Factors Affecting Bioassay of Potato Virus M in Red Kidney Bean

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ABSTRACT

When Carborundum-dusted primary leaves of bean (*Phaseolus vulgaris* 'Red Kidney') were inoculated with potato virus M (PVM), more lesions resulted from 0.057 M phosphate buffer at a range of pH 7.0 - 8.0 as a diluent than from other diluents tested. Plants grown at a light intensity of 5,380 lx (500 ft-c) were most susceptible to PVM. Susceptibility, which was low with juvenile primary leaves, increased as the plant grew, and then declined, showing a plateau 10-12 days after sowing in summer and 14-15 days in winter. A linear relationship existed between dilutions of 10^{-1} to 10^{-4} of infectious leaf sap and the PVM infectivity. Inocula differing in PVM content by 15% or more, yielded statistically significant differences in lesion number when tested on 36 opposite half-leaves.

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Additional key words: local lesion host, PVM infectivity test.

Potato virus M (PVM), a member of the carla virus group (4), occurs singly or as a component of a virus complex, inducing leaf rolling mosaic (16), paracrinkle (10), or interveinal mosaic (1, 2) in potato (*Solanum tuberosum* L.). The symptoms induced by PVM range from very mild to severe, depending on virus strain, potato cultivar, and perhaps also on environmental conditions. The distribution of PVM is world-wide (22). However, our knowledge of quantitative aspects on the biological properties of this virus has been limited because of lack of a suitable assay host. Bean (*Phaseolus vulgaris* L. 'Red Kidney') has been found to be a reliable local lesion host for PVM (6). This paper reports factors influencing the quantitative assay of PVM in Red Kidney bean. A preliminary report of this work and publications on other aspects of PVM lesions in this host plant appeared elsewhere (7, 8, 9, 21).

MATERIALS AND METHODS.—PVM (AP-1) was maintained in 'King Edward' potato tubers (20). Inoculum was obtained either directly from potato or from infected tomato (*Lycopersicon esculentum* Mill. 'Earliana') leaves. Crude juice was obtained by grinding infected leaves, either frozen or fresh, with a mortar and a pestle, and was strained through two layers of fine cheesecloth. In some experiments, suspensions of partially purified PVM were used as inocula. Leaves were homogenized for 5 min in 0.057 M Na_2HPO_4-KH_2PO_4 buffer pH 7.5 containing 0.001 M 4-phenyl-3-thiosemicarbazide, at a ratio of 0.5 g of tissue/ml of buffer. The homogenate was strained through cheesecloth and 8 ml of *n*-butanol/100 ml of extract were added while stirring. Chloroplast coagulate was removed by centrifuging the extract for 30 min at 10,000 *g*. Polyethylene glycol (PEG) 20,000 was added to the resulting supernatant at a rate of 2g/100 ml. The virus pellets were obtained by centrifuging at 5,000 *g* for 15 min after the PEG had completely dissolved. The virus suspension in 0.057 M phosphate buffer (pH 7.5) was clarified at 5,000 *g* for 15 min. The supernatant consisted of a suspension of partially purified virus.

Beans were sown in steam-sterilized U.C. mix II (C) (12) in 12-cm diam clay pots. During winter, supplementary illumination was provided to maintain a 16 h-light period per day. The buds of trifoliate leaves were removed before inoculation. Inoculation was made on Carborundum (600-mesh)-dusted primary leaves by stroking with a cotton swab [Q-tips, Chesebrough-Pond's (Canada) Ltd., Toronto] dipped in inoculum. Inoculated leaves were rinsed immediately after inoculation, since the leaves tended to show injury without rinsing. Necrotic lesions were usually visible within 3-4 days, and were countable 7-8 days after inoculation at 17 ± 2 C (Fig. 1). All inoculation procedures were based on the use of opposite half-leaves.

The relative infectivity of single dilutions of each of two inocula was compared directly by inoculating opposite half-leaves in a balanced pattern. Phosphate buffers were prepared by mixing equimolar solutions of $Na_2HPO_4 \cdot 7H_2O$ and KH_2PO_4. The pH of the buffer solution was determined with a glass electrode. In some experiments, 0.057 M K_2HPO_4, pH 8.5, alone was used for extraction.

