Studies on the Resistance of Cucumber to Cucumber Mosaic Virus (CMV) I. Comparison of Symptom and Virus Multiplication in Leaves of Cucumber Cultivars Infected with CMV

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Studies on the Resistance of Cucumber to Cucumber Mosaic Virus (CMV)

I. Comparison of Symptom and Virus Multiplication in Leaves of Cucumber Cultivars Infected with CMV

Yoshimiki AMEMIYA and Tadao MISAWA

Department of Agronomy, Faculty of Agriculture,
Tohoku University, Sendai, Japan

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Summary

The symptoms and virus infectivity of CMV-infected leaves of three cucumber cultivars were compared.

Chlorotic spots developed on inoculated cotyledons of each cultivar. The degree of resistance of cucumber was determined according to their size and number. In the susceptible cultivar, large and many chlorotic spots developed and they enlarged with the passage of time after inoculation, whereas in the resistant cultivar, they were smaller and fewer in number, and did not enlarge. The mosaic symptom on systemically infected true leaves was also severe in the susceptible cultivar. Virus infectivity of the extracts from the inoculated cotyledon of each cultivar showed a characteristic cyclic pattern in its daily change.

The extracts of the cotyledon and respective true leaves of the susceptible cultivar showed a generally higher virus infectivity at any given interval than that of the corresponding leaves of the resistant cultivar. This paralleled the severity of the symptoms. The upper leaves of resistant and moderately resistant cultivars showed extremely low infectivity.

The extracts obtained from the healthy cotyledon of each cultivar were inhibitive to virus infection. But there was no correlation between the degree of resistance and the inhibitory activity of the extract.

Introduction

Cucumber plants were systemically infected by CMV and their leaves showed the mosaic symptom. The degree of susceptibility also varied among their cultivars (1). The cucumber cultivars distributed in Asia are generally more resistant than European cultivars (2). Moreover, the cucumber cultivars which are cultivated in Japan have different susceptibilities (3). The Aofushinari group of Japanese cultivars is susceptible, and the Zibai group is resistant. The mosaic symptom in resistant cultivars is mild and extracts of their diseased leaves show a low virus infectivity. In general, the difference of susceptibility to virus infection among cultivars has

been recognized in the various plant-virus combinations (4, 5, 6). Comparative studies between resistant and susceptible cultivars were conducted with the quantitative changes of enzyme activities (7, 8, 9, 10), soluble proteins (11, 12), ribonucleic acids (13), and free amino acids (14). But there is no correlation between their changes and the degree of resistance. Therefore, the mechanism of the resistance to virus does not seem to be explainable by the changes of such cellular components.

On the other hand, there is another explanation (15) for the resistance mechanism. It is that the speed of virus movement from cell to cell is different between resistant and susceptible cultivars. Thomas and Fulton (15) showed that the size of local lesions induced by tobacco mosaic virus on the resistant tobacco cultivar was smaller than that on the susceptible cultivar and they presumed that this phenomenon caused by the restriction of virus movement from cell to cell in relation to fewer plasmodesmata.

Several hypotheses have been formulated for this type of resistance, but the mechanism of resistance is not fully understood. In this paper, as the basic research for the study of the resistance mechanism of cucumber to CMV, the symptom and virus multiplication in infected leaves were compared among cucumber cultivars which have different susceptibility to CMV.

Materials and Methods

Plant: Cucumber cultivars used for this experiment were Best Green (susceptible), Risshu (moderately resistant), and Shimoshirazu Zibai (resistant). These plants were grown in autoclaved soil in 4-inch pots and kept in a green house. About ten days after sowing, the cotyledons reached approximately full size and the first true leaves began to appear. At this stage, seedlings of each cultivar were dusted with 400-mesh Carborundum and inoculated by rubbing with a gauze pad dipped into the inoculum.

Virus: The strain of CMV used was the yellow strain (CMV-Y) (16). The virus was maintained in tobacco (*Nicotiana tabacum* L. cv. KY-57). The inoculum was prepared from inoculated tobacco leaves, homogenized in 0.1 M phosphate buffer, pH 7.0 (1:10, w/v), and filtered through a four fold layer of a gauze. When partially purified virus was used, it was prepared by the method of Scott (17). Inoculated tobacco leaves were homogenized in 0.5 M citrate buffer, pH 6.5 (containing 0.1% mercaptoethanol and 0.01 M EDTA) and chloroform (1:1:1, w/v/v). Then the aqueous layer was recovered by low speed centrifugation. After being dialysed against 0.005 M borate buffer, pH 9.0, the preparations were centrifuged for 15 minutes at 78,000×g, stored at −20°C and diluted appropriately before the inoculation.

