Hemolymph as a Reservoir for the Cowpea Strain of Southern Bean Mosaic Virus in the Bean Leaf Beetle

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Partially supported by CSRS Grant 816-15-16.

Accepted for publication 11 December 1970.

ABSTRACT

The hemolymph of the bean leaf beetle, *Cerotoma trifurcata,* contained the cowpea strain of southern bean mosaic virus (CP-SBMV) after an acquisition feed on infected cowpea. Beetles transmitted CP-SBMV following injection of purified virus into the hemocoel. The patterns of transmission by beetles acquiring CP-SBMV by feeding or by injection were similar. Recovery from the hemolymph decreased with time after virus acquisition by injection or feeding. The virus was recovered from all areas of the intestinal tract of dissected beetles. Survival of CP-SBMV in the hemolymph for at least 10 days and the demonstration of the hemolymph as a virus source for transmission indicated that bean leaf beetle hemolymph serves as a CP-SBMV reservoir. Phytopathology 61:538-540.

Insects with chewing mouthparts have long been known to transmit plant viruses, but the virus-vector relationship has not been thoroughly explored. In 1956, Freitag (1) recovered squash mosaic virus (SqMV) from the hemolymph of a small percentage of western striped cucumber beetles, *Acalymma trivittata,* and western spotted cucumber beetles, *Diabrotica undecimpunctata undecimpunctata,* following a virus-acquisition feed. This indicated that a complex virus-beetle relationship exists. A review by Walters (6) discusses the aspects of virus transmission by beetles.

Walters (5) reported the bean leaf beetle, *Cerotoma trifurcata,* to be an efficient vector of the cowpea strain of southern bean mosaic virus (CP-SBMV). Beetles transmitted CP-SBMV in high percentages the first 5 days after acquisition (6), with intermittent transmissions up to 19 days (7).

Studies were conducted to further elucidate relationships between CP-SBMV and the bean leaf beetle. Specifically, we sought to determine (i) whether the virus occurred in the hemolymph of viruliferous beetles; and (ii) if so, how long it persisted; (iii) whether the bean leaf beetle would become viruliferous after injection of purified virus into the hemocoel; and (iv) whether the virus would accumulate in any portion of the intestinal tract.

MATERIALS AND METHODS.—Bean leaf beetles, *Cerotoma trifurcata* (Forst.), were collected from soybeans in the Arkansas River Valley near Alma and on the University of Arkansas Experiment Station at Fayetteville. Collected beetles were allowed to feed on CP-SBMV-infected cowpea, *Vigna sinensis* (Torner) Savi 'Monarch', for 24 hr. Beetle hemolymph was assayed for virus immediately or beetles were transferred to healthy plants for assay at a later date. Beetles were maintained en masse on an immune variety of soybean, *Glycine max* (L.) Merr. 'Lee 68', to obtain large assay numbers with minimum handling; or single beetles were transferred daily to Monarch cowpea plants to correlate transmissions with virus recovery from the hemolymph. Beetles injected with CP-SBMV were always individually transferred daily to Monarch cowpea plants. Hemolymph was assayed for CP-SBMV by inoculating Carborundum-dusted Monarch cowpea. These plants, as well as Monarch cowpea fed on by beetles, were assayed for CP-SBMV on Georgia 21 cowpea, a local lesion host, or by Ouchterlony gel-diffusion tests (2). Monarch and Georgia 21 cowpea seed were obtained from virus-free plants grown in screenhouses.

Hemolymph recovery.—Beetles anesthetized with carbon dioxide were immersed briefly in a 0.525% sodium hypochlorite solution and rinsed in distilled water to preclude any chance of surface contamination from acquisition hosts before collecting hemolymph. Undiluted hemolymph was collected on a scalpel for test plant inoculation after basally severing one or both metathoracic legs. When a second collection was made and metathoracic legs were unavailable, the wings were basally severed with a razor blade.

Injections.—Bean leaf beetles were injected with CP-SBMV to determine whether they could become viruliferous from a virus source located in the hemolymph. Beetles were anesthetized and wings severed. Injections were made dorsally just beneath the integument between the first three to four abdominal segments, using a 32-gauge hypodermic needle with an electrolytically tapered point (Hamilton Co., Whittier, Cal.). Injections were administered with a Model M Microapplicator (Instrumentation Specialties Co., Lincoln, Neb.). The beetles were injected with a maximum dose of 72 µg of purified CP-SBMV in 6 µliters of buffer. Backflow along the needle probably caused a smaller actual dose to be administered. The CP-SBMV preparations were purified by the procedure described by Shepherd & Fulton (3). Preparations were highly infectious and exhibited a single component (110 S) in a Beckman Model E ultracentrifuge.

