

キュウリモザイクウイルス(CMV)の感染性に与えるpHの影響

ウイルス感染に対する表皮RNaseの作用

誌名	日本植物病理學會報 = Annals of the Phytopathological Society of Japan
ISSN	00319473
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発行元	日本植物病理學會
巻/号	63巻6号
巻号補足	
掲載ページ	p. 445-449
発行年月	1997年12月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council
Secretariat



Analysis of the pH Effect on Infectivity of Cucumber Mosaic Virus—A Possible Role of Ribonuclease—

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Abstract

The infectivity of Cucumber mosaic virus (CMV) and CMV RNA on cowpea and tobacco leaves after mechanical inoculation was lower when the inoculum was suspended in buffers at pH 6.0 as opposed to pHs 7.0 or 8.0, with or without EDTA. When leaves of both plants were inoculated with CMV suspended in water and immediately treated with buffers at the above pHs, infectivity did not vary among the treatments, suggesting that pH did not affect cell susceptibility, but did affect the inoculum. Before inoculation, no detectable structural changes in the CMV particles were observed at this pH range. Thus the effect on the inoculum of exuded substances such as degradative enzymes in epidermal cell sap after inoculation was examined on tobacco leaves. The effect of pH on this interaction, as well as the behavior of ribonuclease (RNase) in the epidermal sap correlated well with the infectivities of CMV and CMV RNA. CMV RNA was degraded more by the epidermal RNase at pH 6.0 than at pHs 7.0 or 8.0. The RNase also bound more easily with CMV virions at pH 6.0 than at pH 8.0 without attacking the inner RNA, thereby maintaining their activity; when virions treated with epidermal sap at pH 6.0 were dissociated into the coat protein (CP) and RNA, the RNA was significantly degraded. Thus, RNase exuded from wounded epidermal cells may bind easily with CMV CP at pH 6.0, disrupting virus multiplication in the cells at the infection sites.

(Received April 30, 1997; Accepted September 1, 1997)

Key words: cucumber mosaic virus, infectivity, ribonuclease, pH.

INTRODUCTION

Plant viruses infect host plants mainly through wounds or vector transmission. However, the early infection processes are still unclear. Virus infectivity *via* mechanical inoculation is affected by factors such as pH, ionic strength and cations in the inoculum⁹. Though the optimum pH for virus infectivity differs for virus-host combinations, a pH within 7.0 to 8.0 seems to be favored¹³. In fact, many viruses including tobacco mosaic virus (TMV) have high infectivity when the pH of inoculum is on the basic side. In addition, many plant virus particles swell in slightly basic conditions; viruses, such as TMV, brome mosaic virus and alfalfa mosaic virus, treated at pH 8.0 were able to undergo *in vitro* translation^{1,15}. However whether these phenomena are related to pH effects on infectivity is unknown. No possible explanation for this affect of the inoculum pH level on virus infectivity has been given until now.

While investigating the effect of pH on CMV infectivity, we found that plant ribonuclease (RNase) was closely related to the pH-dependent infectivity. Because CMV easily loses infectivity when exposed to pancreatic RNases^{2,4}, plant RNase may also affect virus infection.

We describe here the effect of pH on CMV infectivity in tobacco and cowpea leaves and a possible role of plant RNase in the early stage of the infection process on tobacco leaves.

MATERIALS AND METHODS

Virus and Plants The ordinary strain of CMV was maintained in tobacco (*Nicotiana tabacum* L. cv. ky 57). Purified virus was prepared essentially by the method of Scott¹¹ followed by sucrose density gradient ultracentrifugation. CMV-RNA was extracted from purified virus with phenol and SDS. Tobacco and cowpea (*Vigna unguiculata* (L.) Endl. var. *sesquipedalis* cv. Kurodanesanjaku) plants were grown in a growth chamber at 25°C under 1.6×10^4 lux illumination using 16 hr photoperiods.

Infectivity was assayed using the fully developed 10th tobacco leaf at the 15-leaf stage and cowpea primary leaves at 10 days after seeding. Leaves were dusted with carborundum (600 mesh) and mechanically inoculated with a cotton swab dipped in inocula.

