ON THE INTERFERENCE OF INACTIVATED PLANT VIRUSES

I. TOBACCO MOSAIC VIRUS

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不活化植物ウイルスの干渉

I. タバコモザイクウイルス

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INTRODUCTION

The phenomena of interference between active and inactivated viruses have been reported not only in animal viruses, but also in bacteriophages. However, the reports of the phenomenon in plant viruses are very few; it is considered that immunization of plants with the inactivated virus may be very difficult. Bawden and Kleczkowski (1953) reported that preparations of tobacco mosaic virus inactivated by ultraviolet rays interfered with infection by active TMV on *Nicotiana glutinosa*. Hirayama (1951) reported that the juice of tobacco plant infected with TMV treated with formalin gave the tobacco plant immunization and decreased the infectivity of the inoculated virus. Thomson (1960) stated that the interfering activity of tobacco mosaic virus was greatly reduced by heating at 55° or 60°C. for 10 minutes and it was lost when the virus was inactivated with ultraviolet radiation. In this paper there are reported interference phenomena of tobacco mosaic virus, inactivated by ultraviolet radiation and heat treatment, against infectivity of the active virus. Experiments were also carried out on the relationship between the interference phenomenon and the antigenicity of the inactivated virus.

MATERIALS AND METHODS

The juice of tobacco plants infected with tobacco mosaic virus (TMV) used in these experiments was purified by means of salting-out by ammonium sulfate and precipitation at isoelectric point. The expressed juice of tobacco plants infected with the ordinary strain of TMV was frozen, and then thawed at room temperature, and precipitates in this juice were removed by centrifugation at 3500 rpm for 20 minutes. Ammonium sulfate was added to the supernatant fluid to make a one-fourth saturation, and the resulting precipitate was suspended with phos-

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phate buffer solution (pH 7.0), and the insoluble materials were removed by centrifugation at 3500 rpm for 20 minutes. After repeating the same procedure twice, the virus suspension was brought to pH 3.3 with diluted HCl; the resulting precipitate was separated by centrifugation at 3500 rpm for 30 minutes, and was resuspended with phosphate buffer solution (pH 7.0), bringing the solution to the volume equal to that of original juice. It was centrifuged again at 3500 rpm for 20 minutes, and the supernatant fluid thus prepared was used in these experiments.

Ultraviolet radiation was carried out by means of Matsuda's germicidal lamp (wave length 2537 Å; intensity of radiation was 2160 $\mu\text{W}/\text{cm}^2$). The irradiation was made at the distance of 10 cm from the center of the lamp to virus solution in Petri dish of 9 cm diameter, with shaking slowly along the axis of the lamp. If the irradiation was carried out for a long time, the volume of juice was decreased by evaporation, so the same volume of water evaporated was added to the Petri dish during the irradiation. Following heat treatment, the virus solution was put into a small glass tube with rubber stoppers, and the glass tube was immersed in hot water of a given temperature, after this treatment the glass tube was instantly cooled with tap water. The infectivity of TMV was determined by the number of local lesions produced on the leaves of *Nicotiana glutinosa* that were inoculated with virus solution. The antigenicity was tested by the precipitation reaction resultant from the mixture of the virus and the antiserum which was prepared by injection of rabbits with purified TMV. The precipitation reaction was carried out in small glass tubes of 0.8 cm diameter and 8 cm length. One-fourth cc of the diluted antiserum, diluted with physiological salt solution, was poured into the glass tubes, and then the same volume of virus solution was added to each one. After shaking the glass tubes, they were incubated in hot water at 37°C. for 2 hours; the resulting precipitate was observed by agglutinoscope; after the tubes were allowed to stand overnight at low temperature room the precipitation reaction was again observed. The number of local lesions shown in the tables indicates the total number of those obtained in several experiments. Usually 32 to 64, rarely up to 128 leaves were inoculated.

RESULTS

A. Ultraviolet radiation

1. Infectivity, antigenicity and interfering activity of TMV exposed to ultraviolet radiation

The degree of infectivity was determined by the number of local lesions produced on the leaves of *Nicotiana glutinosa* and the antigenicity by the precipitation reaction. As a result, it was found that TMV readily lost infectivity by exposure to ultraviolet radiation for 30 minutes, but no remarkable change was recognized in the antigenicity. The antigenicity did not show any remarkable decrease until 8 hours of irradiation, as compared with that of non-irradiated virus. However, as shown in Table 1, 12 hours of irradiation caused a little decrease, 15 hours of irradiation showed a greater decrease of TMV antigenicity, and finally the antigenicity was almost lost after 18 hours of irradiation. In order to ascertain the interference of inactivated TMV on the infectivity of active TMV, the following experiments were carried out. The

