Within-Plant Accumulation of Potato Leafroll Virus by Aggregated Green Peach Aphid Feeding

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ABSTRACT

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Aggregates of 20 green peach aphids placed on noninoculated leaves of plants previously inoculated with potato leafroll virus (PLRV) caused significantly more accumulation of PLRV at their feeding sites than in aphid-free leaves in both potato and *Physalis floridana*. Based on comparable periods of postinoculation, six times more viral antigen was detected by enzyme-linked immunosorbent assay in inoculated leaves of *P. floridana* than of potato. The amount of PLRV detected in inoculated leaves was significantly correlated with the amount of PLRV that accumulated in "aggregate feeding" leaves, indicating that systemic spread of PLRV was titer-dependent. The movement of PLRV to aggregated aphid feeding sites probably followed the path of metabolites directed to the site of aphid damage and/or nutrient withdrawal. The accumulation of PLRV was not specific to the area of aggregated feeding but was evident throughout the leaf supporting the aphid colony. Aphids feeding in aggregates provided a mechanism that optimized virus acquisition from infected plants in which the pathogen was not uniformly distributed.

Additional keywords: nutrient sinks, virus distribution, virus movement.

Potato leafroll virus (PLRV) is a phloem-limited luteovirus (39) circulatively transmitted by several aphid species, the most efficient of which is the green peach aphid, *Myzus persicae* (Sulzer) (34). The amount of PLRV acquired by *M. persicae* feeding on inoculum sources is a function of virus concentration at the feeding site and the length of the acquisition access period (35). Luteoviruses are not uniformly distributed in infected plants (5,24, 28), and the localized availability of PLRV in plant tissues significantly affects green peach aphid transmission efficiency (23,30,37).

Systemic spread of viruses in infected plants occurs through vascular tissues (13,19), in some cases through the xylem (15,22), but most often through the phloem (24). Virus spread, particularly in the phloem, appears to follow the flow of metabolites (6) and may result from passive movement of virus particles through vascular tissues (19) dictated by plant physiological responses to growth, damage, and/or aging.

Aphids can cause nutrient sinks in areas of the plant in which aggregated feeding occurs (11,12,40,41). These sinks are a plant response to aphid feeding damage and/or metabolite removal at the feeding sites (26). Because aphids are primarily phloem feeders, it is possible that aggregated feeding could cause the movement of phloem-limited viruses to feeding sites. In this study, I examine the possibility that aggregated green peach aphid feeding results in accumulation of PLRV at feeding sites.

MATERIALS AND METHODS

Aphids, virus isolate, and test plants. A nonviruliferous clonal colony of *M. persicae* was maintained on mustard (*Brassica juncea* L. 'Florida Broadleaf') in an insectary at 22 ± 2°C with a 16-h photoperiod. A viruliferous colony of the same clone was maintained on PLRV-infected *Physalis floridana* Rydb. in a different insectary room under the same environmental conditions. A severe isolate of PLRV (LR-7; provided by P. E. Thomas, USDA-ARS, Prosser, WA) was maintained by aphid transfers on *P. floridana*. Purified LR-7 virions (supplied by P. H. Berger, Division of Plant Pathology, University of Idaho) were used for quantitative enzyme-linked immunosorbent assay (ELISA).

Potato (*Solanum tuberosum* L. 'Russet Burbank') plants were grown from virus-free tissue-culture seedlings to eliminate the possibility of other potato viruses influencing the accumulation and movement of PLRV (2,3). Potato and *P. floridana* test plants used in all experiments were 6 to 8 weeks old and had sufficient fully expanded leaves in the midrange of the plant to incorporate all treatments. Older, lower leaves and actively expanding leaves in the crown were not used.

Leaf cages. Preliminary experiments showed that large, clip-type cages (25) covering a large area caused chlorosis and inhibited PLRV accumulation when attached to leaves for more than 4 days. Therefore, pin-type cages were constructed of polycarbonate tubing (1.27 cm inside diameter × 1.59 cm outside diameter) cut into 8-mm sections. A closed-cell foam gasket was attached around one edge with double-stick tape. Two stainless steel pins, 1.9 cm long, were hot-glued opposite one another to the side of the cage. A closed-cell foam stopper was used to confine aphids within the cage. The cage was attached to the upper surface of leaves by carefully pushing the pins through the leaf into a polystyrene support held to the under surface. The polystyrene held the pins securely, and the cage gasket was compressed on the upper surface of the leaf. All pin cages were attached to the approximate center of leaves. The cages left no sign of chlorosis or other damage except for two small holes made by the pins.

