Vector Relations

Transmission of Potato Leafroll Virus from Plants and Artificial Diets by *Myzus persicae*

J. F. J. M. van den Heuvel, T. M. Boerma, and D. Peters

Department of Virology, Binnenhaven 11, 6709 PD Wageningen, Netherlands.
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ABSTRACT

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To analyze quantitatively the transmission characteristics of potato leafroll virus (PLRV) by *Myzus persicae*, acquisition from purified virus-containing artificial diets and transmission efficiency were studied. Multiple regression of the data revealed that the amount of viral antigen in the nymphs after feeding on PLRV-containing artificial diets is linearly related to the $\log_{10}$ transformed virus concentration in the diet and the length of the acquisition access period. The percentage of viruliferous nymphs was also linearly related to the logarithm of the virus concentration in the diets. These relationships indicate that the amount of viral antigen present in aphids can be used as a parameter in deducing the amount of virus available in a source for acquisition by aphids. The PLRV concentration in the diets did not influence the length of the median latency period (LP50) in *M. persicae*. However, the LP50 was affected by intrinsic properties of the virus and the age of the *M. persicae* nymphs used. PLRV purified from top leaves of *Physalis floridana* was transmitted with a significantly shorter LP50 than virus purified from bottom leaves. This difference was also observed when aphids acquired the virus from top or bottom leaves, hence, showing that purification did not affect the relative transmissibility of the virus. After acquiring purified PLRV from an artificial diet, 1-day-old *M. persicae* nymphs were more efficient in transmitting the virus than 4-day-old nymphs. To obtain 50% viruliferous nymphs, the virus concentration needed in the diet was significantly lower for young nymphs than for older ones. Furthermore, the LP50 of the virus was significantly shorter in the younger nymphs.

Additional keywords: cocktail-ELISA, enzyme amplification, membrane feeding, persistent virus transmission.

Persistent virus transmission by aphids encompasses the processes of acquisition, circulation, and inoculation. To describe virus transmission in a quantitative manner, the concepts of acquisition access period (AAP), latency period (LP), and inoculation access period (IAP) have been introduced (12). The LP of potato leafroll virus (PLRV) recorded in *Myzus persicae* (Sulz.) ranges from 5 min, measured as the minimum LP, to 123 hr, measured as the maximum LP (7,13,20). Differences in the minimum or maximum values of the LP are difficult to analyze statistically. In these studies, the length of the AAP and IAP, the PLRV source, the test plant species, the number of aphids per test plant, and the age of the vectors involved varied considerably (13). Sylvester (17) introduced the median LP (LP50) of which the fiducial limits are more readily determined. The LP50 can be estimated from repeated aphid transfers (17) and may be affected by the experimental design (21). The LP50 is commonly used to characterize virus isolates with respect to their transmissibility (e.g., 1,8,10). However, there is no consensus as to whether differences in the LP are caused by the virus concentration in the sap ingested (17,18) or injected into the aphids (2), or by intrinsic biological properties of the virus.

Peters (12) showed that the length of the LP50 of PLRV in *M. persicae* nymphs increased with the severity of symptoms on the *Physalis floridana* Rydb. (Soy) plants used as the virus source. Similarly, van den Heuvel and Peters (24) observed that the LP50 was significantly longer when *M. persicae* nymphs acquired this virus from *P. floridana* bottom leaves with pronounced symptoms

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rather than from symptomless top leaves. Additionally, a lower percentage of the nymphs that fed on bottom leaves was viruliferous as compared with those that fed on the top leaves. However, the concentration of viral antigen measured by an enzyme-linked immunosorbent assay (ELISA) was higher in the bottom leaves than in the top leaves (24). These results indicate that the acquisition of the virus by aphids declines with increasing infection age and symptom severity.

