# Regurgitant as a Determinant of Specificity in the Transmission of Plant Viruses by Beetles

R. C. Gergerich, H. A. Scott, and J. P. Fulton

Department of Plant Pathology, University of Arkansas, Fayetteville 72701.
Published with the approval of the director, Arkansas Agricultural Experimental Station.
Accepted for publication 18 January 1983.

## ABSTRACT

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.

Regurgitant from leaf-feeding beetles (*Cerotoma trifurcata, Epilachna varivestis*, and *Diabrotica undecimpunctata*) contains a factor(s) that prevents infection by most viruses, but has no effect on beetle-transmissible viruses. When beetle feeding was simulated by gross wounding of leaf tissue and purified virus was applied during wounding, high levels of transmission of both beetle-transmissible and non-beetle-transmissible viruses were achieved. When inoculum was mixed with beetle regurgitant and applied using the gross wounding technique, however, there was a high level of transmission of beetle-transmissible viruses and a very low level of transmission of non-beetle-transmissible viruses. The regurgitant factor(s), which prevents infection by non-beetle-transmissible viruses, is heat labile, is stable to freezing, and has a molecular weight >50,000 daltons. The regurgitant factor(s) does not irreversibly inactivate non-beetle-transmissible viruses.

*Additional key words*: bean pod mottle virus, *Cerotoma trifurcata, Diabrotica undecimpunctata, Epilachna varivestis,* southern bean mosaic virus, tobacco mosaic virus, tobacco ringspot virus.

There is a high degree of specificity between beetle vectors and the plant viruses they transmit. Early investigators (3,5) suggested that the ability of beetles to transmit plant viruses is related to the regurgitation of virus from the foregut during feeding. Scott and Fulton (4) reported that beetles that had been acquisition-fed on virus-infected tissue deposited both a beetle-transmissible virus (southern bean mosaic virus [SBMV]) and a non-beetle-transmissible virus (the cowpea strain of tobacco mosaic virus [CP-TMV]) on the leaf surface during feeding. Although the host plant was equally susceptible to both viruses when inoculated mechanically, only SBMV was transmitted.

The infectivity of SBMV or CP-TMV in regurgitant remained at the same level as that in plant sap when both were stored for 7 days at room temperature (1). In view of this lack of evidence for a direct inactivation of plant viruses by regurgitant, it seemed possible that beetle regurgitant might interfere with the infection process of non-beetle-transmissible viruses, but have little or no effect on beetle-transmissible viruses. Therefore, we have utilized a virus inoculation technique that mimics the injury of beetle feeding to study the effect of beetle regurgitant on transmission of beetle-transmissible and non-beetle-transmissible viruses. Several properties of the factor(s) in beetle regurgitant that are responsible for the specificity of transmission are described.

## MATERIALS AND METHODS

**Viruses and virus purification.** Two beetle-transmissible viruses (SBMV and bean pod mottle virus [BPMV]) and two non-beetle-transmissible viruses (CP-TMV and tobacco ringspot virus [TRSV]) were used in this study. Southern bean mosaic virus, BPMV, and CP-TMV were purified from infected bean, *Phaseolus vulgaris* L. 'Black Valentine,' harvested 10–14 days after inoculation. Tobacco ringspot virus was purified from cucumber, *Cucumis sativus* L. 'Model,' harvested 6–8 days after inoculation. All viruses except CP-TMV were purified by chloroform-butanol extraction followed by two to three high- and low-speed centrifugations and resuspension in 0.01 M phosphate buffer, pH 7.2. Bean leaves infected with CP-TMV were homogenized in 0.01 M phosphate buffer (pH 7.2) containing 0.1 mM disodium EDTA. The homogenate was clarified by low speed centrifugation, and the virus was precipitated from the supernatant by the addition of 4% PEG (6000 MW) and 0.3 M NaCl. After 30 min of stirring, the virus was pelleted by low-speed centrifugation, resuspended, and purified further by alternate high- and low-speed centrifugation.

