Estimates in the Latent Period of Strawberry Crinkle Virus in the Aphid *Chaetosiphon jacobi* as a Function of the Experimental Design

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ABSTRACT

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Two experimental treatment designs, undisturbed feeding versus serial transfer, were used to estimate the median latent period of the strawberry crinkle rhabdovirus in the aphid, *Chaetosiphon jacobi*, inoculated by injection with infectious insect extract. The serial transfer design resulted in a significantly ($P<0.01$) shorter latent period estimate than that obtained under conditions of undisturbed feeding (5.85 versus 8.24 days). The results support the hypothesis that inoculation of host plants by viruliferous aphids is more effective during active stylet penetration and salivary sheath formation than during feeding.

Transmission of circulative aphidborne plant viruses has been studied continuously since the initial work of McClintock and Smith (5) on spinach blight. Phases of the transmission process including acquisition, latent period, inoculation, and retention have been identified. These are discussed in recent reviews of the circulative and propagative aphidborne plant virus literature (4,9), and a general model has been proposed for estimating the key parameters of these phases of the transmission process (3).

Several parameters have been used to characterize the latent period phase of the transmission process. Since the latent period is manifested as a distribution of time intervals that separate acquisition and inoculation phases of the transmission process, it is generally agreed that the median latent period (LP_{50}) provides the most useful and robust single-parameter measure of the latent period (8).

The methodology used to obtain data sets from which the LP_{50} can be estimated have varied, depending upon the species of aphid and the plant-virus complex being studied. For example, aphids may acquire virus by feeding on a source plant or inoculum can be introduced into the haemocoel by injection. However, inoculation data usually have been generated by using a serial transmission experimental design with test insects transferred at intervals to a series of disease-free test plants. The plants are then held long enough for those that are infected with virus to develop symptoms. The distribution of infected and uninfected plants is used to estimate the LP_{50} as well as parameters associated with individual vector efficiency and the retention period (6).

Periodically, feeding of the aphids is artificially disturbed by aphid transfers with this experimental design. The question of whether interruption of feeding measurably affects the

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transmission process was explored in the tranmission of yellow-net by the green peach aphid, *Myzus persicae* (7). It was shown that transmission efficiency increased when the aphids' feeding was disturbed. More recently (12), estimates of the LP_{50} for pea enation mosaic virus were found to decrease as the post-acquisition transfer intervals were shortened. These results support a hypothesis that inoculation is most likely to take place during stylet penetration to the phloem and salivary sheath formation (7).

An alternative to a serial transmission experimental design, one that minimizes the artificial disturbance feeding activities of the aphids during the inoculation access period, is to use a series of different inoculation access periods for aphids that otherwise are treated alike. Aphids are exposed to the virus; each aphid is then transferred to a single disease-free plant where it is allowed to remain for a predetermined inoculation access period. Subsequently, each aphid is transferred to a final sequence of one or two test plants on which it is allowed to remain for an extended period of time. This experimental design was recently used to study the transmission of pea enation mosaic virus by the pea aphid, *Acrythosiphon pisum* (3).

In the experiments reported here we compared estimates of LP_{50} in the transmission of the strawberry crinkle rhabdovirus, which is propagative in the aphid *Chaetosiphon jacobi*, by analyzing comparative data generated from a serial transmission experiment and a variable inoculation access period experiment.

MATERIALS AND METHODS

A clonal line of *C. jacobi* was reared in growth chambers set for a 12-hr light/dark photophase at approximately 20 and 15 C, respectively, on Alpine strawberry seedlings, *Fragaria vesca* L. var. *semperflorens* (2). Test plants were two- to four-leaf Alpine strawberry seedlings that had been raised from greenhouse-grown seed.

Virus isolate. The isolate of strawberry crinkle virus (SCV) was originally collected from a commercial strawberry cultivar (2) and was maintained in *C. jacobi* infected by feeding or by injection. The virus isolate was previously passed twice through the insect and the donor aphid had been frozen at −65 C for 9 mo before being used to inoculate the donors subsequently used in this work.

Injections. Heads of seven infected donors, which had transmitted SCV to test plants, each were triturated in 5 μl of cold distilled water (11), pooled, and kept at 4 C until used (0.5–7.5 hr).

Recipient young adult *C. jacobi* (average age, 13 days) were prefasted at 4 C for 3–8 hr prior to injection. After anesthetization with CO_2, insects were injected, using glass needles (10), with inoculum from small drops placed on a Parafilm-covered dish containing ice. Groups of 6–12 insects were injected from each droplet and distributed among 12 small plastic boxes. When 15 injected insects had been collected in each box, the entire procedure was repeated to give a total of 30 insects in each box. Forty-six spares also were injected.