This study aims to quantify the amount of virus ingested by the aphids that fed on leaves with different symptoms caused by PLRV and to elucidate whether the dose of virus acquired is responsible for the observed differences in the transmission characteristics. For these purposes, we studied PLRV acquisition and transmission by *M. persicae* from artificial diets with known concentrations of the virus. The amount of viral antigen present in *M. persicae* nymphs, and the transmission characteristics like the LP50 and the percentage of viruliferous nymphs after an AAP on virus-containing artificial diets were compared with those of nymphs after similar AAPs on top or bottom leaves of *P. floridana* infected with PLRV. Furthermore, the transmission of the virus purified from top and bottom leaves of infected *P. floridana* plants and acquired by *M. persicae* from artificial diets was investigated.

MATERIALS AND METHODS

Aphids. *M. persicae* biotype WMp2 (14) was reared on *Brassica napus* L. subsp. *oleifera* (oilseed rape) in a greenhouse compartment at 20 ± 3 C with a photoperiod of 16 hr per day. Cohorts of similar-aged nymphs were produced by daily transfer of mature apterae, confined to leaf cages, to new oilseed rape plants.

Virus maintenance and purification. The Wageningen isolate of PLRV was maintained by repeated aphid transfers on seedlings of *P. floridana*. They were inoculated by one viruliferous *M. persicae* nymph in a 2-day inoculation access period (IAP). After inoculation, seedlings were kept in a greenhouse at 25 ± 3 C for symptom development.

PLRV was purified from *P. floridana* leaf material infected with PLRV by using a modified enzyme-assisted purification procedure (22). Frozen leaf material was homogenized with 0.1 M sodium citrate buffer (2 ml/g), pH 6.0, containing 0.5% cellulase Onozuka R-10, 0.5% macerozyme R-10 (Yakult Honsha Co. Ltd, Tokyo), 0.1% thioglycolic acid, and 0.5% ethanol in a blender. The homogenate was stirred for 4 hr at 25 C and emulsified with one-half of the volume of a 1:1 (v/v) mixture of chloroform and 1-butanol. After low-speed centrifugation, 1% Triton X-100, 8% polyethylene glycol, and 0.4 M sodium chloride were added to the aqueous phase. The precipitate was collected by centrifugation, resuspended in 0.1 M sodium citrate buffer, pH 6.0 (containing 5% ethanol), layered on 30% sucrose, and subjected to high-speed centrifugation. The pellets were resuspended in 0.1 M sodium citrate buffer, pH 6.0, and loaded on a 20–50% sucrose gradient. After high-speed centrifugation, the virus-containing zones were collected and the virus was sedimented by centrifugation. The pellets were resuspended in 0.1 M sodium citrate buffer, pH 6.0, and the virus concentration was measured spectrophotometrically assuming a specific absorbance of 8.6 units at 260 nm (19) for 1 mg of virus per milliliter. When purified PLRV was used in membrane-feeding experiments, the pellets were resuspended in artificial diet MP148 (5).

Virus acquisition from artificial diet and from plants. Membrane-feeding experiments were carried out with purified virus in an artificial diet. To prevent the virus from adhering to the Parafilm membranes, 1% bovine serum albumin was added to the diet. The virus concentration in the diet was determined with an ELISA by comparing a dilution series of the virus in diet with a stock suspension of known PLRV concentration. Sachets made of two stretched Parafilm membranes with 100 μl of the virus-containing diet sealed between them were prepared under sterile conditions. Membrane-feeding chambers were made by attaching these sachets to a plastic ring (2.5-cm-diameter). A group of approximately 30 nymphs was caged in a chamber and placed in a controlled environment at 20 ± 0.1 C, 80% relative humidity, and continuous illumination (8,000 lx) to acquire the virus.

Aphids were allowed to acquire the virus from top leaves that did not show symptoms and from bottom leaves that showed pronounced symptoms; the *P. floridana* plants used were inoculated with PLRV 5 or 12 wk previously, respectively. Groups of about 30 nymphs were put in cages clipped on these leaves and the plants were placed in a controlled environment.