Analysis of virion conformation and coat protein Whole virus conformation was analysed with 1.2% agarose gel electrophoresis⁵, rate zonal sucrose density

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gradient centrifugation²⁾ and electron microscopy by negative staining⁷⁾. To investigate the degradation of coat protein subunits, protein blot analysis was performed as described by Shirako and Ehara¹²⁾.

Tobacco epidermal saps and RNase activity

The cell sap exuded from the tobacco epidermis after rubbing with carborundum was collected; tobacco leaves were dusted with carborundum (200 mg/leaf) and the leaf surface was rubbed with distilled water (2 ml/10 cm square). The water on the leaf was then immediately collected and centrifuged at $10,000 \times g$ for 30 min. The supernatant was used as the cell sap from epidermis (ES I).

In addition, tobacco leaf epidermis was stripped and homogenized with 100 mM sodium acetate buffer, pH 5.5 (containing 0.1% mercaptoethanol) in proportions of 50 mg to 1 ml, respectively. After centrifuging the homogenate at $80,000 \times g$ for 90 min, the supernatant was dialysed against the same buffer (10 mM) overnight, then used as the epidermal homogenate (ES II). RNase activity in the ESs was determined with a spectrophotometer by measuring the increase in low molecular weight 260 nm-absorbing material produced by enzyme action on yeast RNA substrate. The enzyme unit was defined as the amount of enzyme causing an increase in absorbance at 260 nm of $1.0 \text{ unit} \cdot \text{min}^{-1} \cdot \text{ml}^{-1}$ ¹⁴⁾. The degradation of CMV RNA was analysed by 1.5% TBE agarose gel electrophoresis.

ELISA Non-precoated indirect enzyme-linked immunosorbent assay (ELISA) was performed according to Koenig's method⁸⁾. Leaf tissues were ground in 50 mM sodium carbonate buffer, pH 9.6, then incubated in the wells of a polystyrene microassay plate for 60 min at 37°C. After washing, 200 μ l of antisera diluted with TBST (20 mM Tris-HCl, 0.15 M NaCl, pH 7.5, containing 0.05% Tween-20) was added to the wells and incubated for 60 min at 37°C. After washing, 200 μ l of alkaline phosphatase-conjugated specific antibody was added to the wells and incubated for 60 min at 37°C. After washing, the substrate (sodium *p*-nitrophenyl phosphate in diethanolamine buffer) was allowed to react at room temperature, and the reaction was stopped with 3 M

NaOH. Color intensity of each well was measured by reading the absorbance at 405 nm. Plates were washed three times with TBST between each step.

Detection of RNase attached on CMV surface

A CMV suspension (20 μ g/ml) was mixed with 1/10 volumes of ES II with a final pH of 6.0 or 8.0 at 25°C for 30 min. The mixture was then layered on 20% sucrose and centrifuged at $300,000 \times g$ for 90 min. The precipitated virions were dissociated to the CP and RNAs by freezing and thawing in 2 M CaCl₂ and dialysed against 10 mM potassium phosphate buffer, pH 6.0, for 4 hr at 4°C. After dialysis, the CP-RNA mixture was incubated at 37°C for 2 hr. After incubation, the RNA was extracted with SDS and phenol to be analysed by 1.5% agarose gel electrophoresis.

All experimental tools and materials, except those denatured by heating, were autoclaved at 120°C for 40 min.

RESULTS AND DISCUSSION

Effect of pH on the infectivity

Cowpea leaves were inoculated with CMV or CMV RNA (20 μ g/ml each) suspended in 10 mM potassium phosphate and sodium phosphate buffers at pH 6.0, 7.0 or 8.0. The number of local lesions formed on the leaves increased with higher pH. Similar results were obtained with Tris-HCl and citrate buffers (Table 1) and with tobacco leaves inoculated with CMV and CMV RNA under the same conditions. Virus multiplication in tobacco was also lower at pH 6.0 than at pH 8.0 (Fig. 1), and intermediate at pH 7.0 (data not shown). The pH effect on infectivity in both plants did not vary with the addition of EDTA (10 mM).

Thus, the infectivity of both CMV and CMV RNA was higher with higher pHs, regardless of the kind of buffer and host plant.