Virus assay; Virus infectivity was determined by the local lesion method. Virus suspension was inoculated on the Carborundum-dusted primary leaves of

cowpea (*Vigna sinensis* Endl. *var. sesquipedalis*, cultivar Kurodane-sanzyaku). Cowpea plants were used at the same day after sowing throughout the experiment, and 10 leaves were used for each assay.

Determination of virus infectivity of extracts from infected leaves were performed as follows: The cotyledons of twenty plants of each cultivar were inoculated with crude inoculum. Successively everyday for 12 days after inoculation, 20 disks of 8 mm diameter were punched out from the cotyledons of 20 plants of each cultivar. Each plant provides 12 disks over 12 days. The disks from each cultivar were immediately frozen and stored under –20°C until the last sample was obtained, and they were homogenized in 0.1 M phosphate buffer, pH 7.0 (1:10 w/v), before assay. Each extract was inoculated on cowpea leaves as previously described. The relative infectivity was expressed as mean lesion number per leaf. The infectivity of true leaves of each position was estimated at intervals of 3 days over a period of 15 days respectively when such leaves were of a suitable size for removing disks. Two disks (10 mm in diameter) were punched out from each leaf of twenty plants of each cultivar. Combined disks were frozen, homogenized, and assayed as above cited.

Results

Symptoms

Chlorotic spots developed on the inoculated cotyledon of each cultivar at 3 to 4 days after inoculation. Striking differences were observed among three cultivars in number and size of chlorotic spots. The number of chlorotic spots was about two times greater and its size was about 2.5 times larger in Best Green (susceptible) than in Shimoshirasu Zibai (resistant) as indicated in Table 1. Chlorotic spots on the cotyledon of Risshu (moderately resistant) were approximately the same number as those on the susceptible cultivar, but their sizes were similar to the resistant cultivar. The chlorotic spots on the susceptible cultivar enlarged gradually with time and became a diffused chlorosis. On the contrary, the spots on the resistant

TABLE 1. *Number and Size of Chlorotic Spots on CMV-inoculated Cotyledon*[a]

Cultivar	number of chlorotic spots per cotyledon[b]	diameter of chlorotic spot (mm)[c]
susceptible	167	1.17
moderately resistant	162	0.46
resistant	82	0.46

a: Chlorotic spot number and size were counted at 5 days after inoculation.
b: Average number for 20 leaves from 10 plants.
c: Average for 30 spots, 5 from each 6 cotyledons.

and the moderately resistant cultivars did not enlarge at all. Similar results were also observed in the inoculated true leaves.

In the systemically infected true leaves, chlorotic spots appeared beside the vein. These spots on the susceptible cultivar enlarged and fused with each other, and developed into severe mosaic symptom. These systemically infected true leaves did not show any malformation. The leaf length of each position was measured at intervals of 3 days after inoculation. As indicated in Fig. 1, the infected leaves from each location on the susceptible cultivar were much smaller than the corresponding healthy leaves. In the upper leaves, considerable difference was observed from the early stage of unfolding. One of factors affecting this difference seemed to be that leaf appearance was delayed owing to the growth inhibition caused by the infection. On the other hand, in the resistant cultivar, the symptom was mild and leaf expansion and plant growth were not so suppressed. In the moderately resistant cultivar, plant growth was only slightly suppressed, although the symptom was relatively severe in the first true leaf.