Virus transmission. After acquisition from the virus-containing sachets or from *P. floridana* infected with PLRV, the nymphs were individually transferred to seedlings of *P. floridana* to determine the percentage of nymphs that transmitted PLRV in an IAP of 5 days.

In parallel experiments, the LP of the virus in the nymphs was determined by transferring them at constant intervals of 24 hr to new seedlings of *P. floridana*. The LP was defined as the time interval from the start of the AAP to the end of the IAP in which the first transmission occurred. A log-probit transformation was used to estimate the LP50, which represents the time at which 50% of the nymphs that transmitted the virus completed their LP (17). The LP50 and its 95% fiducial limits were calculated following Finney (4) by using the SAS program (SAS Institute Inc., Cary, NC).

Recording honeydew excretion. The number of honeydew droplets excreted by 1-day-old *M. persicae* nymphs feeding on sachets and on different leaves of *P. floridana* infected with PLRV was recorded with a honeydew clock (24). The volume of the honeydew droplets excreted by these nymphs was estimated by measuring the diameter of the approximately spherical droplets after collecting them in petri dishes filled with mineral oil.

Cocktail-ELISA and enzyme amplification. The amount of viral antigen present in nymphs after acquisition on the virus-containing artificial diet or from *P. floridana* infected with PLRV was determined by using enzyme amplification preceded by cocktail-ELISA (23). Nunc-Immunoplate Maxisorp F96 plates (Nunc, Roskilde, Denmark) were coated by adding 250 μl of 2 μg/ml of anti-PLRV rabbit γ-globulin in coating buffer (0.05 M sodium carbonate, pH 9.6) to each well. After an incubation period of 3 hr at 37 C, plates were washed with PBS-Tween (0.02 M sodium phosphate buffer, pH 7.4, containing 0.14 M NaCl, 2 mM KCl, and 0.05% Tween 20). Nymphs were individually homogenized in 100 μl of sample buffer (PBS-Tween containing 2% polyvinylpyrrolidone and 0.2% ovalbumin) and the extract was added to the wells with 1 μg/ml of anti-PLRV mouse γ-globulin. The plates were incubated overnight at 4 C, and washed with PBS-Tween; 100 μl of a 5,000-fold diluted goat anti-mouse-alkaline phosphatase conjugate (Sigma Chemical Co., St. Louis, MO) was added to the wells. After 3 hr of incubation at 37 C, plates were washed again and 100 μl of 0.2 mM β-nicotinamide adenine dinucleotide phosphate monosodium salt in 0.05 M diethanolamine buffer, pH 9.5, was added to each well. After a 45-min incubation period at 20 C, remaining alkaline phosphatase activity was blocked by the addition of 15 μl of 0.5 M 4-nitrophenyl disodium orthophosphate (BDH Chemicals Ltd., Poole, England) in 0.025 M phosphate buffer, pH 7.0, to each well. Subsequently, 150 μl per well of the amplification mixture, consisting of 700 units of alcohol dehydrogenase, 100 units of lipoamide dehydrogenase, 3% (v/v) ethanol, and 1 mM *p*-iodonitrotetrazolium violet in 15 ml of 0.025 M phosphate buffer, pH 7.0, was added.

Absorbance values were read on a Titertek Multiskan colorimeter (Flow Laboratories Ltd., Irvine, Scotland) at 492 nm (A_{492nm}). A dilution series of purified PLRV was incorporated in each plate.

RESULTS

Quantification of virus acquisition by *M. persicae* nymphs. The amount of virus acquired by *M. persicae* nymphs was analyzed after an AAP of 1–5 days on PLRV-containing artificial diets and on top and bottom leaves of *P. floridana* infected with PLRV. PLRV was purified from 7-wk-old infected *P. floridana* plants

and the artificial diets contained 3.75, 7.5, 15, 30, 60, and 120 μg of virus per milliliter. From each combination of virus concentration and AAP, 20 nymphs were individually tested for their virus content by cocktail-ELISA followed by enzyme amplification. The average absorbance values are presented in Figure 1.