Assay. Injected insects were caged singly, in cloth-capped acetate-butyrate cylinders, on *F. vesca* test seedlings 20 min to 7.3 hr after injection; dead or injured aphids were replaced by spares. Ten treatments were set up with 90 temperature-preconditioned plants in the first treatment (serial transfers) and 30 in each of the other nine (variable access periods) treatments. Remaining spares also were set up. All caged plants then were placed in a growth chamber kept at 25 C and constantly lighted at 8,600–11,000 lux at plant level. Subsequent transfers to fresh test plants were made as follows: For treatment 1, aphid transfers to fresh test plants were made serially every 24 hr. For treatment 2, each aphid was allowed to feed for 24 hr on a test plant, then was transferred to a second test plant for a 9-day inoculation access period, and then to a third test plant for 10 more days. In treatments 3–10, each aphid was allowed an extra 24-hr inoculation access period on the first test plant, and 24 hr less on the second test plant. Thus, insects in treatment 2 were moved after an initial inoculation access period of 23.8 hr and in treatment 10, after a 216.45-hr access period. The access time on the third set of test plants was 10 days in all treatments. This final transfer, which was made 10 days after the start of the experiment, insured that the aphids' feeding was disturbed at least once during the final tests for aphid infectivity in treatments 2 to 10, at a time when the virus latent period was almost certain to have been completed.

At the time of the first change, 23.8-hr after the injection, when the aphids in treatments 1 and 2 were transferred, all dead insects were replaced with the spares. The remaining spare insects were then added to the 10 treatments series, seven in the first series, and four in each of the remaining nine. After each transfer or removal of the test aphids, the plants were fumigated with nicotine, placed in a greenhouse, and observed for symptom development. An unexpected infection of any of hundreds of test seedlings or colony stock plants, either before or after colonization with virus-free aphids, has yet to be observed. SCV is inefficiently acquired by *C. jacobi*, with an expected maximum infectivity rate of about 10% with aphids reared on virus-infected strawberry plants.

TABLE 1. Cumulative inoculation frequency and log-probit analysis of data from the serial transmission experiment

Time t (days)	Cumulative inoculation frequency, Y_t	ln(t)	Probit (Y_t)
0.9	0/86	...[a]	...
1.0	0/86	...	...
2.9	0/86	...	...
3.9	0/86	...	...
4.9	11/86	1.59	3.86
5.9	42/86	1.77	4.97
6.9	77/86	1.93	6.26
7.9	83/86	2.07	6.81
8.9	85/86	2.19	7.27
9.9	86/86	...	...

Linear least squares fit of Probit (Y_t) = $a + b \ln(t)$, in which $r^2 = 0.98$, $a = -5.297$, and $b = 5.828$.

Corresponding log-normal distribution,
$Y_t = [1/\sqrt{2\pi}\,\sigma] \int_0^t e^{-(1/2)[(\ln\tau - \ln(LP_{50}))/\sigma]^2} d\tau$: $LP_{50} = 5.85$; $\sigma = 0.17$.

[a] ... Means probit transformation cannot be used.

RESULTS AND DISCUSSION

It took about 6 hr to inject the approximately 400 aphids used in the experiment. However, for calculation purposes, each aphid was regarded as having been inoculated in the middle of this period. For the group of aphids inoculated first and last, this represented only a small percentage displacement with respect to the estimates obtained for the LP_{50}. Since the aphids, after inoculation, were placed at random in the various treatment groups, the error introduced was distributed in an unbiased manner among the treatments and does not invalidate the conclusions reached.

For the serial transmission treatment, 97 injected aphids were placed on plants and transferred to new plants at 0.9 days, 1.9 days, and every day thereafter up to 9.9 days when they were finally transferred to the readout set of plants on which they were allowed to remain for another 10 days. Three aphids died before transmitting and eight aphids did not transmit to any plant including those in the final readout set. All transmitting aphids inoculated plants prior to being placed on the final set of readout plants. Thus, 86 of the 97 (88.7%) were shown to have acquired virus.

The time period in which each aphid first transmitted, as indicated by symptoms on the test plants, was noted and used to derive the cumulative frequencies listed in Table 1. A log-probit analysis provided an excellent regression (98% of the variation was accounted for by the fit) and a value of 5.85 days was obtained for the LP_{50} (Fig. 1).

The data from the variable inoculation access period trial paralleled those obtained for the serial transmission treatment in that inoculation access periods of 0.9, 1.9, 2.9, ..., 8.9 days were used. The results are listed in Table 2. A log-probit analysis of these data again gave an excellent regression fit (96% of the variation was

TABLE 2. Cumulative inoculation frequency, a log-probit analysis and a demonstrated acquisition analysis of the variable inoculation access period data

Length of IAP t (days)	Number of: Inoculations during IAP (n_i)	Number of: Aphids acquiring (n_a[a])	Number of: Aphids tested (n)	$\ln(t)$	Probit of cumulative inoculation frequency [Probit $(Y_t - n_i/n_a)$]	Percent acquisition (n_a/n)
0.9	0	25 (15)	34	...[b]	...	74.5
1.9	0	31 (21)	34	...	...	91.2
2.9	0	29 (18)	34	...	...	85.3
3.9	0	26 (22)	33	...	...	78.8
4.9	0	27 (20)	34	...	...	79.4
5.9	2	27 (22)	33	1.77	3.55	81.8
6.9	7	27 (25)	31	1.93	4.35	87.1
7.9	7	20 (19)	30	2.07	4.62	66.7
8.9	16	24 (23)	33	2.19	5.43	72.7
						Mean = 79.8
						s = 7.65

Linear least squares fit of Probit $(Y_t) = a + b \ln(t)$, in which $r^2 = 0.96$, $a = -3.877$, $b = 4.203$.