Plant inoculation and aggregated aphid feeding. A midrange leaf on each test plant was randomly selected for inoculation (inoculated leaf) and two fourth-instar or adult viruliferous *M. persicae* were placed in an attached leaf cage for a 48-h inoculation access period. The aphids and any nymphs laid then were removed, and the cage was left on the leaf. Leaf cages were at-

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tached to the first and second leaves above the inoculated leaf and were designated the empty cage and aggregate treatments, respectively. The leaf immediately below the inoculated leaf was designated the no-cage treatment. This arrangement was designed to maximize the chance that PLRV would accumulate in empty and no-cage treatment leaves before the aggregate leaf because none of the treatments were on the same parastychy (38,42), resulting in the aggregate leaf being the farthest vascular distance from the inoculated leaf.

After removal of the viruliferous aphids from the inoculated leaf cage, 20 nonviruliferous fourth-instar and adult *M. persicae* were placed in the aggregate cage. Aggregate cages were examined daily throughout each experiment, and nymphs were removed to maintain 20 aphids per cage. Aggregated feeding was allowed to proceed for 5 to 11 days before the experiments were terminated. There were 8 to 15 replications (plants) per experiment arranged in a randomized complete block design. All experiments were conducted in an insectary room at 22 ± 2°C and a 16-h photoperiod with a combination of fluorescent and incandescent lights for normal plant growth (8).

Two control experiments were conducted to monitor PLRV movement in inoculated plants without aggregated aphid feeding. These experiments were conducted as described above, except that no aphids were placed in any of the cages. For each experiment, 15 *P. floridana* plants were inoculated, and treatment leaves from 5 plants were taken for virus quantification at 8, 9, and 10 days after the beginning of the inoculation access period.

Viral antigen quantification and statistical analysis. At the end of the postinoculation periods in control experiments or after the aphids were removed from the aggregate cages, leaf areas under all cages and an area in the center of the no-cage treatment were cut from the leaves with a No. 7 cork borer that just fit the inner diameter of the cages. Each leaf disk was weighed, ground in a mortar with a pestle at a 1:20 (wt/vol) dilution in extraction buffer (0.02 M phosphate [pH 7.4], containing 0.14 M NaCl, 2 mM KCl, 0.05% Tween 20, 2% polyvinylpyrrolidone, and 0.2% ovalbumin), and the extracts were tested by double-antibody sandwich (DAS)-ELISA (9). The plates were read in a Bio-Tek EL-312 microplate reader (Bio-Tek Instruments, Winooski, VT) and blanked against wells containing extracts of uninfected potato or *P. floridana*, as appropriate. Each plate used to assay viral antigen in the leaf disks included a 10-step dilution series of 10 to 100 ng of purified LR-7 virions per well. To quantify viral antigen in the leaf disks, absorbance values obtained from the dilution series were used to calculate a standard curve for each ELISA plate using TableCurve 1.0 software (Jandel Scientific, San Rafael, CA).

In the control and first three aggregated feeding experiments, only the leaf area under the cage was excised and tested. In four subsequent experiments, leaf disks were cut from under the cage