On all virus sources tested, either artificial diets or infected leaves, an increase of the AAP resulted in a greater amount of viral antigen in individual nymphs. The amount of virus detected in the aphids increased with increasing PLRV concentration in the artificial diets. Multiple regression of the data showed that the A_{492nm} was linearly related to the $\log_{10}$ transformed virus concentration (μg/ml) in the diet and the length of the AAP (days). The regression equation was

$$A_{492nm} = -0.507 + 0.491X + 0.129Z \ (r^2 = 0.82)$$

in which X represents the $\log_{10}$ transformed virus concentration (μg/ml) in the diet, ranging from 3.75 to 120 μg/ml; and Z is the AAP, ranging from 1 to 5 days.

Nymphs that had fed on bottom leaves of 12-wk-old, PLRV-infected plants with pronounced symptoms acquired considerably less of the virus in the same AAP than nymphs from diets with the lowest concentration of the virus. The amount of virus detected in nymphs on top leaves equaled that in nymphs on artificial diets containing 3.75 or 7.5 μg of purified PLRV per milliliter.

Positive control samples of 0.5, 1, 2, 4, and 8 ng of purified PLRV by cocktail-ELISA and enzyme amplification gave A_{492nm} values of 0.02, 0.12, 0.32, 0.64, and 1.30, respectively. Control virus-free aphids yielded A_{492nm} values ranging from 0.00 to 0.02.

Relationships among virus concentration, vector age, and virus transmission. Membrane-feeding experiments were carried out to determine the effect of the PLRV concentration in the diet on the percentage of nymphs transmitting the virus and on the LP50. The virus concentrations in the artificial diets were as mentioned before. For each virus concentration, four sachets were used on which two cohorts of 1-day-old and two cohorts of 4-day-old nymphs were placed. On each sachet about 50 *M. persicae* nymphs were allowed to acquire virus for a period of 24 hr. The percentage of nymphs transmitting PLRV was determined by placing 30 nymphs of each cohort individually on test plants for 5 days. To estimate the LP50, another group of 30 nymphs was transferred to fresh test plants five times at intervals of 24 hr. The data are presented in Table 1. Young nymphs transmitted the virus more efficiently than older nymphs, as was reflected in a shorter LP50 and a higher percentage of virus-transmitting nymphs at each virus concentration tested. The virus concentration in the diet at which 50% of the nymphs transmitted PLRV was 14 μg/ml (95% fiducial limits [f.l.]: 11–18 μg/ml) and 45 μg/ml (95% f.l.: 35–60 μg/ml) for young and old nymphs, respectively. The percentage of nymphs transmitting (Y) was linearly related to the $\log_{10}$ transformed virus concentration in the diet (X). The regression equations were

$$Y = 60X - 20 \ (r^2 = 0.90), \text{ and } Y = 55X - 39 \ (r^2 = 0.94)$$

for 1- and 4-day-old *M. persicae* nymphs, respectively. The length of the LP50 was independent of the virus concentration present in artificial diets.

Transmission of virus purified from bottom or top leaves. PLRV was purified from top leaves that didn't have any symptoms and from bottom leaves showing pronounced interveinal chlorosis of *P. floridana* plants that had been inoculated with PLRV 8 wk previously. The percentage of nymphs that transmitted the virus and the LP50 were determined after feeding for 24 hr on an artificial diet containing 60 μg of purified virus per milliliter from either top or bottom leaves. One-day-old *M. persicae* nymphs were transferred to fresh *P. floridana* seedlings every 12 hr for 5 days. The results are presented in Figure 2.

All nymphs transmitted PLRV from either preparation. However, the LP50 of nymphs that had been feeding on virus purified from top leaves was significantly shorter ($P < 0.05$) than that from nymphs feeding on virus purified from bottom leaves.