Effect of pH treatment after inoculation on cell susceptibility

Cowpea leaves were inoculated with CMV (20 μ g/ml) suspended in distilled water, then immediately immersed for 5 seconds in 10 mM sodium phosphate buffer at pH

Table 1. Number of local lesions on cowpea leaves after inoculation with either CMV or CMV RNA (20 μ g/ml each) at pHs 6.0, 7.0 and 8.0

Inoculum	Buffer ^{b)}	Number of local lesions/leaf ^{a)}		
		pH 6.0	pH 7.0	pH 8.0
CMV virion	K-phosphate	91 $\pm$ 19	212 $\pm$ 23	270 $\pm$ 29
	K-phosphate (+ EDTA)	116 $\pm$ 19	201 $\pm$ 17	251 $\pm$ 24
	Na-phosphate	150 $\pm$ 17	215 $\pm$ 18	247 $\pm$ 21
	Na-citrate	97 $\pm$ 12	179 $\pm$ 20	— ^{c)}
	Tris-HCl	— ^{c)}	113 $\pm$ 22	147 $\pm$ 17
CMV-RNA	K-phosphate	37 $\pm$ 4	127 $\pm$ 14	191 $\pm$ 17
	K-phosphate (+ EDTA)	39 $\pm$ 7	90 $\pm$ 12	176 $\pm$ 21
	Na-phosphate	65 $\pm$ 11	131 $\pm$ 24	241 $\pm$ 19

a) Data show the mean values and standard deviations for 10 leaves from at least three experiments.

b) The concentrations of buffering reagents and EDTA are 10 mM.

c) Not tested.

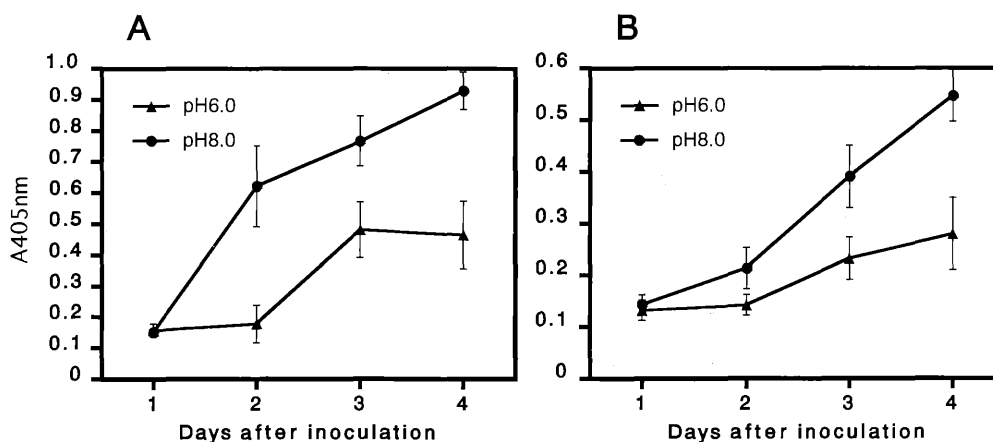


Fig. 1. CMV coat protein accumulation in tobacco leaves inoculated with either 20 µg/ml CMV (A) or CMV RNA (B) suspended in 10 mM potassium phosphate buffer at pH 6.0 or 8.0 as determined by ELISA (absorbance at 405 nm). Each point is the average with standard deviation of three experiments.

Table 2. Number of local lesions on cowpea leaves after treated with sodium phosphate buffers at pHs 6.0, 7.0 and 8.0 after inoculation with CMV (20 µg/ml) suspended in distilled water

Buffer pH ^{a)}	Number of local lesions/leaf ^{b)}
6.0	152 ± 26
7.0	133 ± 8
8.0	141 ± 17

a) The leaves were immersed for 5 sec in 10 mM sodium phosphate buffers of pHs 6.0, 7.0 or 8.0 immediately after inoculation.

b) Data show the mean values and standard deviations for 10 leaves from at least three experiments.

6.0, 7.0 or 8.0. The number of local lesions on inoculated leaves was not changed significantly by any pH treatment immediately after inoculation (Table 2). When the same experiment was carried out with tobacco leaves, virus multiplication was unaffected by the pH treatment (Fig. 2).