RESULTS.—*Increase in local lesion number after inoculation.*—When local lesions are used to estimate virus infectivity, sufficient time should be allowed for assay plants to fully develop conspicuous necrotic lesions. This not only makes it easy to count minute lesions, but also increases the accuracy of the assay. To determine an appropriate time of counting, the rate of local lesion increase was investigated by counting PVM lesions on the primary leaves. A few, very minute, water-soaked lesions started to appear 3 days after inoculation. At this stage, the lesions were visible only from the lower side of the leaves, when viewed by transmitted light. Necrotic lesions increased in size and number, and became readily recognizable 4 days after inoculation. There was a very rapid increase in number of local lesions per half leaf between the 3rd and 4th day. The local lesion number started to level off after 6-7 days and thereafter up to 12 days after inoculation the increase in local lesion number was very small (Fig. 2-A). Fig. 2-B shows the actual increase in the local lesion number per half leaf based on the data obtained from the same experiment. It is apparent that there is a drastic decline in the lesion increase after a rapid initial increase during the period

between the 3rd and 4th day.

Age.—Primary leaves of different ages, from 6-14 days after seeding were inoculated with a single inoculum of PVM.

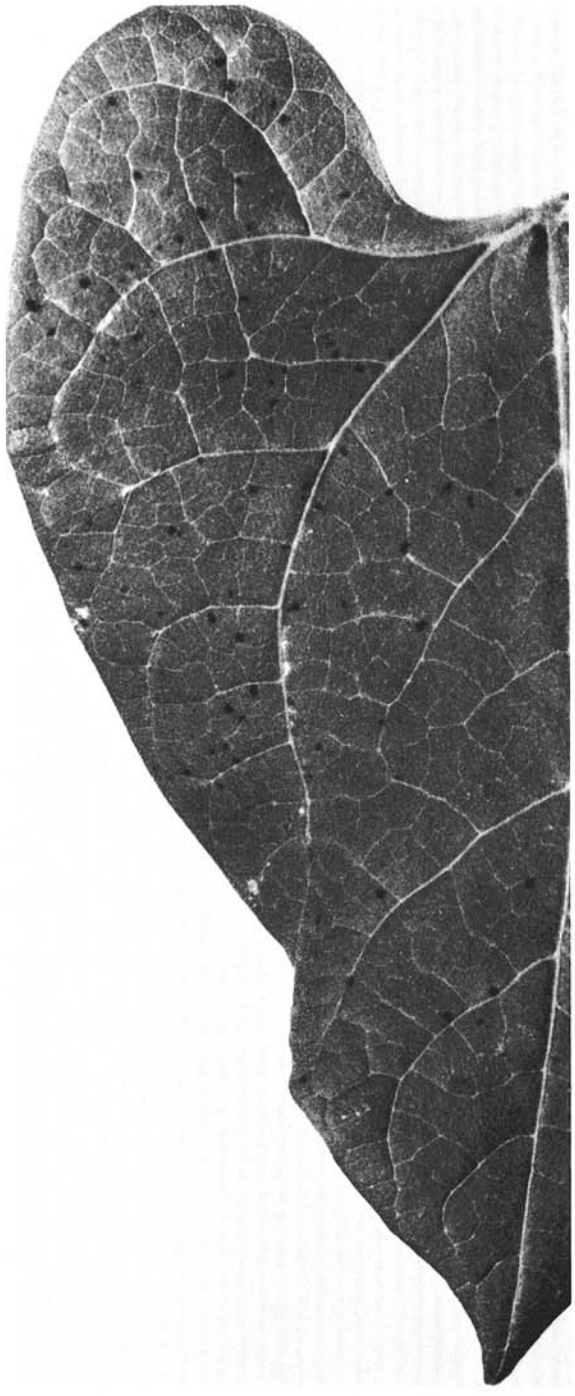


Fig. 1. A primary half-leaf of bean cultivar Red Kidney showing lesions induced by potato virus M 8 days after inoculation (× 1.5). (Photograph by T. Tribe).

The most lesions were obtained 10-12 days after seeding in summer and 14-15 days after seeding in winter under our greenhouse conditions (Fig. 3). The leaves of the plants older than 3 wk after seeding were resistant to PVM and were not suitable for assay.