TABLE 1
Antigenicity of ultraviolet irradiated TMV

Duration of irradiation (hrs.)	Final dilution of antiserum					Control
	16	32	64	128	256	
not irradiated	++	+	+	+	±	—
4	++	++	+	+	±	—
6	++	++	+	+	+	—
8	++	+	+	+	+	—
12*	+	+	+	+	+	—
15**	+	+	+	+	+	—
18	—	—	—	—	—	—
20	—	—	—	—	—	—

*: A little decrease of reaction

**: A weak reaction

active TMV and the inactivated one were mixed together in a ratio of 1 and 99, and the active TMV and phosphate buffer solution (pH 7.0) were mixed in the same ratio as the control. Each mixture was inoculated on the leaves of *Nicotiana glutinosa* by the half-leaf method. As a result in Table 2 show, the number of local lesions produced by the mixture of active and inactivated TMV was smaller than those produced by the control, so it was found that the inactivated TMV interfered with the infectivity of active TMV. However, the interference phenomenon was recognized only on the inactivated TMV exposed to ultraviolet radiation from 30 minutes to 12 hours and still having antigenicity. Furthermore, the inactivated TMV exposed to ultraviolet radiation for 15 hours showed a weak reaction, but still interference phenomenon was observed. TMV inactivated by 18 hours or more of irradiation almost lost antigenicity and interfering activity.

TABLE 2
Interference of ultraviolet irradiated TMV

Duration of irradiation (hrs.)	Number of local lesions		Percent. of A/B
	Mixture of active and inactivated virus (A)	Mixture of active virus and phosphate buffer sol. (control) (B)	
0.5	254	444	57.6*
1.5	257	405	62.4*
3	1220	1954	62.3*
4	962	1386	69.4*
6	1228	1780	68.9*
8	343	547	62.7*
12	170	270	62.9*
15	231	287	80.4*
18	490	523	93.6
20	231	228	101.6

*: The data have a significant difference to population ratio 0.5.

2. Interfering activity and quantity of TMV inactivated by ultraviolet radiation

When the ratio of mixture of the inactivated TMV exposed to ultraviolet radiation for 3 hours was increased to 10^2 , 10^3 , 5×10^3 and 10^4 against 1 portion of the active TMV, the interfering activity was promoted in accordance with the increase of the quantity of inactivated TMV as shown in Table 3.

TABLE 3
Interfering activity and quantity of TMV inactivated by ultraviolet radiation

Active virus : inactivated virus	Number of local lesions		Percent. of A/B
	Mixture of active and inactivated virus (A)	Control (B)	
1 : 10^2	2717	3849	70.6*
1 : 10^3	1091	1830	59.7*
1 : 5×10^3	326	649	50.2*
1 : 10^4	159	458	34.7*

To concentrate the amount of the inactivated TMV in the solution, the inactivated TMV was again precipitated by centrifugation at the isoelectric point, and the precipitate was suspended by means of a phosphate buffer solution (pH 7.0) of a half or a quarter volume of the original solution to double or quadruple the concentration of the inactivated TMV. The ratio of quantity of active and inactivated virus solution was 1:99. Interfering activity of the materials was compared with that of the original one. As shown in Table 4, the interfering activity was promoted by the increase of concentration of the inactivated TMV.

TABLE 4
Interfering activity and concentration of TMV inactivated by ultraviolet radiation

Concentration of inactivated TMV	Number of local lesions		Percent. of A/B
	Mixture of active and inactivated virus (A)	Control (B)	
original conc.	1028	1304	78.8*
double conc.	774	1335	58.0*
quadruple conc.	793	1997	39.7*

As mentioned above, the interfering activity had a relationship to the quantity and concentration of the inactivated TMV.

3. Pre-inoculation with the inactivated TMV

Experiments were carried out in order to determine whether pre-inoculation with the inactivated TMV on *N. glutinosa* may produce the interference phenomenon or not, when the inoculation was followed by the use of active TMV on the same leaves. The half-leaves of *N. glutinosa* were at first inoculated with the inactivated TMV exposed to ultraviolet radiation for 3 hours and the other half-leaves were rubbed with phosphate buffer solution (pH 7.0) as the control. After this inoculation, the active TMV solution diluted to one-hundredth with phosphate buffer solution was inoculated on each surface of half-leaves at intervals of 1, 3, 5, 7, 9,

and 11 days. The number of local lesions produced on the half-leaves was compared with that on the control. As shown in Table 5, it was found that the number of local lesions on the half-leaves previously inoculated with the inactivated TMV was less than that on the control when the leaves were inoculated with the active TMV at one day after inoculation. However, when the intervals were 3 days or more, no distinct interference was recognized, as the number of local lesions on the half-leaves inoculated previously with the inactivated TMV did not show any considerable decrease as compared with that of the control. On the basis of the results above noted, the inactivated TMV did not show interference when the intervals were 3 days or more after the inoculation of inactivated TMV.