Infectivity of extracts from the chlorotic tissue and its surrounding green tissue on cotyledons were assayed. Disks (2 mm in diameter) were cut from the chlorotic tissue and its surrounding green tissue at 5 days after inoculation. Twenty five disks obtained from 5 plants of each cultivar were homogenized in

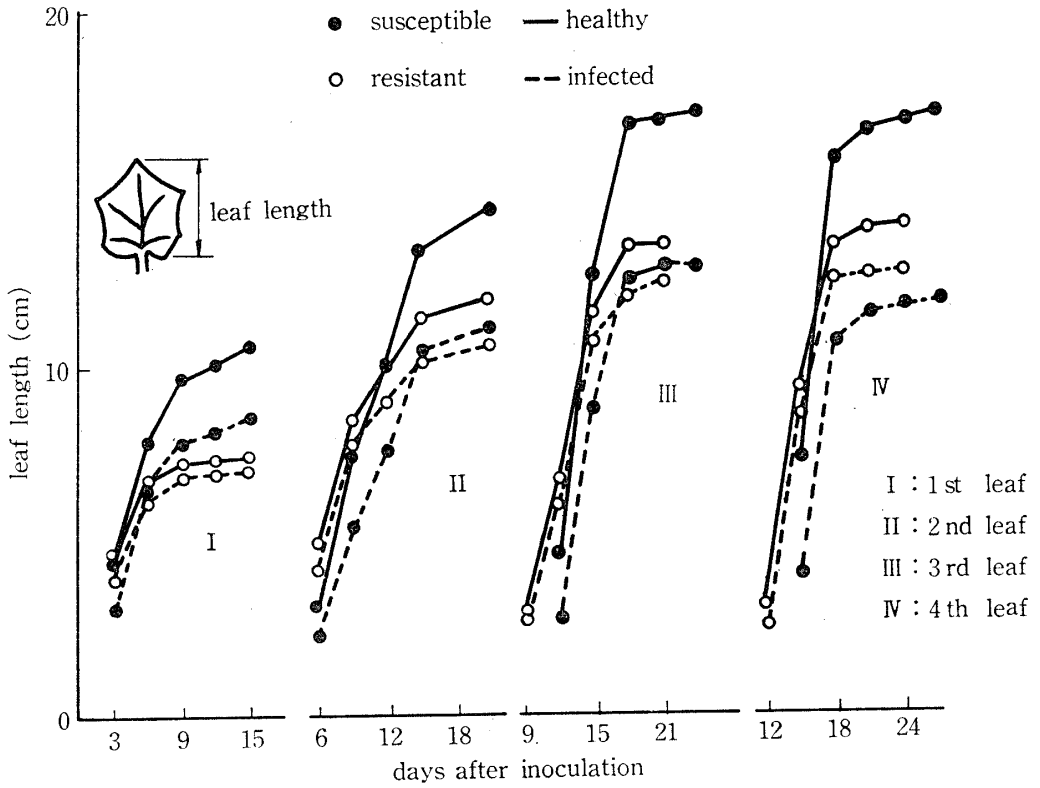

FIG. 1. Growth of true leaves of healthy and CMV-infected cucumber

0.5 ml of 0.1 M phosphate buffer, pH 7.0, separately, and its sap was inoculated on the primary leaves of cowpea. As in Table 2, a much higher infectivity was observed in the chlorotic tissue than in the green tissue of both cultivars. The infectivity in the chlorotic tissue of the susceptible cultivar was higher than in that of the resistant cultivar.

TABLE 2. *Virus Infectivity of Extracts from Chlorotic Spot and Green Part of Cotyledon*

Cultivar	Tissue used for assay[a]	Virus infectivity[b]	
		exp. 1	exp. 2
susceptible	Chlorotic spot	963	1262
	Green part	22	34
resistant	Chlorotic spot	551	473
	Green part	33	35

a: Twenty-five disks from 5 cotyledons were homogenized with 0.5 ml of phosphate buffer, pH 7.0, and inoculated on primary leaves of cowpea.
b: Mean lesion number per cowpea leaf.