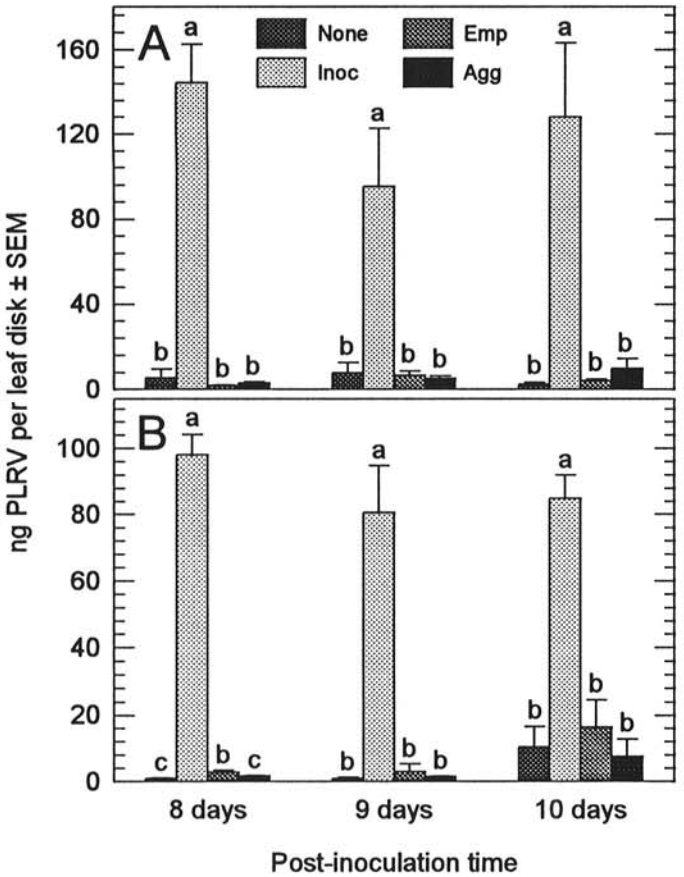

Fig. 1. Control experiments measuring the normal movement of potato leafroll virus in *Physalis floridana* after a 2-day inoculation access period (IAP) by viruliferous *Myzus persicae*. Inoculative aphids were destroyed immediately after the IAP. **A and B,** Each graph represents a separate experiment with five replications testing only under-cage leaf disks. Postinoculation times of 8, 9, and 10 days are from the beginning of the IAP. Treatments with the same letter are not significantly different according to the Student-Newman-Keuls test at $\alpha = 0.05$. Inoc = inoculated leaf; Emp = leaf 1 above inoculated leaf corresponding to empty-cage leaf in other experiments; Agg = leaf 2 above inoculated leaf corresponding to aggregated feeding leaf in other experiments; None = leaf 1 below inoculated leaf with no cage. Vertical bars indicate standard error.

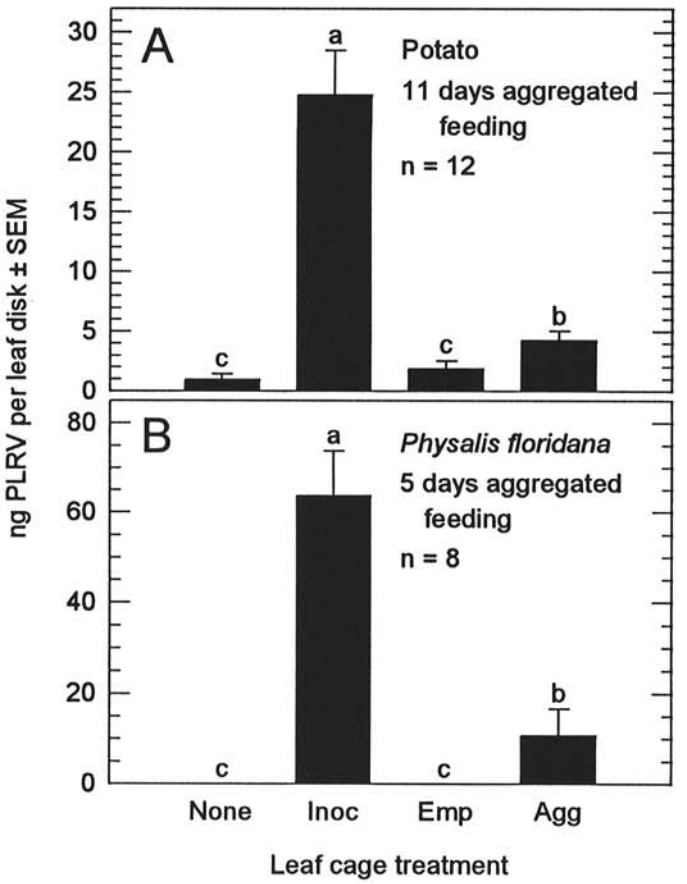

Fig. 2. Influence of aggregated green peach aphid feeding on the accumulation of potato leafroll virus in **A,** infected potato and **B,** *Physalis floridana*. Aggregated feeding by 20 nonviruliferous aphids began after a 2-day inoculation access period (IAP) by viruliferous *Myzus persicae*. Inoculative aphids were destroyed immediately after the IAP. **A and B,** Each graph represents a separate experiment testing only under-cage leaf disks. Treatments with the same letter are not significantly different according to the Student-Newman-Keuls test at $\alpha = 0.05$. Inoc = inoculated leaf; Emp = leaf 1 above inoculated leaf with empty cage; Agg = leaf 2 above inoculated leaf with cage containing 20 nonviruliferous aphids; None = leaf 1 below inoculated leaf with no cage. Vertical bars indicate standard error.