Corresponding log normal distribution, $Y_t [1 \sqrt{2\pi}\sigma] \int_0^t e^{-(1/2)[(\ln\tau - \ln(LP_{50}))/\sigma]^2} d\tau$: $LP_{50} = 8.26$ days, $\sigma = 0.23$.

[a] The figures in parentheses are data from the second test plants alone. The combined data from the second and third test plants were used in the probit analysis. All aphids were transferred to the third test plant 10 days after injection.
[b] ... Means probit transformation cannot be used.

explained by the regression). This treatment, however, yielded a substantially larger value for the LP_{50}, 8.24 days versus 5.85 days (a 41% increase). The two sets of cumulative inoculation periods were clearly different when examined visually (Fig. 1).

In the variable inoculation access period treatment (Table 2), an average of 79.8% of the aphids acquired virus. These data also yielded an unbiased estimate of the population variance, $s^2 = 58.6$ ($s = 7.64$) where $s^2 = [n/(n-1)] \times$ sample variance. To obtain these estimates the fact was ignored that the sample size for each inoculation access period was not the same (due to one or more aphid fatalities in some samples). The differences were sufficiently slight, however (30–34 aphids per sample), that they had less effect on the estimates than did the error incurred by rounding off values to two significant figures.

These acquisition data were obtained by examining both the second and third sets of test plants in treatments 2 to 10. Using the data from the first three treatments of the variable access time (Table 2) and comparing, respectively, the acquisition data obtained from the second test plants only, and the second and third test plants combined, we see that 31 of 85 (36.5%) infective aphids did not transmit virus within the initial 10 days of feeding. Comparable figures for the last three treatments are four of 71 (5.6%). The essential difference between the first and the last three treatments listed in Table 2 is that in the former, all aphids were transferred to the second test plant well before the serial transmission LP_{50} (5.85 days) had been completed, while in the last three treatments all aphids were transferred after this LP_{50} was over. Thus, even though the aphids fed on the second test plant until well after the undisturbed-feeding LP_{50} (8.26 days) had occurred, it appears that the probability of virus transmission, given that the aphid had acquired virus, was strongly dependent on whether the transfer took place before or after the LP was completed. These results certainly are compatible with the "inoculation during stylet penetration and salivary sheath formation" hypothesis.

Evidence for a difference in the percentage of inoculative aphids realized in the variable inoculation experiment (79.8%) compared to that obtained in the serial transmission experiment (88.7%) was not significant (adjusted $\chi^2 = 3.36$, df 1, $P > 0.05$).

The question now remains as to the significance of the evidence for a difference between the estimates of the LP_{50} as obtained from the two experimental treatment designs. The standard analysis for obtaining the confidence interval around a regression line (1) can be used to obtain points, $A = 6.4$ days and $B = 6.8$ days (depicted in Fig. 1), in which A and B are such that

$$\text{prob}\,[LP_{50} > A = 6.4 \text{ days} \mid \text{serial transmission data}] < 0.01$$

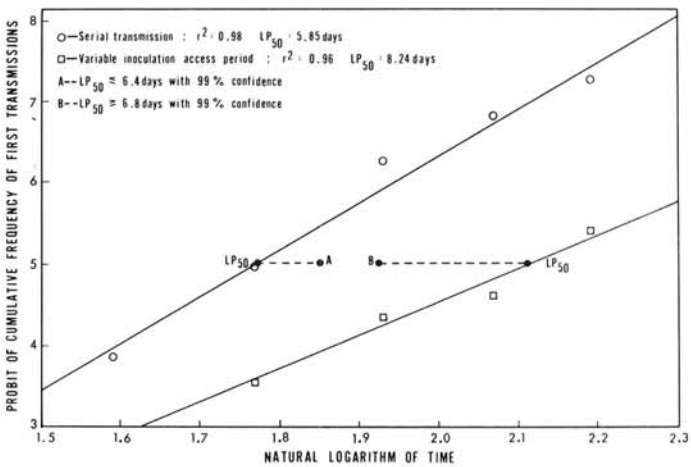

Fig. 1. Log-probit analysis of the cumulative frequency of first transmissions of strawberry crinkle virus to Alpine strawberry by injected *Chaetosiphon jacobi* aphids, using two different experimental designs.

and

$$\text{prob}\,[LP_{50} < B = 6.8 \text{ days} \mid \text{differential IAP data}] < 0.01,$$

thus the difference between the two estimates of the LP_{50} are highly significant.

The experimental results strongly support the hypothesis that successful inoculation of a plant is more likely to occur during stylet penetration and salivary sheath formation than during feeding. Furthermore, since the variable inoculation access period experimental design is a better approximation to reality than is the serial transmission experimental design for sedentarily feeding aphids (since aphids are periodically artifically disturbed in the latter), the former experimental design may yield a more reliable estimate of the LP_{50} for the field situation.

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