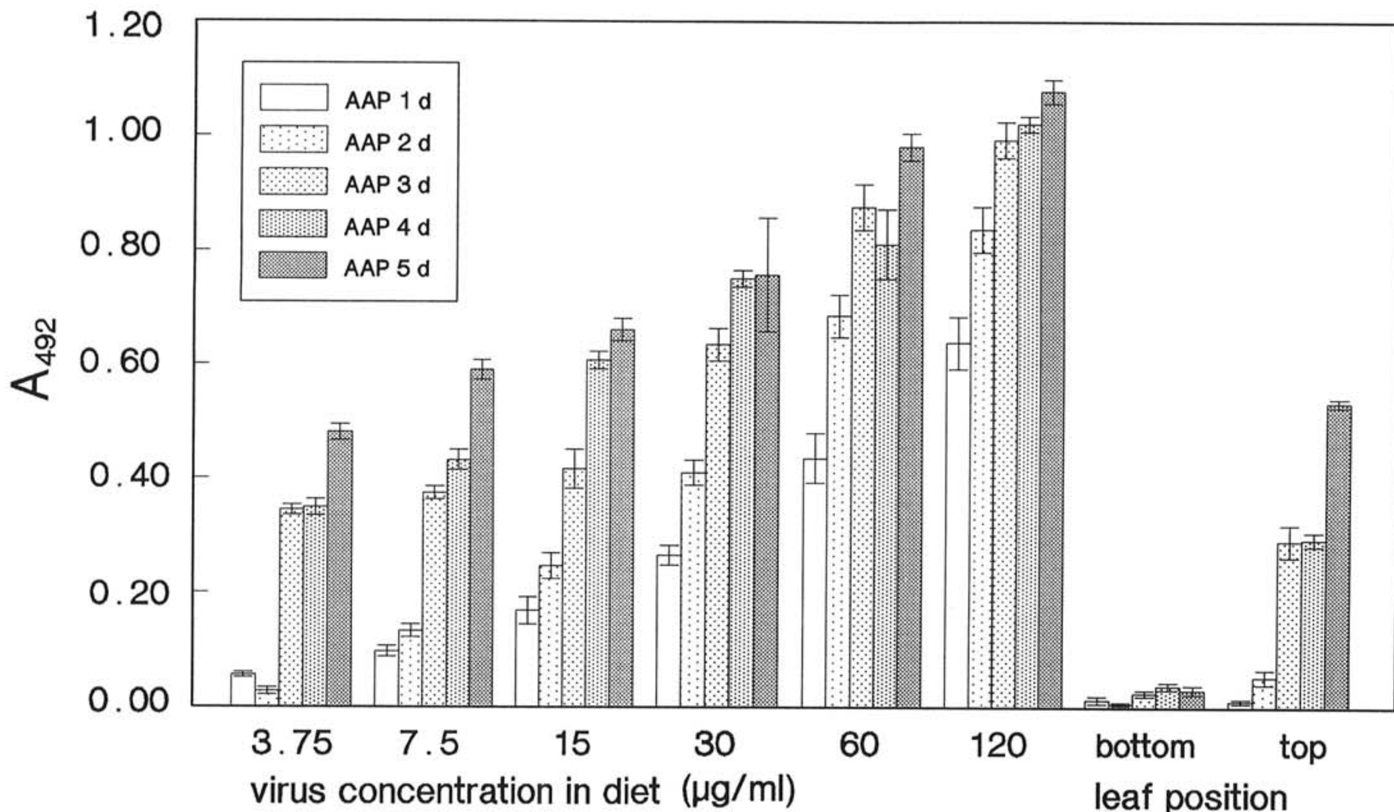

Fig. 1. Virus acquisition by *Myzus persicae* nymphs during an acquisition access period of 1–5 days on artificial diets that contained increasing amounts of potato leafroll virus (PLRV), and on leaves of *Physalis floridana* infected with PLRV. The nymphs were individually tested by cocktail-ELISA followed by enzyme amplification. Color development was measured at 492 nm (A_{492nm}) after 30 min. Each bar represents the mean value for 20 nymphs. The standard error of a mean is indicated in each bar.