and immediately adjacent to the petiole to determine whether PLRV movement was feeding site specific or generalized to the aggregate leaf. If PLRV was not detected in any leaf disk, particularly the inoculated leaf disk, the plant was considered uninfected, and the replicate was not included in the analysis. Data were expressed as nanograms of PLRV per leaf disk and transformed to $\log_{10}(x + 1)$ to stabilize variances. Leaf treatments were compared by one-way (first three experiments) or two-way (last four experiments) analysis of variance followed by Student-Newman-Keuls mean comparison test at $\alpha = 0.05$ (10). Correlation analysis was used to examine the relationship between the amounts of PLRV in inoculated and aggregate leaves for all potato and *P. floridana* replicates with detectable amounts of PLRV in aggregate treatments (14).

RESULTS

In all experiments, none of the plants showed symptoms of PLRV infection, indicating minimal random systemic movement (24) within the experimental periods. The first control experiment indicated that mean PLRV antigen accumulation was significantly higher only in inoculated leaf disks at 8, 9, and 10 days postinoculation (Fig. 1A). In the second control experiment, along with inoculated leaf disks, mean PLRV antigen accumulation was significantly higher in the empty-cage leaf disks than those in no-cage or aggregate leaf disks at 8 days postinoculation; PLRV accumulation was significantly higher only in inoculated leaf disks at 9 and 10 days postinoculation (Fig. 1B).

In all experiments, there was significantly more viral antigen in inoculated leaf disks than in any other treatment disks (Figs. 1–3). To compare viral antigen accumulation in inoculated leaves among experiments that differed in postinoculation periods, the amount of viral antigen determined was divided by the number of days postinoculation. Based on these calculations, 0.61 ± 0.13 (13) and 1.91 ± 0.29 (12) ng of PLRV (mean ± SEM [*n*]) were determined for inoculated leaf disks from two potato experiments. In five *P. floridana* experiments, the corresponding values in inoculated leaf disks were 9.10 ± 1.44 (8), 13.35 ± 1.65 (11), 4.38 ± 0.86 (11), 6.08 ± 0.90 (12), and 6.26 ± 0.74 (11) ng of PLRV.

In the first experiment with potato, which was terminated after 7 days of aggregated feeding, PLRV antigen was detected only in inoculated leaf disks, and no PLRV was found in any other leaf disk. The second potato experiment was terminated after 11 days of aggregated feeding, and there was significantly more PLRV detected in the aggregate disk than in empty and no-cage treatments (Fig. 2A).

Only under-cage leaf disks were tested in the first aggregated feeding experiment with *P. floridana*. Apart from the inoculated leaf, PLRV was detected only in aggregate disks after 5 days of aggregated feeding (Fig. 2B). In four subsequent experiments, significantly more PLRV was detected in aggregate disks than in empty and no-cage treatments, except in the final experiment in which the no-cage and aggregate treatments were not significantly different (Table 1; Fig. 3A–D). There was more PLRV detected in petiole-side than in under-cage leaf disks, but the difference was not significant until 8 days of aggregated feeding (Table 1; Fig. 3C–D). Correlation analysis revealed a significant positive relationship between nanograms of PLRV per leaf disk in inoculated leaves and nanograms of PLRV in aggregate leaves ($r = 0.523$, $P = 0.0021$, $n = 32$).