Thus, the pH effect on infectivity was not a result of a change in host cell susceptibility, but of an alteration in the inoculum.

Effect of pH on virion conformation

To examine conformational changes of CMV particles at various pHs, particles treated with 10 mM potassium phosphate buffer at pH 6.0, 7.0 or 8.0 were analysed by 1.2% agarose gel electrophoresis, rate zonal sucrose density gradient centrifugation and electron microscopy. No differences were found in the electrophoreses, sedimentation profiles and fine structures among CMV particles treated at different pHs (data not shown). Thus, the conformation of CMV virions did not change in the pH range of 6.0 to 8.0 during the experimental period. Similar results have already been obtained^{6,10}. The cause of this pH-dependent CMV infectivity was further examined on tobacco leaves as follows.

Effect of epidermal sap on the stability of CMV and CMV RNA

During mechanical inoculation, the epidermal cell

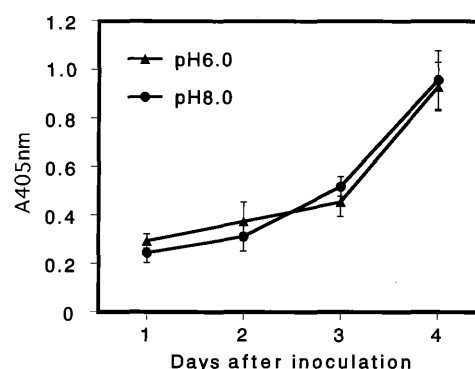


Fig. 2. CMV coat protein accumulation in tobacco leaves inoculated with CMV (20 µg/ml) suspended in distilled water and immediately treated with 10 mM sodium phosphate buffer at pH 6.0 or 8.0 as determined by ELISA (absorbance at 405 nm). Each point is the average with standard deviation of three experiments.

walls at infection sites were broken by rubbing with abrasive. As a result, the epidermal cell sap, which was mainly vacuolar, was exuded to the outside⁹. Thus, CMV or CMV RNA may come in contact with the cell sap. As the vacuole contains many degradative enzymes such as protease and RNase, the enzymatic actions of epidermal sap on CMV and CMV RNA was examined.

CMV and CMV RNA (final concentration 20 µg/ml each) were suspended in 10 mM potassium phosphate buffer at pHs 6.0, 7.0 and 8.0, mixed with a 1/10 volume of solution containing the cell sap from the epidermis (ES I) or epidermal homogenate (ES II) of tobacco leaves. Thereafter, the degradation of CMV and CMV RNA in the mixture was examined. Protein blotting analysis of CMV did not show any bands originating from the degradation of CP subunits (data not shown). CMV RNA was extracted and analysed by agarose gel electrophoresis. In both samples treated with ES I and ES II, the degradation of CMV RNA was greater after the incubation at pH 6.0 than at pH 8.0, and was intermediate at pH 7.0 (Fig. 3). More degradation was found in

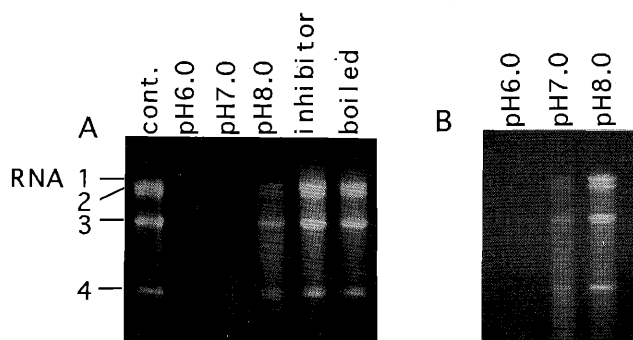


Fig. 3. Agarose gel electrophoresis of CMV RNA which was suspended in 10 mM potassium phosphate buffer and incubated with 1/10 volumes of tobacco epidermal sap (A) or epidermal homogenate (B) at pHs 6.0, 7.0 and 8.0. Incubation condition was 37°C for 30 min (A) or 10 min (B). The incubation was also carried out with the epidermal sap which was boiled for 30 min or was mixed with RNase inhibitor (1 unit/ μ l) before incubation. As a control, CMV RNA was incubated without the sap at 37°C for 30 min.