Dissections.—The intestinal tract of dissected bean leaf beetles was assayed in four parts: head and foregut, anterior midgut, posterior midgut, and hindgut. Dissections were made in a solution of physiological saline. Extracted tissues were dipped in a 0.525% sodium hypochlorite solution followed by a distilled water rinse. The externally cleansed tissues were ground on microscope slides by flattened glass rods before inoculation to Monarch or Georgia 21 cowpeas.

RESULTS.—*Virus recovery from hemolymph.*—Hemo-

lymph assays from single beetles immediately following a 24-hr acquisition feed infected 32 of 64 Monarch cowpeas in a series of four tests. Virus recovery decreased with time after acquisition. Ten of 113 plants became infected from beetle hemolymph assayed after 5 days, and 1 of 92 plants became infected when inoculated with hemolymph recovered 10 days after acquisition. No plants became infected by hemolymph from 82 beetles checked individually after 20 days, or from 9 beetles checked individually after 30 days.

Virus recovery from the hemolymph of injected beetles followed the same trend as recovery following natural acquisition. Twenty-five of 27 plants inoculated with beetle hemolymph 24 or 48 hr after injection of the beetles with CP-SBMV became infected. Ten days after injection, virus was recovered from 4 of 15 beetles.

Transmission relationships.—Transmission rates following a 24-hr acquisition feed corresponded to reports by Walters (6) that bean leaf beetles efficiently transmit CP-SBMV for at least 5 days (Table 1). Fifty-six of 59 beetles that fed on an acquisition host for 24 hr transmitted. Transmission rates fell from 80% the first day to 22% the 5th day. Only scattered transmissions were observed after 7 days.

The pattern of transmission by beetles injected with CP-SBMV was remarkably similar to that of beetles which had acquired virus by natural means (Table 2). Thirty-five of 58 injected beetles that fed transmitted at least once, and many transmitted to several plants. Most beetles transmitted within the first 24 hr; however, one beetle did not transmit until the 8th day. Transmission became sporadic after 4 days. Twenty of the original 80 injected beetles died without feeding. Presumably, death was due to septicemia.

It might be argued that transmission following injection resulted from rupture of the gut wall. Our observations, however, indicated that when gut rupture occurred the beetles died quickly. Injections of distilled water made with the hypodermic needle extending deep into the body cavity of 20 beetles killed all beetles within 1 hr. Also, several beetles which had transmitted were dissected, and no evidence of gut ruptures was found.

Location of virus in beetle tissue.—Examination of the beetle intestinal tract to define possible areas where virus particles might accumulate or pass into the hemocoel produced no clear results. Virus was recovered from all sections of the intestinal tract up to 8 days following a 24-hr acquisition feed. In two isolated cases, 1 of 40 beetles dissected after 10 days and 1 of 36 beetles dissected after 20 days, the posterior midgut only contained virus. Similarly, all sections of the beetle intestinal tract were found to contain virus 24-48 hr after injection.

Discussion.—Bean leaf beetle hemolymph serves as a CP-SBMV reservoir. The injection results show that the bean leaf beetle may become viruliferous from CP-SBMV contained in the hemolymph, and that the virus can be transmitted by the beetle at separated intervals. During the first few days after a virus-acquisition feed, virus contained within the crop of the bean leaf beetle is probably sufficient for the high levels of transmission. But, for a bean leaf beetle to transmit 19 days following an acquisition feed, there must be a place in the intestinal tract or the hemocoel where the virus may remain free and capable of being used to infect a new host. Therefore, it seems likely that the bean leaf beetle draws on the virus reservoir in the hemolymph during sporadic periods of transmission.

