with 8% butanol at 4 C. After centrifugation for 20 min at 13,000 *g*, the virus was concentrated by adding 8% polyethylene glycol (PEG), stirring for 1 hr, recovering the precipitated virus by centrifugation at 13,000 *g* for 10 min, and resuspending in 0.05 M potassium phosphate-0.01 M EDTA buffer, pH 7.5. The virus was reprecipitated with 5% PEG and 0.3 M NaCl, collected by low-speed centrifugation (13,000 *g* 10 min) and resuspended in the phosphate-EDTA buffer. The virus was further purified by centrifugation in a CsCl density gradient (13) at 130,000 *g* for 18 hr. Following fractionation, the virus was reconcentrated by high-speed centrifugation (105,000 *g*, 90 min). Virus pellets were resuspended in phosphate-EDTA buffer.

Beetles and regurgitant collection. Mexican bean beetles, *Epilachna varivestis* Mulsant, were reared in the greenhouse on caged bean, *Phaseolus vulgaris* L. 'Pinto.' Bean leaf beetles, *Cerotoma trifurcata* (Forster), were collected from soybean fields and maintained in petri dishes on detached primary leaves of Pinto bean. Beetles were induced to regurgitate by holding the beetle between thumb and forefinger and teasing the mouthparts with a capillary tube, which was used to collect the emitted regurgitant. The pooled regurgitant (obtained from 50–200 beetles) was stored at −20 C in closed glass capillary tubes.

Gross wounding technique of inoculation. The effect of regurgitant on virus transmission was determined using the gross wounding technique (4). A single hole was bored in a leaf or cotyledon of the test plant using the fractured edge of a glass cylinder with an outside diameter of 7 mm and an inside diameter of 6 mm. Immediately before cutting the hole, the edge of the glass cylinder was dipped into a mixture of equal volumes of virus and regurgitant. Control plants received the same treatment, but the inoculum consisted of virus mixed with an equal volume of buffer in which the final purified virus pellet had been suspended. After 14 days plants inoculated with SqMV, CSMV, BMV, CCMV, and TMV were assayed for virus by the Ouchterlony double diffusion technique. Double diffusion tests for ZYMV were conducted in sodium dodecyl sulfate agar gels (12) 14–21 days after inoculation.

Test plants. Plants used in all experiments were systemic hosts of the viruses. Cotyledons of seedlings of Early Prolific Straightneck squash were used for SqMV and ZYMV inoculations. Cowpea chlorotic mottle virus and CSMV were inoculated to Pinto or Black Valentine bean, respectively, and to Monarch cowpea at the primary leaf stage. Plants of Rosen wheat at the three- to five-leaf stage were inoculated with BMV. *Nicotiana tabacum* L. 'Kentucky 16,' at the three- to four-leaf stage were used for TMV inoculation.

Recovery of virus from virus:regurgitant mixtures. Two types of tests were conducted to determine if the factor(s) in Mexican bean beetle regurgitant inactivated CCMV and ZYMV, an inefficiently beetle-transmissible virus and a virus not transmissible by beetles, respectively. The first type of test involved diluting a CCMV:regurgitant mixture 1:25, 1:50, and 1:100 and a ZYMV:regurgitant mixture 1:5, 1:10, and 1:30 in the appropriate buffers. These suspensions were inoculated onto systemic hosts using the gross wounding technique.

In the second type of test, CCMV: and ZYMV:regurgitant mixtures were fractionated by centrifugation in CsCl density gradients (13) and the viruses reconcentrated by high-speed centrifugation. As controls, equal amounts of the viruses were diluted in their appropriate buffers instead of regurgitant and subjected to CsCl centrifugation. The suspensions were tested for infectivity using the gross wounding technique.