ES II than ES I (RNase activity in ES I and ES II was 3.4 and 5.8 unit/ml at pH 7.5, respectively). CMV RNA was degraded also by adding protease inhibitor (1 mM phenylmethyl-sulfonyl fluoride, data not shown), but not by adding RNase inhibitor (Takara, 1 unit/ μ l) or by boiling the sap for 30 min (Fig. 3A). Thus, CMV RNA appears to be easily degraded by the RNase in epidermal sap at pH 6.0. We have not observed a positive participation of protease in pH-dependent infectivity.

Effect of epidermal sap on the infectivity

The effect of the epidermal sap on CMV and CMV RNA infectivities was also examined. CMV and CMV RNA were mixed with the sap (ES I) at pH 6.0 and 8.0 as described above and incubated for 0–20 min at 25°C (Fig. 4). All samples were then adjusted to pH 7.0 with 100-fold (v/v) potassium phosphate buffer (100 mM, pH

7.0) before the inoculation of cowpea leaves. The infectivity of CMV immediately after being mixed with sap at pH 6.0 was about 20% less than that at pH 8.0, suggesting that CMV was affected more at pH 6.0 than at pH 8.0 by a component(s) in the sap immediately after being mixed. Infectivity continued to decrease more at pH 6.0 than at pH 8.0 with increasing incubation times (Fig. 4A). The infectivity of RNA extracted from the CMV virion which was incubated with ES I in the experiment was almost the same as that of untreated CMV (data not shown). Therefore, the internal RNA was probably not affected by incubation with the sap; infectivity loss was probably caused by a substance(s) such as RNase that bound to the virion surface.

Immediately after mixing, CMV and CMV RNA infectivity at different pHs was not very different. The infectivity of CMV RNA incubated at pH 8.0 changed little within 10 min in comparison with that at pH 6.0. After 20 min incubation, the infectivity of RNA incubated at pH 6.0 had decreased more than that at pH 8.0 (Fig. 4B), again suggesting the participation of enzymatic action (RNase).

Such a significant loss in infectivity was not observed when boiled sap was used (data not shown). Similar results were also obtained with ES II.

Possible binding of epidermal RNase with CMV particles

The results above suggested that epidermal RNase was closely related to the pH-dependent infectivity loss not only for CMV RNA but also for the CMV virion. Therefore, the relationship of CMV virions and epidermal RNase was examined. The CMV virion incubated with ES II at pH 6.0 or 8.0 for 30 min was dissociated to CP and RNA (as described in Materials and Methods). After dialysis, the CP-RNA mixture was incubated at 37°C for 0–2 hr, and the RNA was extracted for agarose gel electrophoresis. CMV RNA in the CP-RNA mixture from the virion treated with ES II at pH 6.0 was degraded more than when incubated at pH 8.0 (Fig. 5).

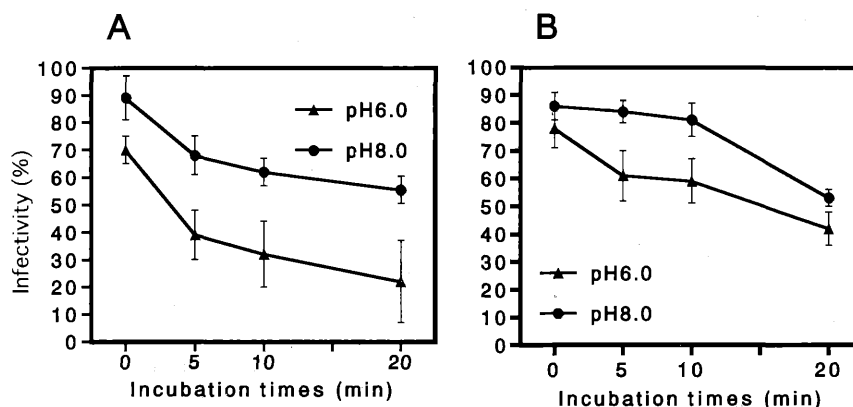


Fig. 4. Effect of epidermal sap on the infectivity of CMV (A) and CMV RNA (B). CMV and CMV RNA (20 μ g/ml each) were suspended in 10 mM potassium phosphate buffer and incubated with 1/10 volumes of epidermal sap (ES I) for 0–20 min at 25°C. Immediately after incubation, the samples were adjusted to pH 7.0 for inoculating cowpea leaves. Infectivity is the percentage of that of the CMV and CMV RNA which were incubated without the sap. Each point is the average with standard deviation of three experiments. 0 min: immediately after being mixed.