DISCUSSION

Based on ELISA results, these experiments indicate there was significant PLRV accumulation at primary inoculation sites before viral antigen was detected in other parts of the plant. Other workers measuring systemic virus spread from primary inoculation sites have incubated excised plant parts to increase virus titers and, subsequently, used infectivity assays to detect the pres-

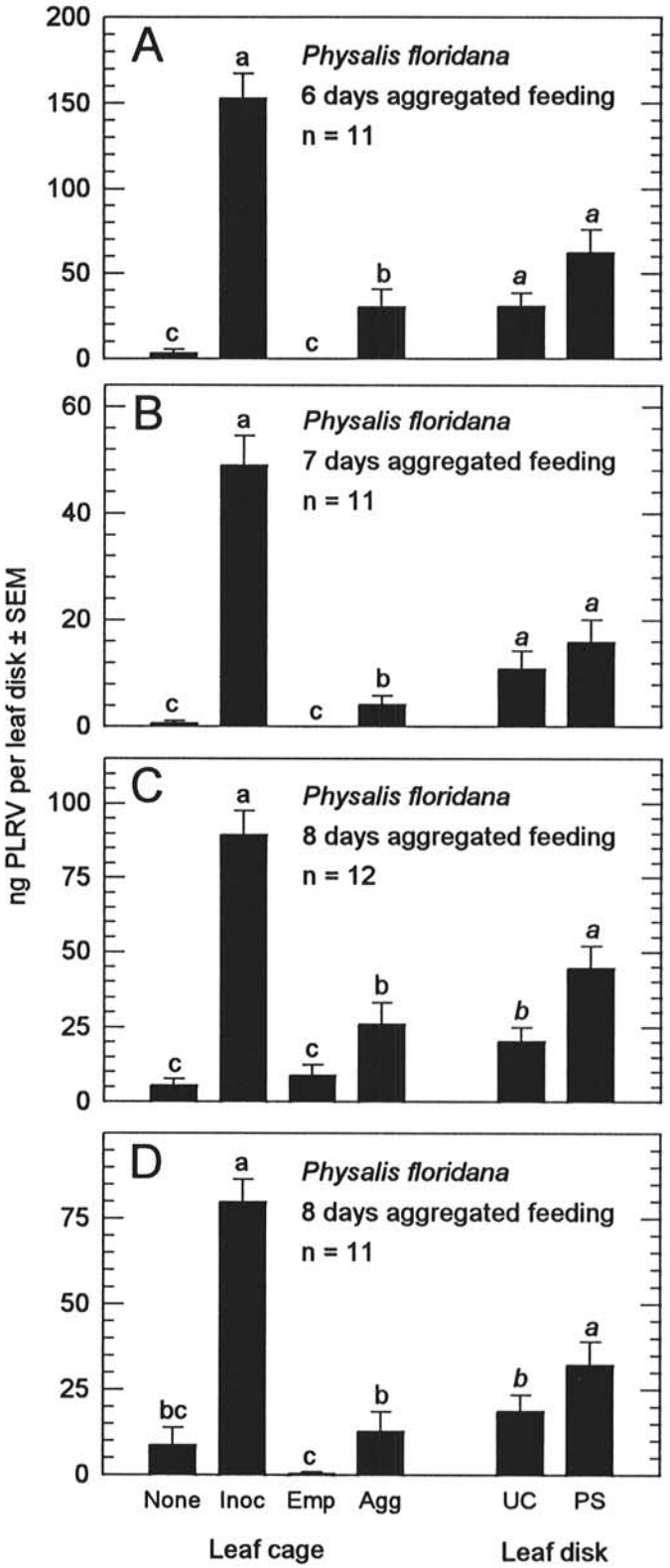

Fig. 3. Influence of aggregated green peach aphid feeding on the accumulation of potato leafroll virus in infected *Physalis floridana*. Aggregated feeding by 20 nonviruliferous aphids began after a 2-day inoculation access period (IAP) by viruliferous *Myzus persicae*. Inoculative aphids were destroyed immediately after the IAP. **A–D,** Each graph represents a separate experiment testing under-cage leaf disks (UC) and petiole-side leaf disks (PS). There were no cage/disk interactions. Treatments with the same letter are not significantly different according to the Student-Newman-Keuls test at $\alpha = 0.05$; normal letters apply to leaf cage treatments and italicized letters to leaf disks. Inoc = inoculated leaf; Emp = leaf 1 above inoculated leaf with empty cage; Agg = leaf 2 above inoculated leaf with cage containing 20 nonviruliferous aphids; None = leaf 1 below inoculated leaf with no cage. Vertical bars indicate standard error.

ence of virus (21,27,32). Some of the noninoculated leaf treatments in the first experiment with potato may have been PLRV infected at levels below ELISA detection limits. This may have important implications for the epidemiology of PLRV relative to when an infected plant becomes an inoculum source for subsequent virus spread by aphid vectors, because successful acquisition of luteoviruses requires titers well within the detection range of ELISA (16,28,35). It appears that, following inoculation with PLRV, it takes 9 to 12 days before a potato plant may serve as an inoculum source. This is not the case for *P. floridana*, which showed detectable amounts of PLRV within 7 days postinoculation, the shortest test period used in these experiments.