**Beetles and regurgitant collection.** Mexican bean beetles, *Epilachna varivestis* Muls., and bean leaf beetles, *Cerotoma trifurcata* (Forst.), were reared in the laboratory. Spotted cucumber beetles, *Diabrotica undecimpunctata howardii* (Barber), were collected from the field. Beetles were induced to regurgitate by holding the beetle between thumb and forefinger and teasing the mouthparts with a capillary glass tube, which was used to collect the emitted regurgitant. Regurgitant was used immediately or was stored at −20 or 4 C in closed glass capillary tubes until use.

**Inhibitory activity of regurgitant: local lesion assay.** Cultivar Pinto bean and cowpea, *Vigna unguiculata* (L.) Walp. subsp. *unguiculata* 'Georgia 21,' were used as local lesion hosts for SBMV and TRSV, respectively. The inhibitory effect of Mexican bean beetle regurgitant on SBMV or TRSV was tested by mixing purified viruses with twofold dilutions of regurgitant and making half-leaf comparisons on corundum-dusted indicator plants. A predetermined concentration of purified virus that gave 100–150 local lesions per half-leaf of cowpea or Pinto bean was used in these experiments. Half-leaves were inoculated with virus solution that had been mixed with buffer (0.01 M phosphate, pH 7.2) or with regurgitant.

**Selective inhibitory activity of regurgitant: Gross wounding inoculation technique.** The traditional method of inoculating plants using corundum as an abrasive produces a virus infection site in a manner quite different from the wounds produced by beetles during feeding (Fig. 1A,B). For this reason, a gross wounding technique was developed in an effort to simulate the type of damage done by beetles during feeding. Inoculation was accomplished by boring a leaf disk out of a leaf using a glass cylinder with an outside diameter of 7 mm and inside diameter of 6 mm. Immediately before cutting the disk the edge of the glass cylinder was dipped into an inoculum mixture (Fig. 1C). The effect of regurgitant on virus transmission was determined by adding regurgitant (or buffer as a control) to purified virus and inoculating a systemic host, cultivar Black Valentine bean, by using the gross wounding technique. After 2 wk all test plants were indexed for virus serologically by using the gel-diffusion technique.

**Centrifugation in membrane cones and dialysis.** Centrifugation in membrane cones, in addition to dialysis, was used to determine

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

whether the infectivity-affecting factor(s) in beetle regurgitant is a macromolecule. Mexican bean beetle regurgitant was centrifuged in Centriflo Type CF50A membrane cones (Amicon Corporation, Lexington, MA 02173), which are designed to retain molecules with molecular weights greater than 50,000 daltons. Mexican bean beetle regurgitant (100 μl) was diluted 1:10 with 0.01 M phosphate buffer (pH 7.2) and centrifuged in a cone until the volume was reduced to 100 μl. This sample was then washed by diluting 1:10 with the same buffer and centrifuging as above until the volume was again 100 μl. The resulting sample was mixed with purified CP-TMV and SBMV and tested for activity.

Mexican bean beetle regurgitant was dialyzed overnight against 0.01 M phosphate buffer (pH 7.2) at 4 C. The dialyzed regurgitant was mixed with purified CP-TMV and SBMV and tested for selective inhibitory activity by using the gross wounding technique of inoculation.

## RESULTS

**Inhibitory activity of regurgitant: Local lesion assay.** Mexican bean beetle regurgitant completely suppressed local lesion formation by both SBMV and TRSV when the regurgitant in the inoculum was diluted 1:20 or less. The dilution endpoint at which regurgitant no longer had an effect on local lesion formation of SBMV or TRSV was between 1:320 and 1:640 for both viruses.