Virus multiplication in CMV-infected leaves

(1) *Virus infectivity of extracts of inoculated cotyledon*

Changes in the infectivity of the cotyledon extracts and the effect of temperature on virus multiplication were investigated. Experiments were performed at 20, 24 and 30°C. The infectivity of leaf extracts were determined for 12 days after inoculation. Results were shown in Fig. 2, 3, and 4. A cyclic pattern which showed increasing and decreasing of virus infectivity was observed in the inoculated cotyledons of each cultivar. This pattern was particularly evident in the susceptible cultivar, showing 2 or 3 peaks during the 12 days. But in the resistant and moderately resistant cultivars, this cyclic pattern was not so evident as in the susceptible cultivar. The rate of virus increase in cotyledon extracts was faster at higher temperatures than at lower temperatures. The highest infectivity was obtained at 2 days after inoculation at 30°C. The infectivity rapidly decreased after reaching a peak. There was little infectivity in the cotyledon extracts of the resistant cultivar at 10 days after inoculation. The time required to reach maximum infectivity was delayed under lower temperatures. In the susceptible cultivar, the highest infectivity was obtained on the 6th day at 24°C. and on the 9th day at 20°C, respectively. Appearance of chlorotic spots also tended to be delayed at lower temperatures. Under every examined temperature, the infectivity of the inoculated cotyledon extracts was always higher in the susceptible cultivar than in the resistant cultivar. The order of relative infectivity of three cultivars was susceptible$\gg$moderately resistant$>$resistant.

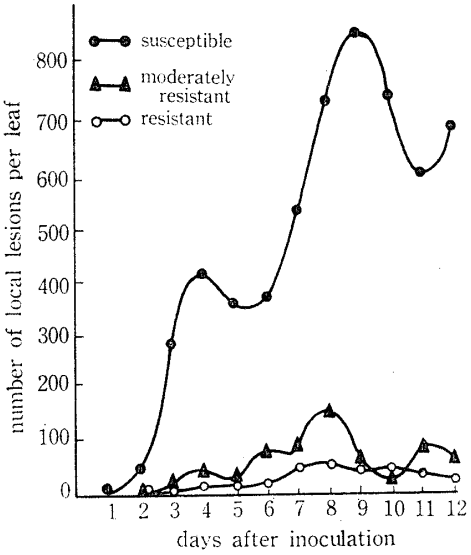

FIG. 2. Changes in virus infectivity of extracts of CMV-inoculated cotyledon from each cultivar at 20°C.

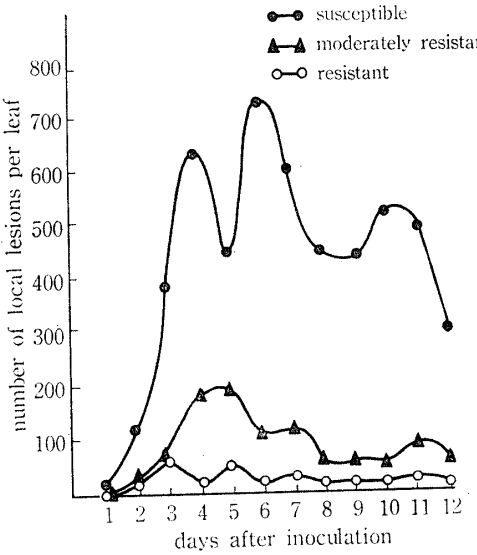

Fig. 3

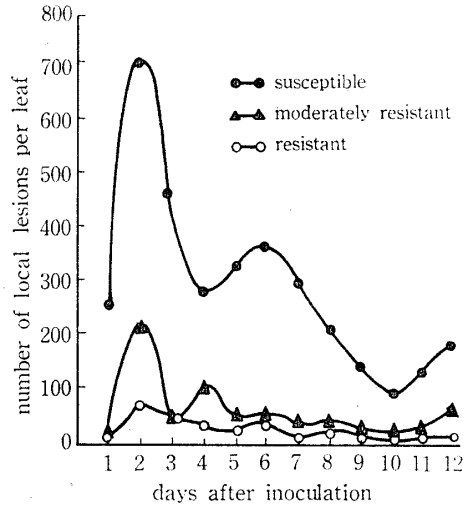

Fig. 4

FIG. 3. Changes in virus infectivity of extracts of CMV-inoculated cotyledon from each cultivar at 24°C.

FIG. 4. Changes in virus infectivity of extracts of CMV-inoculated cotyledon from each cultivar at 30°C.