P. floridana is a better host for PLRV replication and/or systemic movement than potato. Based on comparable periods postinoculation, there was approximately six times more PLRV antigen in inoculated leaves of *P. floridana* than in potato leaves. Because there was a significant correlation between the amount of PLRV in inoculated leaves and the amount accumulating in aggregate leaves, it is possible that systemic movement of PLRV is titer-dependent. The relatively lower PLRV titers in inoculated potato leaves, possibly due to restricted viral replication relative to *P. floridana* (4,5), could account for the delayed detection of PLRV in other leaves.

Aggregated aphid feeding clearly influenced the movement and/or accumulation of PLRV in both potato and *P. floridana*. Virus accumulation in aggregated feeding leaves may have resulted from PLRV being directed to the feeding site along with metabolites. It is unlikely, however, that PLRV accumulation is feeding site specific, because petiole-side leaf disks always showed more virus than under-cage disks (Fig. 2). The leaves of the potato plants used in these experiments were still unifoliolate. In more mature plants in which the leaves are multifoliolate, more than one leaflet may accumulate PLRV due to aggregated aphid feeding on another leaflet of the same leaf. In addition, it is possible that leaves on the same parastychy as aggregated feeding leaves may accumulate virus in the same manner.

Newly inoculated plants were used in these experiments to measure virus accumulation due to aphid feeding prior to systemic spread of PLRV. Although the disease process affects the vascular system of PLRV-infected plants (33), these aggregated feeding results have implications as to the availability of PLRV for aphid vectors in older, systemically infected plants where uneven virus distribution results in some areas of the plant being virus-free. In addition to virus-free areas, localized luteovirus titers can be highly variable, significantly influencing aphid acquisition (5,16,17,29, 35,36). In areas of the plant that are virus-free or in which titers are low, aggregated aphid feeding provides a mechanism to insure acquisition through virus accumulation at feeding sites. Moreover, PLRV infection increases *M. persicae* growth and fecundity on both potato and *P. floridana* (1,8,20,31). Such increases can lead to the formation of aggregated aphid colonies on infected plants, which, in turn, can assure PLRV acquisition and its subsequent transmission by *M. persicae*.

The spread of PLRV in the field is related to virus incidence (percentage of infected plants) and the size of the vector population (7,18). Limiting the spread of PLRV through vector control is usually based on the number of aphids detected in field samples (7) and is generally effective during nonepidemic years. However, epidemics are often associated with cyclical outbreaks of aphid vectors, and aggregated colonies often form as aphid populations increase. As indicated by the results presented here, aggregated colonies may represent more efficient virus-acquisition environments for individual aphids within the aggregate. The contribution of aggregated colonies to viral spread in an epidemic may be related more to their distribution prior to and during acquisition than to the actual size of the population.

TABLE 1. Analyses of variance of the effect of aggregated green peach aphid feeding on the accumulation of potato leafroll virus in *Physalis floridana*

Exp.[a]	Source	df	MS	*F*	*P*
A	Leaf cage	3	20.623	85.44	0.0000
	Disk position	1	0.925	3.83	0.0543
	Cage × position	3	0.165	0.68	0.5647
	Error	70	0.241	...	...
B	Leaf cage	3	12.082	146.68	0.0000
	Disk position	1	0.268	3.26	0.0755
	Cage × position	3	0.074	0.90	0.4468
	Error	70	0.082	...	...
C	Leaf cage	3	12.476	35.86	0.0000
	Disk position	1	5.314	15.28	0.0002
	Cage × position	3	0.136	0.39	0.7590
	Error	77	0.348	...	...
D	Leaf cage	3	14.620	84.15	0.0000
	Disk position	1	1.533	8.83	0.0041
	Cage × position	3	0.349	2.01	0.1203
	Error	70	0.174	...	...

[a] Letters correspond to experimental designations in Figure 3.

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