RESULTS

Selective inhibitory effect of beetle regurgitant. Regurgitant from Mexican bean beetles and bean leaf beetles reduced or prevented infection by ZYMV and TMV (not transmissible by beetles) and had little effect on infection by SqMV (beetle-transmissible) when tested with the gross wounding technique (Table 1). The reaction of CSMV to the presence of regurgitant in the inoculum was dependent on the test host. When this beetle-transmissible virus was inoculated with regurgitant to Monarch cowpea, transmission occurred. When Black Valentine bean was used as a host, however, the regurgitant prevented infection. The inefficiently beetle-transmissible bromoviruses varied in their responses to the presence of regurgitant in the inoculum. Beetle

TABLE 1. Effect of beetle regurgitant on transmission of squash mosaic (SqMV), cowpea severe mosaic (CSMV), brome mosaic (BMV), cowpea chlorotic mottle (CCMV), zucchini yellow mosaic (ZYMV), and the common strain of tobacco mosaic (TMV) viruses using the gross wounding technique[a]

		Proportion of infected plants	
Viruses	Test host	Mexican bean beetle regurgitant[b]	Bean leaf beetle regurgitant
Beetle-transmissible			
SqMV	Early Prolific Straightneck squash	41/45[c] (43/45)[d]	15/33 (32/33)
CSMV	Monarch cowpea	15/44 (39/44)	30/35 (35/35)
CSMV	Black Valentine bean	0/20 (18/20)	··· ···
Inefficiently beetle-transmissible			
BMV	Rosen wheat	43/43 (43/43)	30/30 (30/30)
CCMV	Monarch cowpea	1/43 (31/45)	0/30 (16/30)
CCMV	Pinto bean	0/36 (20/36)	··· ···
Not transmissible by beetles			
ZYMV	Early Prolific Straightneck squash	2/49 (30/49)	0/30 (22/30)
TMV	Kentucky 16 tobacco	4/49 (43/43)	0/20 (18/19)

[a] Total number of plants used in two experiments performed independently with two virus preparations. Concentration of viruses: SqMV = 18 or 19 mg/ml, CSMV = 12 or 26 mg/ml, BMV = 5 or 7.5 mg/ml, CCMV = 7.5 or 32 mg/ml, ZYMV = 10 or 19 mg/ml, TMV = 0.2 or 2 mg/ml.

[b] Inocula consisted of equal volumes of purified virus and beetle regurgitant.

[c] Ratio of plants that became infected to total plants in trial.

[d] Figures in parentheses are the ratios for the controls in which the inoculum consisted of equal volumes of each of the purified viruses and the appropriate buffer.

regurgitant had no effect on the transmission of BMV, whereas the behavior of CCMV in both bean and cowpea resembled that of a virus not transmissible by beetles.

Recovery of virus from virus:regurgitant mixtures. When CCMV: or ZYMV:regurgitant mixtures were diluted before inoculation of plants using the gross wounding technique, the inhibitory effect of beetle regurgitant was still evident. When plants were inoculated with diluted virus:buffer mixtures, low levels of infection also occurred (Table 2). Apparently the virus concentrations used did not permit a level of dilution high enough to sufficiently reduce the inhibitory effect of regurgitant. An alternative explanation, however, is that the viruses were inactivated by regurgitant components.

To test this possibility CCMV and ZYMV were mixed with regurgitant, repurified in CsCl density gradients, and inoculated to test plants by the gross wounding technique (Table 3). Infectivity of both viruses was recovered indicating that regurgitant had little or no irreversible effect on the virus particles in vitro.

DISCUSSION

Earlier work by Gergerich et al (4) showed that infection of Black Valentine bean by beetle-transmissible viruses such as southern bean mosaic and bean pod mottle occurs in the presence of regurgitant, whereas transmission of viruses not transmissible by beetles, tobacco ringspot, and the cowpea strain of TMV is prevented when the gross wounding technique, which mimics beetle feeding, is used. The work reported here describes results obtained by applying the gross wounding technique to previously untested non-transmissible viruses, ZYMV and TMV, and suggests that the action of beetle regurgitant on viruses not transmissible by beetles is a general phenomenon.