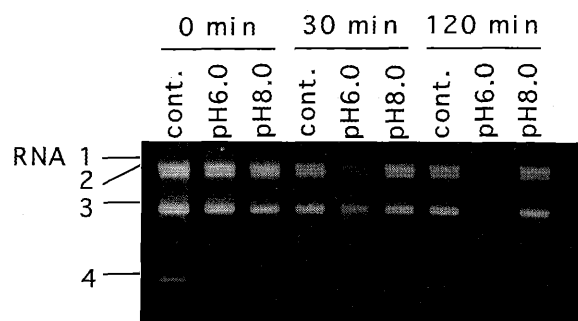


Fig. 5. Agarose gel electrophoresis of CMV RNA after incubation of the coat protein and RNA which were dissociated from CMV virions treated with 1/10 volumes of epidermal homogenate at pHs 6.0 or 8.0 for 30 min at 25°C. The virion treated with water instead of sap was used as a control. The coat protein and RNA mixture were incubated for 0, 30 and 120 min at 37°C.

Such degradation of CMV RNA was not detected when boiled epidermal sap was used or RNase inhibitor (1 unit/ μ l) was added before incubation (data not shown). The results suggested the coat protein fraction of the virus treated with epidermal sap at pH 6.0 had higher RNase activity than at pH 8.0.

Accordingly, pH-dependent binding of epidermal RNase with CMV CP was proven in the present experiment; such bindings have also been assumed for pancreatic RNase²⁾. Thus, the pH-dependent CMV and CMV RNA infectivities after mechanical inoculation may be related to the degree of binding and digestion by RNase, respectively.

The epidermal RNase probably originated from the vacuolar solution exuded from wounded epidermal cells. Epidermal RNase may attach to the virus, preventing some processes necessary for initiation of infection or attacks on the uncoated RNA. Thus, the epidermal RNase may act against virus infection.

We thank Dr. Hideo Nagayama, Department of Science and Technology, Ishinomaki Senshu University, for useful suggestions and discussions. We are also grateful to Drs. Teruyoshi Hashiba and Akira Karasawa for their interest. A part of this work was supported by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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和 文 摘 要

大野 浩・長谷 修・江原淑夫：キュウリモザイクウイルス (CMV) の感染性に与える pH の影響—ウイルス感染に対する表皮 RNase の作用

ササゲ (品種：黒種三尺) およびタバコ (品種：ky 57) に対する CMV (普通系) の感染性は懸濁緩衝液の種類、EDTA 添加に関係なく pH が 6.0, 7.0, 8.0 の順に高い。この傾向は CMV RNA を用いた場合も同様であった。滅菌水に溶解したウイルスを接種後、直ちに接種葉を pH 6.0-8.0 の緩衝液に浸漬した場合、感染性に影響はないことから、pH は植物細胞の感受性に影響を与えるのではなく、接種源に影響するものと考えられた。しかし、この pH 範囲でウイルス粒子の形態に変化は認められないことから、摩擦接種の際表皮から滲出する物質との関係が注目され、タバコ葉を用いて検討した。その結果、表皮細胞液 (表皮細胞滲出液、または汁液) 中の RNase の挙動と感染性における pH との関係に相関が認められた。すなわち表皮細胞液の RNase による CMV RNA の分解は pH 6.0 > 7.0 > 8.0 の関係であった。またこの RNase は粒子内部のウイルス RNA を不活化することなくウイルス表面に結合し、その結合量 (RNase 活性) は pH 6.0 > 8.0 の関係にあった。したがって pH 6.0 における CMV の感染性低下の原因の一つには接種の際滲出する表皮細胞液中の RNase の関与が考えられ、宿主細胞侵入後ウイルス粒子に付着した RNase が増殖に阻害的に働くことが推定された。