TABLE 1. Efficiency of potato leafroll virus (PLRV) transmission of 1- and 4-day-old *Myzus persicae* nymphs after an acquisition access period (AAP) of 24 hr on artificial diet containing purified PLRV[a]

PLRV (μg/ml)	1-day-old nymphs Transmitted (%)	1-day-old nymphs LP50[b] (hr)	1-day-old nymphs 95% f.l.[c] (hr)	4-day-old nymphs Transmitted (%)	4-day-old nymphs LP50 (hr)	4-day-old nymphs 95% f.l. (hr)
3.75	8	ND[d]	ND	0	ND	ND
7.5	23	79	66–95	10	ND	ND
15	70	58	37–70	17	94	75–124
30	76	71	60–87	31	99	90–114
60	83	45	24–57	60	98	89–113
120	97	61	45–73	83	94	83–107

[a] After the acquisition access period (AAP), the aphid nymphs were transferred five times to fresh *Physalis floridana* seedlings at inoculation access periods (IAP) of 24 hr to determine the percentage of nymphs that transmitted the virus and the period after which the first virus transmission occurred.
[b] Median latency period (LP50): the time interval at which 50% of the nymphs completed their latency period (LP). The LP is defined as the period of time between the start of the AAP and the end of the IAP in which the first virus transmission was accomplished.
[c] Fiducial limits of the LP50.
[d] Not determined.

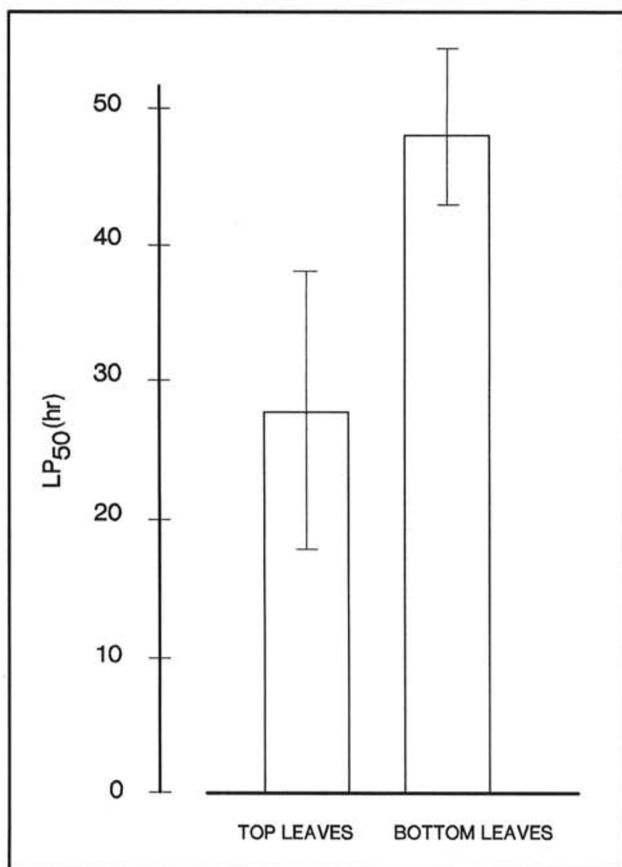

Fig. 2. The median latency period (LP50) of potato leafroll virus (PLRV) in 1-day-old *Myzus persicae* nymphs that have been feeding on an artificial diet that contains 60 μg of PLRV per milliliter, purified from top or bottom leaves of infected *Physalis floridana* plants. After the acquisition access period, the nymphs were transferred at constant intervals of 12 hr to new *P. floridana* seedlings. The 95% fiducial limits are presented with each bar.