**Selective inhibitory activity of regurgitant: Gross wounding inoculation technique.** Regurgitant from three species of leaf-feeding beetles prevented infection by CP-TMV and TRSV (non-beetle-transmissible viruses), but did not prevent infection by BPMV or SBMV when regurgitant was added to mixtures of these viruses and plants were inoculated by using the gross wounding technique (Table 1). When a regurgitant-virus mixture containing both CP-TMV and SBMV was diluted prior to inoculation, however, CP-TMV was also infectious (Table 2). When regurgitant was diluted prior to mixing with purified CP-TMV and the resulting mixture was inoculated by using the gross wounding technique, the inhibitory effect was not evident if the concentration of regurgitant in the inoculum was below 8%.

**Stability of regurgitant factor.** The inhibitory activity of Mexican bean beetle regurgitant against non-beetle-transmissible viruses was not affected by storage of the regurgitant for 20 days at 4 or −20 C in closed capillary tubes (Table 3). When regurgitant was heated for 3 min at 100 C, however, the inhibitory activity of the regurgitant was destroyed (Table 3).

**Macromolecular nature of regurgitant factor(s).** The factor(s) in Mexican bean beetle regurgitant, which affects the activity of CP-TMV, was retained by the Amicon Centriflo Type CF50A cone (Table 4). Since these cones are designed to retain molecules with molecular weights ≥50,000 daltons, this is evidence for the macromolecular nature of the regurgitant factor(s). Dialysis of Mexican bean beetle regurgitant did not reduce the specific inhibitory activity of the regurgitant against CP-TMV, which is further evidence for the macromolecular nature of the regurgitant factor(s).

TABLE 1. Effect of regurgitant upon transmission of two beetle-transmissible viruses (southern bean mosaic virus [SBMV] and bean pod mottle virus [BPMV]) and two non-beetle-transmissible viruses (the cowpea strain of tobacco mosaic virus [CP-TMV] and tobacco ringspot virus [TRSV]) to plants inoculated by using the gross wounding technique

| Virus[a] pairs in inoculum mixture[b] | Regurgitant in inoculum mixture | | |
|---|---|---|---|
| | Mexican bean beetle | Bean leaf beetle | Spotted cucumber beetle |
| SBMV | 28/30[c](29/29)[d] | 23/31(31/31) | 21/29(30/30) |
| CP-TMV | 0/30(19/29) | 1/31(18/31) | 1/29(17/30) |
| SBMV | 28/34(30/35) | ND[e] | ND |
| TRSV | 0/34(28/35) | ND | ND |
| BPMV | 33/46(46/47) | ND | ND |
| CP-TMV | 3/46(22/46) | ND | ND |

[a] Concentration of viruses in 0.01 M phosphate buffer (pH 7.2): SBMV = 30 mg/ml, CP-TMV = 30 mg/ml, TRSV = 15 mg/ml, and BPMV = 20 mg/ml.
[b] Inoculum consisted of equal parts of the two purified viruses and beetle regurgitant.
[c] Ratio of plants of cultivar Black Valentine bean 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 parts of each of the purified viruses and buffer (0.01 M phosphate, pH 7.2).
[e] Not determined.

TABLE 2. Effect of dilution on infectivity of CP-TMV[a] and SBMV[b] mixed with Mexican bean beetle regurgitant

| Dilution[c] of inoculum mixture[d] | Transmission of | |
|---|---|---|
| | SBMV | CP-TMV |
| Undiluted regurgitant + virus mixture | 27/28[e] | 0/28 |
| 1:66 dilution of regurgitant + virus mixture | 30/31 | 10/31 |
| 1:100 dilution of regurgitant + virus mixture | 27/28 | 7/28 |

[a] The legume isolate of tobacco mosaic virus, 30 mg/ml in 0.01 M phosphate buffer (pH 7.2).
[b] Southern bean mosaic virus, 24 mg/ml in 0.01 M phosphate buffer (pH 7.2).
[c] Diluted with 0.01 M phosphate buffer (pH 7.2).
[d] Inoculum mixture consisted of equal parts of purified CP-TMV, SBMV, and Mexican bean beetle regurgitant.
[e] Ratio of plants of cultivar Black Valentine bean that became infected to total plants in trial.