Dark treatment before inoculation.—At various time intervals the effect of dark treatment on susceptibility was determined. The alternative half of each primary leaf was covered on both sides with a piece of aluminum foil and the other half was exposed to light until inoculation. The average lesion numbers obtained from 12 half-leaves were: 6 h dark, 68; light, 92: 16 h dark, 38; light, 38: 17 h dark, 68; light, 51: and 22 h dark, 77; light, 63. These results suggested that the dark treatment by this method did not affect the susceptibility of Red Kidney bean to PVM.

Light intensity.—Experiments were carried out in a growth room equipped with fluorescent lamps providing light intensities of 5,380, 10,760, 16,140, 21,520 lx (500, 1,000, 1,500 and 2,000 ft-c), respectively. The average lesion number from 12 half-leaves were as follows in order of the light intensities: Experiment I - 122, 118, 44, 13 lesions; and Experiment II - 75, 47, 36, 19 lesions. These results indicated that the light intensity of 5,380 lx was most satisfactory for PVM assay.

Concentration of phosphate buffer.—It was shown earlier (23, 24) that the highest local lesion number in Pinto bean was obtained with certain plant viruses extracted in 0.057 M K_2HPO_4. In this study, PVM was extracted in various molar concns of Na_2HPO_4-KH_2PO_4 buffer at pH 7.0 and 7.5, and K_2HPO_4 buffer at pH 8.5, respectively. The highest lesion number was obtained with inoculum prepared in 0.057 M phosphate buffer at three pHs used (Fig. 4).

pH of phosphate buffer.—The pH of phosphate buffer used for extracting PVM from leaves also greatly influenced local lesion number. The results (Fig. 5) suggest that the range of pH 7.0-8.0 was optimal for PVM assay. In acidic buffers, the number of local lesions produced was extremely low. On the alkaline side, local lesion numbers decreased as pH increased.

Effect of additives.—The following experiments were carried out to determine the effect on PVM infectivity of additives, some of which were known to stabilize the infectivity of certain unstable viruses (3, 5). Infected potato or tomato leaf tissue was homogenized respectively at the ratio of 1:10 (w/v) in 1-phenylthiosemicarbazide, 4-phenyl-3-thiosemicarbazide, sodium diethyldithiocarbamate, sodium diethyldithiocarbamate-cysteine HCl, K_2HPO_4, at the concns listed (Table 1). Distilled water was used in the control experiment. The resulting sap samples were tested immediately. The inoculum prepared in 0.057 M K_2HPO_4 produced significantly higher numbers of lesions than did that prepared in other additives (Table 1).

Dilution curve.—To determine dilution curve of PVM, crude sap was diluted in 0.057 M Na_2HPO_4 buffer pH 7.5, 0.057 M K_2HPO_4 buffer 8.5, or in distilled water, pH 7.5, respectively, and assayed on bean leaves. In all cases, lesion numbers were essentially inversely proportional to dilution in the ranges tested (Fig. 6). These results suggested that bean should be useful for the quantitative estimation of PVM infectivity.