(2) *The infectivity of extracts of systemically infected true leaves*

The infectivity of extracts from systemically infected leaves of each cultivar were determined. In this experiment, the first, the second, the third, and the fourth true leaves of the cotyledon inoculated cubumber were assayed separately. Plants were grown and inoculated at 24°C, and the infectivity of the extracts of

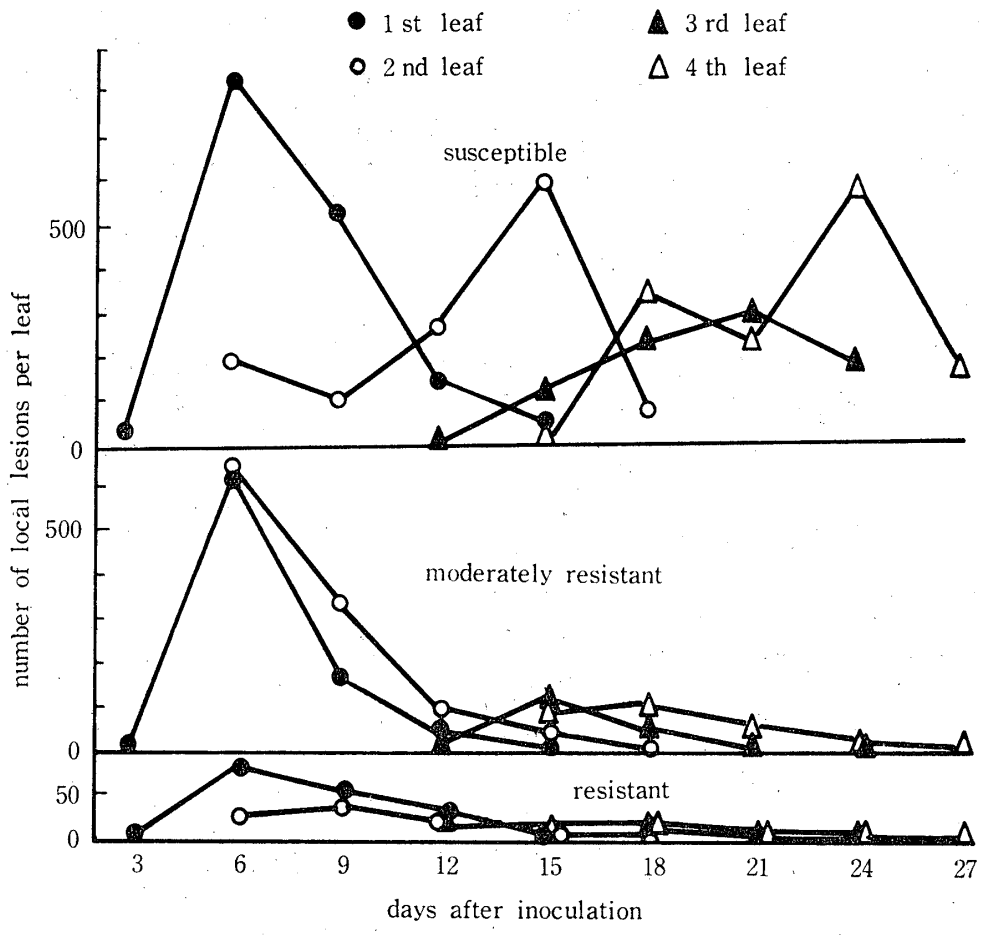

FIG. 5. Changes in virus infectivity of extracts of systemically infected leaves from each cultivar, following cotyledon inoculation with CMV.

each leaf position was determined at intervals of 3 days over a period of 27 days. Results were shown in Fig. 5. Extracts from the first leaves of each cultivar, in which the symptoms developed more clearly than in other leaves, had a higher infectivity than those from other leaf positions. Especially, the extracts of the moderately resistant cultivar had an infectivity as high as that of the susceptible cultivar. The infectivity of the first leaf reached the first peak on the 6th day after infection, and then rapidly decreased. The infectivity of extracts from the first leaves of resistant and moderately resistant cultivars were almost negligible on the 15th day after infection. Extracts from the second leaves of moderately resistant cultivar had a high infectivity on the 6th day after infection, and then its infectivity decreased rapidly until no infectivity was observed on the 18th day. The tested leaves of the resistant cultivar always showed a lower infectivity than the corresponding leaves of the susceptible cultivar. Extracts from the upper leaves of resistant and moderately resistant cultivars had lower infectivity than from the lower leaves, whereas extracts of the susceptible cultivar always showed a high infectivity in the upper leaves.

The cyclic pattern of virus infectivity shown in the inoculated cotyledon was not obvious in the systemically infected ture leaves. Presumably this is because samples were collected at intervals of 3 days in this experiment.