Infectivity of CCMV, a member of the bromovirus group, shown by Hobbs and Fulton (6) to be an inefficiently beetle-transmissible virus, was also inhibited by the regurgitant factor(s). There were no differences between CCMV and the viruses not transmissible by beetles, ZYMV and TMV, in their response to regurgitant when the gross wounding technique was used. Also, no differences were detected between Mexican bean beetle and bean leaf beetle regurgitants, although the Mexican bean beetle has been reported to be a less efficient vector of CCMV (6). Brome mosaic virus, another member of the bromovirus group, which is inefficiently transmitted by beetles (11), exhibited very different behavior, since regurgitant from either beetle did not suppress BMV infectivity. This difference in the effect of beetle regurgitant on viruses within the same group might be related to the type of test host, since the gross wounding technique has not previously been applied to monocotyledonous plants.

Recovery of infectivity of a virus not transmissible by beetles by dilution of virus:regurgitant mixtures was accomplished with the cowpea strain of TMV in earlier work (4). Similar dilution experiments using CCMV and ZYMV were unsuccessful. When these viruses were re-isolated from virus:regurgitant mixtures by fractionation in CsCl density gradients, infectivity was recovered. This demonstrates that regurgitant components do not have an inactivating effect on a potyvirus or a bromovirus, and, as postulated by Gergerich et al (4), it is likely that the inhibitory activity of the regurgitant factor is an effect on the host or on the interaction of the virus with the host.

When purified CSMV was mixed with regurgitant and inoculated to two different host plants, a very pronounced host effect was apparent (Table 1) in that the virus was transmitted to Monarch cowpea but not to Black Valentine bean. When these two hosts are mechanically inoculated by rub-inoculation to carborundum-dusted leaves, both hosts are susceptible. The use of an inoculation procedure that imitates the natural transmission process has potential application for detecting resistance to plant viruses.

The interpretation of comparative beetle-transmission tests using different beetles, acquisition hosts, or test plants has been hampered by the variability of virus concentration in acquisition hosts as well as differences in the amount of feeding done by various species of leaf-feeding beetles. Use of the gross wounding technique in comparative transmission tests offers several advantages. Because purified virus is used in the inoculum mixture, there is no variability due to virus concentration differences in acquisition hosts. Also, test plants are all wounded in the same manner, avoiding the problem of variation in the amount of beetle feeding done on the test plant. The potential of the gross wounding technique to detect host differences in susceptibility that are not demonstrable using rub-inoculation with carborundum warrants further investigation.

TABLE 2. Effect of dilution on infectivity of zucchini yellow mosaic virus (ZYMV) or cowpea chlorotic mottle virus (CCMV) mixed with Mexican bean beetle regurgitant and inoculated using the gross wounding technique

Virus	Dilution of inoculum mixture[a]	Proportion of infected plants	
		Regurgitant	Buffer
ZYMV	Undiluted[b]	1/24[c]	13/24
	1:5[d]	0/15	8/15
	1:10	0/15	4/15
	1:30	0/15	3/15
CCMV	Undiluted[e]	2/20[f]	11/20
	1:25[g]	0/20	2/20
	1:50	0/20	5/20
	1:100	2/20	3/20

[a] Undiluted inoculum mixture consisted of equal parts of purified virus and regurgitant or buffer.
[b] ZYMV concentration = 10 mg/ml.
[c] Ratio of Early Prolific Straightneck squash plants that became infected to total of plants in trial.
[d] Diluted with 0.05 M potassium phosphate-0.01 M EDTA buffer, pH 7.5.
[e] CCMV concentration = 7.5 mg/ml.
[f] Ratio of Monarch cowpea plants that become infected to total plants in trial.
[g] Diluted with 0.1 M sodium acetate, pH 5.0.