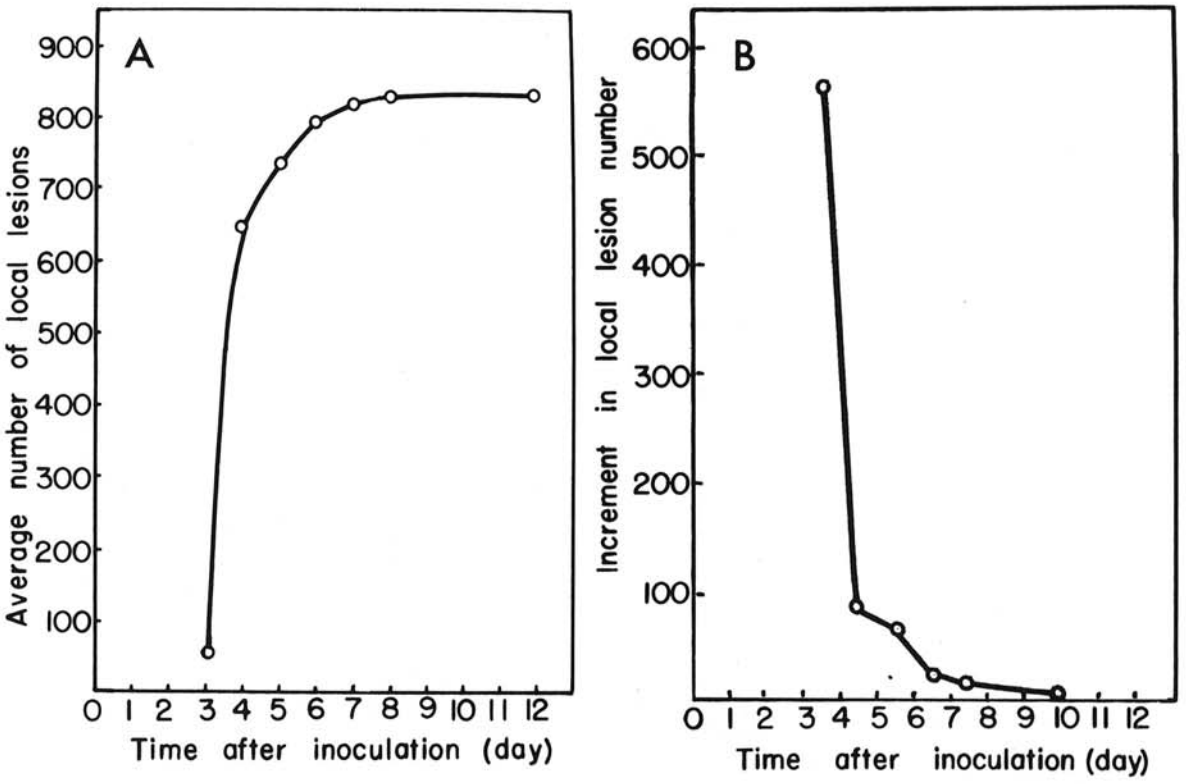


Fig 2-(A,B). Graphs showing the local lesion number obtained in Red Kidney bean inoculated with potato virus M. **A)** Total number of local lesions at different time intervals after inoculation. **B)** The net difference in local lesion number obtained in a given period between each time of lesion counting.

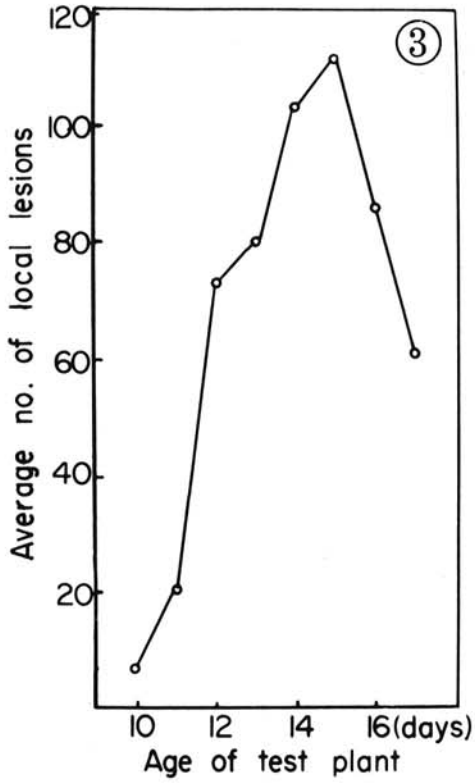
TABLE 1. Effect of additives on average local lesion number of potato virus M in the primary leaves of Red Kidney bean