Table 1. Transmission patterns by bean leaf beetles given a 24-hr acquisition feed on cowpea southern bean mosaic virus

Transfers[c]	Transmissions by individual beetles[a]										Total transmissions[b]
	A	B	C	D	E	F	G	H	I	J	
1	+	+	−	+	+	+	0	+	−	+	39/49
2	+	+	−	−	−	+	+	+	−	+	37/54
3	+	+	−	−	−	+	−	+	−	+	19/49
4	+	+	+	−	−	+	−	+	−	−	21/47
5	−	+	+	−	−	−	−	+	−	+	10/46
6	+	+	+	+	−	−	−	−	−	−	10/55
7	+	+	−	+	−	−	−	−	+	−	5/49
8	−	−	−	−	−	−	0	−	−	−	1/47
9	−	−	−	−	−	−	+	−	−	−	3/50
10	+	−	−	+	−	−	−	−	−	−	2/45
H[d]	−	−	−	−	−	+	−	−	−	−	1/52
11	−	−	−	−	−	−	−	−	0	−	0/34
12	−	−	−	−	−	0	−	−	−	−	0/42
13	−	−	−	−	+	×	−	−	−	−	1/43
14	−	−	−	−	−		−	−	−	−	0/42
15	−	−	−	−	−		−	−	−	−	0/42
16	−	−	−	−	−		−	−	−	−	0/41
17[e]	−	+	−	−	−		−	−	−	−	1/39
H[d]	−	−	−	−	−		−	−	−	−	0/40

[a] A-J are selected beetles demonstrating the variation in infection patterns. + = infected plant; − = no infection; 0 = no feeding; × = death of beetle.

[b] Numerator is number of beetles that transmitted; denominator is number of beetles that fed.

[c] First transfer to Monarch cowpea following 24-hr acquisition feed. Subsequent transfers at 1-day intervals.

[d] Beetle hemolymph was assayed for virus after 10 days and again after 20 days.

[e] Transfers continued for 3 more days with no further transmission.

TABLE 2. Transmission patterns by bean leaf beetles injected with purified cowpea southern bean mosaic virus

Transfers[c]	Transmissions by individual injected beetles[a]																			Total transmissions[b]
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	
1	−	+	−	0	0	−	−	+	−	−	−	−	−	+	−	+	−	+	+	−	16/44[d]
2	+	+	−	+	0	−	0	−	+	+	−	−	+	+	−	+	−	−	+	+	15/37[d]
3	−	+	−	−	0	−	0	−	−	+	+	−	+	×	−	+	+	+	0	−	7/27
4	−	+	0	−	+	−	0	×	+	−	−	−	+		+	−	−	−	−	+	6/24
5	−	×	−	+	−	−	+		−	−	−	−	−		−	−	−	×	−	×	2/22
6	−		+	−	−	+	0		−	−	−	−	−		−	−	−		−		2/20
7	−		0	×	+	−	+		−	−	−	−	0		−	−	−		−		2/17
8	×		×		−	+	+		−	−	−	+	−		−	+	−		−		4/18
9					×	+	0		−	−	−	−	−		−	−	−		−		1/14
10						−	0		−	−	−	−	−		−	−	−		−		0/14
H[e]						−	−		−	−	−	+	−		−	+	+		+		4/15

[a] A-T are selected beetles demonstrating variations in infection patterns. + = infection; − = no infection; 0 = no feed; × = death of beetle.

[b] Numerator is number of beetles that transmitted; denominator is number of beetles that fed.

[c] First transfer to Monarch cowpea immediately following injection of virus. Subsequent transfers at 1-day intervals.

[d] Transmissions by beetles dissected 24 or 48 hr after injection are included. Ten of 18 beetles dissected after 24 hr, and 5 of 7 beetles dissected after 48 hr, transmitted.

[e] Beetle hemolymph was assayed for virus after the 10th day.

The mechanism by which virus particles pass between the intestinal lumen and hemocoel is unknown. Movement of digested food particles into the hemocoel primarily takes place within the midgut region of the intestinal tract of insects (8). Recently, Tanada & Leutenegger (4) showed virus particles in the midgut basement membrane of an insect host. Therefore, the midgut would seem the most likely place for movement of virus particles. In this study, however, virus activity was associated with all portions of the gut for several days after virus acquisition.

Decreased transmission by beetle vectors with time following virus acquisition indicates that CP-SBMV replication in bean leaf beetles does not occur. The reduced amount of CP-SBMV in bean leaf beetle hemolymph with time also indicates a lack of multiplication. It would be interesting to determine the mechanism promoting the gradual reduction of virus particles in the hemolymph. Phagocytosis by hemocytes, a reversed diffusion gradient, or gradual inactivation of virus particles are possibilities.

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