TABLE 3. Transmission of cowpea chlorotic mottle (CCMV) and zucchini yellow mosaic (ZYMV) viruses mixed with Mexican bean beetle regurgitant, repurified in CsCl density gradients, and inoculated using the gross wounding technique[a]

Viruses[b]	Proportion of infected plants	
	Virus repurified from:	
	Virus:regurgitant mixture[c]	Virus:buffer mixture[d]
CCMV	12/20[e]	15/20
ZYMV	14/20	15/20

[a] Purified virus preparations mixed with regurgitant or buffer, repurified in CsCl density gradients, concentrated by high-speed centrifugation, resuspended in the appropriate buffer to the volume of the original virus mixture, and inoculated using the gross wounding technique.
[b] Concentration of viruses: CCMV = 32 mg/ml, ZYMV = 32 mg/ml. Test plants were Monarch cowpea and Early Prolific Straightneck squash for CCMV and ZYMV, respectively.
[c] Virus preparations mixed with an equal volume of regurgitant.
[d] ZYMV and CCMV were mixed with an equal volume of 0.05 M potassium phosphate-0.01 M EDTA, pH 7.5, and 0.1 M sodium acetate, pH 5.0, respectively.
[e] Ratio of plants that became infected to total plants in trial.

LITERATURE CITED

1. Bancroft, J. B. 1971. Cowpea chlorotic mottle virus. No. 49 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst., Assoc. Applied Biologists., Kew, Surrey, England. 4 pp.
2. De Jager, C. P. 1979. Cowpea severe mosaic virus. No. 209 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst., Assoc. Applied Biologists., Kew, Surrey, England. 5 pp.
3. Freitag, J. H. 1956. Beetle transmission, host range, and properties of squash mosaic virus. Phytopathology 46:73-81.
4. Gergerich, R. C., Scott, H. A., and Fulton, J. P. 1983. Regurgitant as a determinant of specificity in the transmission of plant viruses by beetles. Phytopathology 73:936-938.

5. Gonsalves, D., and Ishii, M. 1980. Purification and serology of papaya ringspot virus. Phytopathology 70:1028-1032.
6. Hobbs, H. A., and Fulton, J. P. 1979. Beetle transmission of cowpea chlorotic mottle virus. Phytopathology 69:255-256.
7. Lane, L. C. 1977. Brome mosaic virus. No. 180 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst., Assoc. Applied Biologists, Kew, Surrey, England. 4 pp.
8. Lastra, R., and Munz, K. 1969. Purification and electron microscopy of squash mosaic virus. Phytopathology 59:1429-1435.
9. Lin, M. T., Anjos, J. R. N., and Rios. G. P. 1981. Serological grouping of cowpea severe mosaic virus isolates from central Brazil. Phytopathology 71:435-438.
10. Lisa, V., Boccardo, G., D'agostino, G., Dellavalle, G., and D'aquilio, M. 1981. Characterization of a potyvirus that causes zucchini yellow mosaic. Phytopathology 71:667-672.
11. Proeseler, G. 1978. Ubertragung des trepenmosaik-virus durch Getreidehahnchen (Coleoptera, Chrysomelidae) (Kurtze Mitteilung). Arch. Phytopathol. Pflanzenschutz 14:267-268.
12. Purcifull, D. E., and Batchelor, D. L. 1977. Immunodiffusion tests with sodium dodecyl sulfate (SDS)-treated plant viruses and plant viral inclusions. Fla. Agric. Exp. Stn. Tech. Bull. 788. 39 pp.
13. Purcifull, D. E., and Hiebert, E. 1979. Serological distinction of watermelon mosaic virus isolates. Phytopathology 69:112-116.
14. Zaitlin, M., and Israel, H. W. 1975. Tobacco mosaic virus (type strain). No. 151 in: Descriptions of Plant Viruses. Commonw. Mycol. Inst., Assoc. Applied Biologists, Kew, Surrey, England. 5 pp.