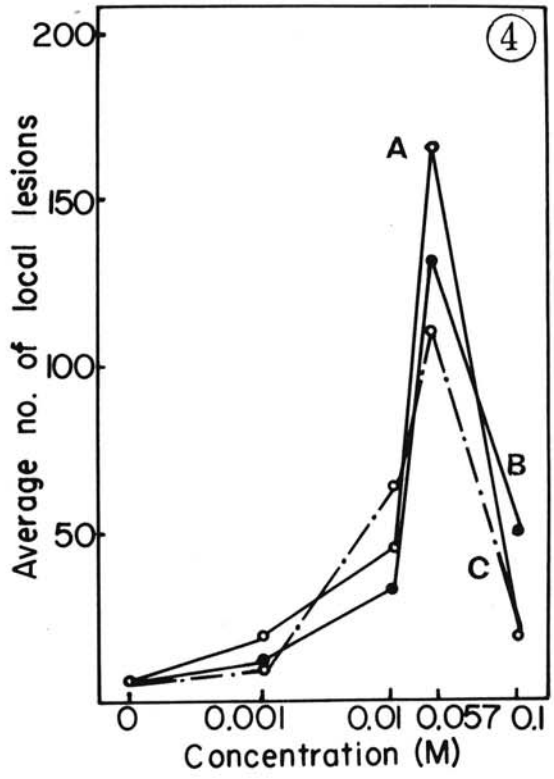
Compound	Concn (M)	pH	No. of local lesions[a] Experiment I	II	III	IV	V	VI	VII	VIII
Distilled water	...	7.0	86	18	26	18	...	...	...	...
K_2HPO_4	0.057	8.5	163	405	170	252	47	110	306	201
NaDIECA	0.01	7.0	86	53	30	23	3	55	...	...
NaDIECA + Cysteine-HCl	0.01	7.0	...	...	...	...	...	...	202	103
1-PTC	0.001	6.8	90	89	33	27	44	48	194	93
4-PTC	0.001	6.8	...	...	...	...	5	82	203	120

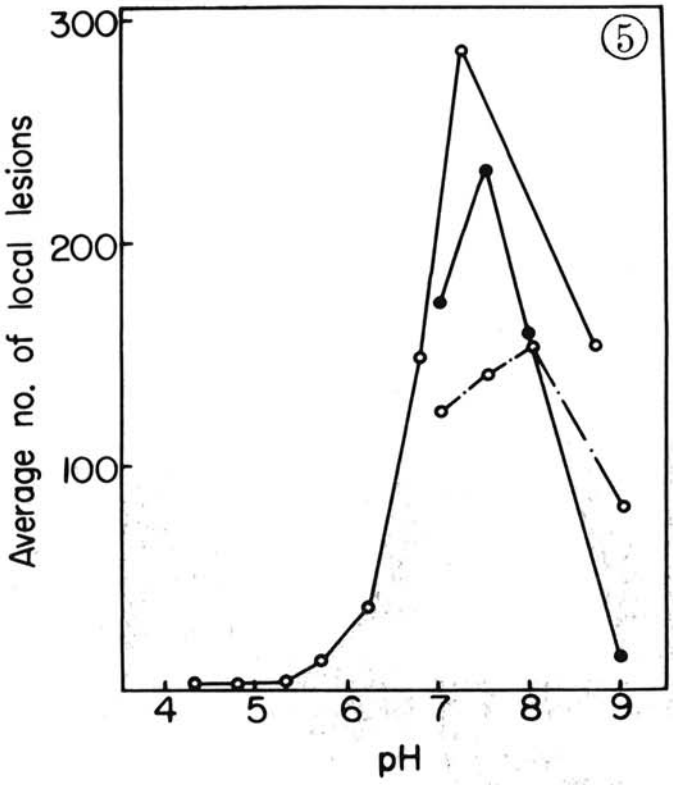
[a]Average number for 12 half-leaves (I-IV) or 8 half-leaves (V-VIII). The results are comparable only within each experiment. NaDIECA = sodium diethyldithiocarbamate, 1-PTC = 1-phenylthiosemicarbazide, 4-PTC = 4-phenyl-3-thiosemicarbazide.

Differences in infectivity of two inocula.—For quantitative work it is essential to know the sensitivity of a given local lesion host for detection of differences between inocula. To determine the sensitivity of Red Kidney bean to PVM, two inocula, a "standard" and an "unknown", were prepared in 0.057 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.0, from a single sample of partially purified PVM. The "unknown" was diluted so as to be either equal to the "standard" in content of PVM or to differ by a known factor. The relative infectivities of the two inocula were then tested on 36 half-leaves. Student's *t-test* was used to analyze the lesion numbers obtained (17). It was possible to distinguish two inocula differing 15% or more in PVM content, if lesion numbers of the "standard" were in the range of 20-50 lesions per half-leaf (Table 2).