Effect of cucumber extracts on virus infectivity

It is well known that the extracts of many plants are highly inhibitive to virus infection (18, 19, 20). The relationship between the inhibitive effect of cucumber extracts and the degree of resistance was investigated. Healthy cotyledons of each cultivar were homogenzied in five fold (v/v) of 0.1 M phosphate buffer, pH 7.0. Crude extracts were mixed with partially purified CMV solution (1:1, v/v), and the mixture was inoculated on primary leaves of cowpea. On the opposite leaves, CMV solution plus buffer mixture (1:1, v/v) was inoculated to serve as a control. Inhibition percentage is represented as follows:

$$\text{Inhibition (\%)} = \left(1 - \frac{\text{mean lesion number per leaf produced by the virus-extract mixture}}{\text{mean lesion number per leaf produced by the virus-buffer mixture}}\right) \times 100$$

The results were shown in Table 3. More than 60% inhibition was observed in the extracts of each cultivar. But there was no correlation between the degree of inhibition of each extract and the resistance of each cultivar.

TABLE 3. *Effect of Cucumber Extracts on the Infectivity of CMV*

Cultivar	Virus infectivity[a]		inhibition (%)
	buffer+virus	extract+virus	
susceptible	1049	353	66
moderately resistant	1107	366	67
resistant	865	343	60

a: Mean lesion number per cowpea leaf.

Discussion

The symptoms which developed on CMV-inoculated cotyledons and systemically infected true leaves were severe in the susceptible cultivar, but mild in the resistant cultivar. Shifriss *et al.* (2) reported that the presence or absence of chlorosis on the cotyledons determined whether the tested plant is susceptible or resistant to CMV. But every cultivar used in this experiment developed chlorotic spots on the inoculated cotyledons. Therefore, the degree of resistance was determined by the size and the amount of chlorotic spots. The changes in infectivity of cotyledon extracts from the three cucumber cultivars showed a

characteristic cyclic pattern, particularly remarkable in the susceptible cultivar having 2 or 3 peaks within 12 days. This cyclic pattern of virus multiplication has already been shown in the combination of CMV and cucumber (2, 7, 8, 9, 21, 22), tobacco (23, 24), and spinach (25). But the meaning of this phenomenon is not yet understood. The pattern of growth cruve of infectious virus was affected by temperature. The increasing rate of infectivity after inoculation was faster at higher temperatures than at lower temperatures. Similar results were also reproted by Havránek (22). The extractable infectivity of infected leaves showed a remarkable difference among cultivars in parallel with their symptoms, namely, the infectivity of the susceptible cultivar was always high compared to the resistant cultivar. But the order of relative infectivity of three cultivars was not reversed by changing the temperature. The infectivity of extracts from systemically infected true leaves of susceptible cultivar was much higher than of resistant cultivar at each leaf position. It was characteristic that the extracts from the upper leaves of resistant and moderately resistant cultivars showed extremely low infectivity, although the extracts from corresponding leaves of susceptible cultivar always had a high infectivity. Therefore, in these cultivars, the suppressing mechanism of virus multiplication may be induced in the later stage of infection.

It had previously been shown (26) that the extracts of healthy cucumber leaves was highly inhibitive to virus infection. Extracts of cucumber cotyledons of each cultivar used here showed a similar high inhibitory effect. But there was no correlation between virus resistance and degree of inhibition. It may be postulated that the difference of the infectivity of extracts determined by bioassay represents a difference of virus amount in the extracts, not caused by the effect of their inhibitor.

Chlorotic spots on the susceptible cultivar were large and fused with each other with the passage of time after infection, but they did not enlarge on the resistant and the moderately resistant cultivars. Extracts from chlorotic tissue showed a much higher infectivity than that from other green tissue of the cotyledon. The infectivity detected in the extracts from green tissue is presumably either derived from viruses which spread from the chlorotic tissue or from viruses present in the tissue which did not become a visble chlorotic spot though the virus invaded and multiplied. The virus infectivity detected in the green tissue of the resistant cultivar seems to be of the latter case. This can be said because, by the our other experiment (27), the extract from the tissue near the inoculated zone on the cotyledon of the resistant cultivar had no infectivity, although the extract from the same part of the susceptible cultivar had a high infectivity.

From these results, it may be assumed that the low infectivity of the extracts from the resistant cultivar is caused by having fewer virus multiplication sites.

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