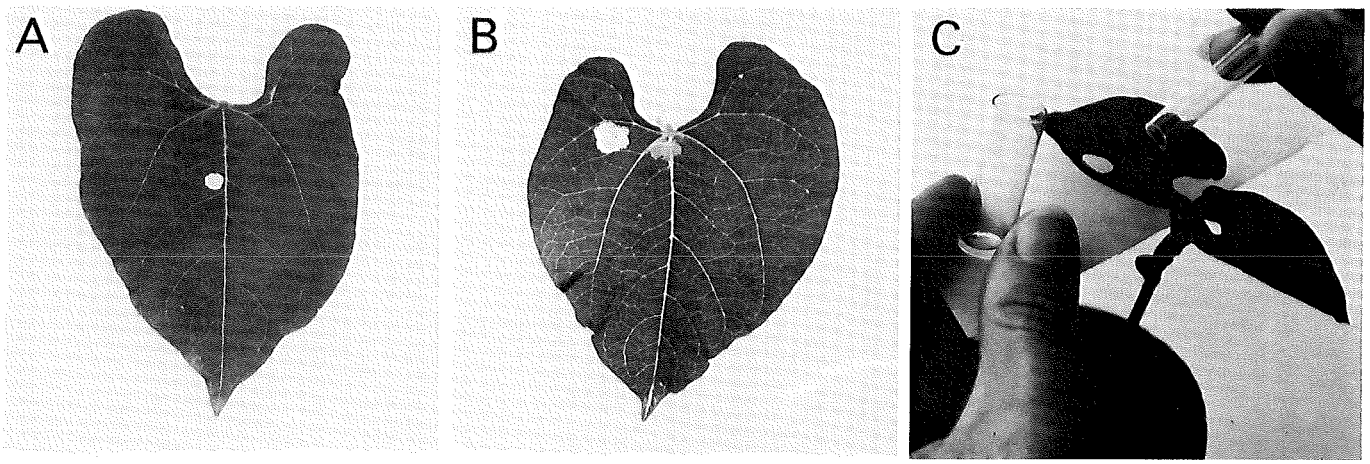


**Fig. 1.** Typical feeding damage caused on cultivar Pinto bean leaves by: **A,** bean leaf beetle; **B,** Mexican bean beetle. **C,** Simulation of feeding damage by using the gross wounding technique.

TABLE 3. Stability of Mexican bean beetle regurgitant factor(s)

| Inoculum[a] | Transmission of SBMV | Transmission of CP-TMV |
|---|---|---|
| Regurgitant + virus mixture | 23/26[b] | 0/26 |
| Frozen regurgitant[c] + virus mixture | 11/12 | 0/12 |
| Refrigerated regurgitant[d] + virus mixture | 12/12 | 0/12 |
| Heated regurgitant[e] + virus mixture | 28/30 | 14/30 |
| Buffer (0.01 M phosphate, pH 7.2) + virus mixture | 25/25 | 17/25 |

[a] Inoculum consisted of equal parts of purified southern bean mosaic virus (SBMV), the cowpea strain of tobacco mosaic virus (CP-TMV), and regurgitant or buffer.
[b] Ratio of plants of cultivar Black Valentine bean that became infected to total plants in trial.
[c] Regurgitant was stored in closed capillary tubes at −20 C for 20 days.
[d] Regurgitant was stored in closed capillary tubes at 4 C for 20 days.
[e] Regurgitant in a thin-walled glass tube was heated for 3 min in boiling water.