DISCUSSION.—The usefulness of bean plant as a bioassay host has been firmly established with many plant viruses (13, 14, 18, 19, 23, 24). The advantages of using the primary leaves of Red Kidney bean for quantitative infectivity test of PVM were obvious from the results presented in this paper. The use of uniformly sized, symmetrical leaves undoubtedly permits accurate estimation of relative infectivity. Evenly flat leaves are


③
Average no. of local lesions
120
100
80
60
40
20
10
12
14
16(days)
Age of test plant


④
Average no. of local lesions
200
150
100
50
A
B
C
0
0.001
0.01
0.057
0.1
Concentration (M)


⑤
Average no. of local lesions
300
200
100
4
5
6
7
8
9
pH

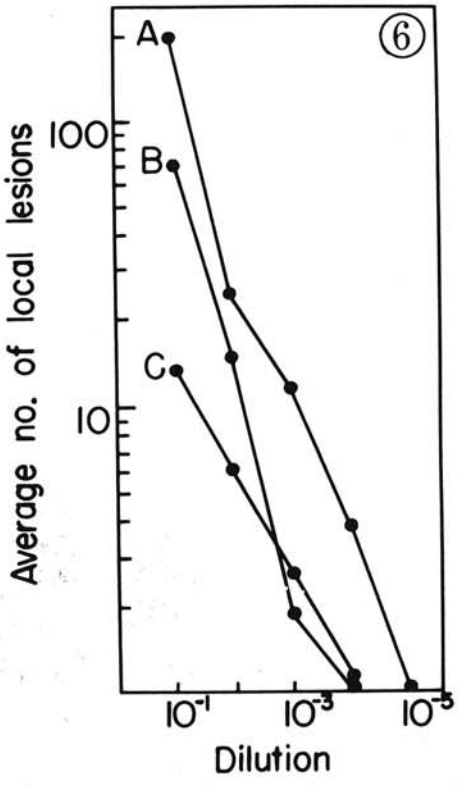

⑥
Average no. of local lesions
A
B
C
100
10
10^{-1}
10^{-3}
10^{-5}
Dilution

Fig. 3-6. 3) Relation of the age of bean cultivar Red Kidney (represented by the number of days after sowing) to the number of potato virus M local lesions produced. **4-(A-C)**] Effect of molar concn of phosphate buffer used to obtain extract from potato virus M-infected tissue upon the number of local lesions. **A**) pH 7.0, **B**) pH 7.5, **C**) pH 8.5. **5**) Relation of pH of 0.057 M phosphate buffer used to obtain extract from potato virus M-infected tissue to the number of local lesions. The results of three experiments are shown. **6-(A-C)**] Dilution curves obtained in bean cultivar Red Kidney with three preparations of potato virus M. Crude sap from Earliana tomato leaves was extracted and diluted either in **A**) 0.057 M phosphate buffer pH 7.5, **B**) 0.057 M K_2HPO_4 pH 8.5, or **C**) in distilled water pH 7.5.

TABLE 2. Sensitivity of opposite half-leaf assay on Red Kidney bean in detecting differences between two potato virus M inocula diluted in 0.057 M Na_2HPO_4-KH_2PO_4 buffer pH 7.0

Virus content of "Unknown" (% of "Standard")	No. of lesions per half-leaf[a]	
	"Standard"	"Unknown"[b]
100	45	46
100	59	52
100	46	44
90	54	49
85	48	36**
85	44	34**
80	40	30**
80	38	29**
60	36	23**
40	33	15**

[a]The average for 36 half-leaves.
[b]** indicates significant difference between the "standard" and "unknown", $P = 0.01$.

large enough for use of the half-leaf method. The bean plant is also easy to raise. The growing period required is about 10-14 days in the greenhouse, depending on seasonal factors and it may be possible to standardize the growth conditions by using growth facilities that provide controlled environment. In contrast, it would take at least 4-8 wk to raise other less-sensitive assay plants such as *Datura metel*, *Nicotiana debneyi*, and *Chenopodium quinoa*.

One disadvantage of using this assay host is that the primary leaves remain susceptible only for the limited period of time and the suceptibility fluctuates as the leaves mature. Similar cases were reported also with other plant viruses tested on *Phaseolus vulgaris* (11, 15, 19). This difficulty can be overcome by raising Red Kidney bean regularly and by using only the leaves which are in the same growth stage optimal for the assay.

When the factors described in this paper are controlled carefully, the bean test would prove to be a convenient and reliable method for the quantitative investigation of PVM.

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