TABLE 2. Honeydew excretion by 1-day-old *Myzus persicae* nymphs feeding on artificial diets and on different leaves of *Physalis floridana* infected with potato leafroll virus

Source	Number of nymphs	Honeydew excretion rate droplets per nymph per hour
Artificial diet	20	0.6
Top leaves	28	1.3
Bottom leaves	28	1.9

Honeydew excretion. The number of honeydew droplets produced by nymphs feeding on artificial diets and on different leaves of *P. floridana* infected with PLRV was recorded during a defined period (Table 2). Nymphs on bottom leaves excreted three times as many and nymphs on top leaves about twice as many honeydew droplets as those feeding on the diet. The volume of the collected honeydew droplets varied between 9–11 nl and did not depend on the source.

DISCUSSION

When PLRV was acquired by *M. persicae* from virus-containing artificial diets, the amount of viral antigen detected by ELISA in the nymphs was a function of the $\log_{10}$ transformed virus concentration and the length of the AAP. There is also a linear relationship between the percentage of transmitting nymphs and the logarithm of the virus concentration in the diet. These relationships indicate that the virus charge in the aphid can play a role as a parameter in estimating the amount of virus available in a source. A dosage dependency of the LP, as suggested by others for persistent virus transmission (2,17,18), could not be confirmed in the membrane-feeding experiments in which different amounts of virus were offered to the aphids (Table 1).

Previous work on the relationship between PLRV transmission and feeding behavior of *M. persicae* nymphs on comparable leaves of infected *P. floridana* plants, in which the honeydew excretion of aphids was used as an indicator of the amount of PLRV imbibed, showed that aphids in which the virus had a long LP excreted as much honeydew as aphids in which the virus had a short LP (24). This observation confirms the present findings that the LP does not depend on the amount of virus acquired by an aphid or present in its body. Indeed, it is unlikely that the number of particles will determine the speed of circulation within the aphid, unless cooperative action between virus particles is required for initiating or completing virus circulation.

The LP seems to be influenced by the age of the *M. persicae* nymphs involved and the intrinsic properties of the virus. Younger nymphs are more efficient virus transmitters than older ones (9,11,15), although some observed the contrary (7) or did not notice any difference (3,6). In our experiments, 4-day-old *M. persicae* nymphs were less efficient at transmitting the virus than 1-day-old nymphs (Table 1), this being reflected in a longer LP50 and a lower percentage of transmitting nymphs.

PLRV, purified from top leaves of infected *P. floridana* plants and given to nymphs in the artificial diet, had a shorter LP50 than PLRV purified from bottom leaves (Fig. 2). This observation is in accordance with the results of PLRV transmission with intact leaves as the virus source (24), and indicates that the virus purification did not change the relative transmissibility of the virus. As similar amounts of virus were fed to the aphids, it is likely that changes in the coat protein at the surface of the viral capsid

or proteins purified along with PLRV affected the transmissibility of the virus.

The availability of PLRV for acquisition by *M. persicae* in infected plants was determined by comparing the acquisition of the virus by the aphids on top and bottom leaves of infected *P. floridana* during an AAP of 1–5 days with that of aphids on artificial diets containing purified PLRV (Fig. 1). The amount of viral antigen detected in nymphs feeding on top leaves matched well with that of those on a diet containing 3.75–7.5 μg of virus per milliliter. However, in making an estimation of the actual virus concentration available in these leaves, it should be noted that nymphs feeding on top leaves excreted twice as much honeydew as those on artificial diets (Table 2). Although the nymphs that had fed on bottom leaves excreted three times as much honeydew as those on the artificial diets, the amount of viral antigen present in these nymphs fell below that of nymphs from diets containing 3.75 μg of PLRV per milliliter. This was not expected as the viral antigen concentration in bottom leaves of *P. floridana* infected with PLRV was higher than in top leaves of the same plants (24). This finding and the presence of more viral antigen in nymphs that fed on top leaves than in those that fed on bottom leaves of *P. floridana* (Fig. 1) suggest that the amount of virus available for acquisition by aphids is considerably lower in bottom leaves than in top leaves. This conclusion emphasizes that the virus charge of the aphid is a more reliable parameter for determining the potency of a plant as a virus source than the concentration of viral antigen in the source itself.

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