TABLE 4. Effect of centrifuging Mexican bean beetle regurgitant in membrane cones that retain molecules with molecular weights ≥50,000 daltons

| Inoculum mixture[a] | Transmission of SBMV | Transmission of CP-TMV |
|---|---|---|
| Untreated regurgitant + virus mixture | 26/30[b] | 3/30 |
| Centrifuged regurgitant[c] + virus mixture | 30/30 | 3/30 |
| Buffer (0.01 M phosphate, pH 7.2) + virus mixture | 29/30 | 24/30 |

[a] Inoculum consisted of equal parts of two purified viruses: Southern bean mosaic virus (SBMV), 24 mg/ml, and the cowpea strain of tobacco mosaic virus (CP-TMV), 30 mg/ml, and regurgitant or buffer.
[b] Ratio of plants of cultivar Black Valentine bean that became infected to total plants in trial.
[c] Regurgitant (100 μl) was diluted 1:10 with 0.01 M phosphate buffer (pH 7.2) and centrifuged in a Centriflo Type CF50A membrane cone until the volume was reduced to 100 μl. The concentrate was washed once with buffer and centrifuged in a cone until the volume was 100 μl.

## DISCUSSION

It appears that beetle regurgitant contains at least one factor that selectively prevents infection of plants by non-beetle-transmissible viruses and whose activity can only be demonstrated with the gross wounding technique and not with the conventional local lesion assay method. We believe that this selective inhibition by beetle regurgitant at least partially explains why beetles are capable of transmitting only certain types of plant viruses.

Very little is known about the chemical constituents of beetle regurgitant or about the origin of the regurgitant within the beetle. Leaf-feeding beetles do not have salivary glands, but they do have gnathal glands in the cephalic region, which may contribute to the contents of the regurgitant (6). The bulk of the regurgitant, however, probably originates from the gut. Kopek and Scott (2) showed that Mexican bean beetles that had fed on bromophenol blue-impregnated tissue produced blue coloring in induced regurgitant and deposited bromophenol blue on leaves during feeding. Bromophenol blue was observed in the gut of these beetles but not in the hemocoele, indicating that the source of the regurgitant is (at least in part) the beetle gut.

The regurgitant factor(s) is unlike other described inhibitors of virus infection in that it is ineffective against certain types of viruses, namely, viruses that are transmitted by beetles. An important feature of this inhibitor is that its selective nature is only evident if the gross wounding method of inoculation is used. This method of inoculation is quite different and less efficient than rub-inoculation with corundum. It resembles the type of inoculation that is done by beetles during feeding in that the inoculum is applied during perforation of the leaf, much like viruliferous regurgitant is applied by the beetle as it feeds.

The selective inhibitor from beetle regurgitant does not irreversibly bind to non-beetle-transmissible viruses because the original virus regains its infectivity when the regurgitant:virus mixture is diluted prior to inoculation with the gross wounding technique. In view of this lack of evidence for a direct interaction of the regurgitant factor(s) with CP-TMV, we believe that the factor(s) must function either by affecting the interaction of the virus with the host or by having an effect on the host itself.

At the present time, there is no explanation why the gross wounding inoculation technique is necessary to demonstrate the selective nature of this inhibitor in beetle regurgitant. Perhaps the sites of infection that are made available by the gross wounding technique of inoculation are different from the sites of infection made available by rub-inoculation. If this is so, the regurgitant factor(s) may be effective only for the particular sites of infection made available by the gross wounding procedure.

## LITERATURE CITED


1. Fulton, J. P., Scott, H. A., and Gamez, R. 1980. Beetles. Pages 115–132 in: Vectors of Plant Pathogens. K. F. Harris and K. Maramorosch, eds. Academic Press, New York. 467 pp.
2. Kopek, J. A., and Scott, H. A. 1983. Southern bean mosaic virus in Mexican bean beetle and bean leaf beetle regurgitants. J. Gen. Virol. (In press).
3. Markham, R., and Smith, K. M. 1949. Studies on the virus of turnip yellow mosaic. Parasitology 39:330-342.
4. Scott, H. A., and Fulton, J. P. 1978. Comparison of the relationships of southern bean mosaic virus and the cowpea strain of tobacco mosaic virus with the bean leaf beetle. Virology 84:207-209.
5. Smith, C. E. 1924. Transmission of cowpea mosaic by bean-leaf beetle. Science 60:268.
6. Srivastava, U. S. 1959. The maxillary glands of some Coleoptera. Proc. R. Entomol. Soc., Lond. (A). 34:57-62.