TABLE 5
Interference by the pre-inoculation of inactivated TMV

Intervals after inoculation of inactivated TMV (days)	Number of local lesions		Percent. of A/B
	Pre-inoculation by inactivated virus (A)	Control (B)	
0	250	461	54.2*
1	292	483	60.5*
3	225	229	98.3
5	91	107	85.0
7	326	374	87.2
9	100	107	93.4
11	252	253	99.6

B. Heat treatment

1. Infectivity, antigenicity and interfering activity of TMV subjected to high temperature

The TMV treated by heating at 90° and 95°C. for 10 minutes completely lost infectivity and antigenicity, but when it was heated at 85°C. for 10 minutes it still had a low infectivity and weak precipitation reaction. These heat-treated TMV samples were used in order to determine whether it possess interfering activity or not by the same methods as in the case of ultraviolet irradiated TMV. The mixture of active and treated TMV was made in the ratio of 1 to 99; the mixture of active TMV and phosphate buffer solution was made in the same ratio. While TMV heated at 85°C. showed low infectivity, the treated TMV was independently ino-

TABLE 6
Antigenicity of heat-treated TMV

Temperature of treatment	Final dilution of antiserum					Control
	16	32	64	128	256	
not treated	++	++	+	+	±	--
85°C	+	+	+	+	±	--
90	—	—	—	—	—	--
95	—	—	—	—	—	--

TABLE 7
Interference of heat-treated TMV

Temperature of treatment	Number of local lesions				Percent. of A-B/control
	Mixture of active and treated TMV (A)	Treated TMV (B)	A-B	Control	
85°C	236	94	142	346	41.0*
90	768	0	768	792	97.0
95	758	0	758	763	99.3

culated on the leaves of *N. glutinosa* to ascertain its infectivity. The number of local lesions was deducted from that produced by the mixture of active and treated TMV, and then the remainder was compared with that of control. As the results exhibited in Table 6 and 7 show, TMV subjected to temperatures of 90° and 95°C. had almost lost its antigenicity and also interfering activity. However, TMV heated at 85°C. did not completely loss infectivity and antigenicity, but showed the interference phenomenon.

DISCUSSION AND CONCLUSION

It is known that the infectivity of TMV exposed to ultraviolet radiation is destroyed without the loss of antigenicity (Stanley, 1936; Bawden, 1950; Miyamoto, 1954; Shikata, 1955). In the present experiments it was found that the infectivity of TMV was destroyed by ultraviolet radiation (intensity of radiation: $2160 \mu\text{W}/\text{cm}^2$) for 30 minutes, but no decrease of antigenicity was recognized till after 8 hours of irradiation. The antigenicity was reduced after the exposure to ultraviolet radiation for 12 hours, and it was completely lost after 18 hours or longer of irradiation. The inactivated TMV produced an antagonistic reaction that interfered with active TMV infectivity when the mixture of active and inactivated TMV at the ratio of 1 to 99 was inoculated on the leaves of *N. glutinosa*. The interference was demonstrated by decrease of the number of local lesions produced on the leaves as compared with that of the control. The interference phenomenon was recognized only for the inactivated TMV exposed to ultraviolet radiation from 30 minutes to 12 hours and still possessing antigenicity. However, TMV irradiated for 18 hours or more and not having antigenicity, did not show interfering activity. The interference was related to the antigenicity. The interference was affected by the ratio of the inactivated TMV to the active TMV; because the greater the quantities or the higher the concentrations of inactivated TMV to active TMV, the greater the resultant interfering activity. A similar interference phenomenon was recognized on the leaves of *N. glutinosa* that were reinoculated with the active TMV at intervals of one day after the inoculation of the inactivated TMV. It was found that the inactivated TMV did not produce an obvious immunization on the inoculated plants at the intervals of 3 days or more after the inoculation of inactivated TMV.

TMV subjected to high temperatures of 90° and 95°C. for 10 minutes completely lost its infectivity and antigenicity, and that treated at the temperature of 85°C. for 10 minutes lost some of them. These treated TMV samples were used to determine whether the material had interfering activity or not, by the inoculation of mixtures of active and inactivated TMV on *N.*

glutinosa. The results show that the inactivated TMV heated at 90° and 95°C. did not produce the interference reaction, while TMV heated at 85°C. had the interfering activity.

Thomson (1960) reported that the interfering activity of the virus in the unfractionated sap from tobacco leaves infected with TMV was greatly reduced by heating at 55° or 60°C. for 10 minutes and lost when the virus was inactivated with ultraviolet light. He suggested that two factors responsible for interference occurred in unfractionated sap; one was more unstable than the virus and inhibited infection and the other was always associated with infectivity and inhibited virus synthesis or movement after infection, producing smaller lesions. According to the electron microscopic observation reported by Shikata (1955), TMV lost its infectivity as a result of exposure to ultraviolet radiation for a short time, but did not show any morphological changes. As the irradiation was continued, the particles were cut into short rods and the longer the irradiation the shorter the particles. The particles smaller than 20 m μ coagulated in deposited form and had no antigenicity. In the present experiment, TMV was caused to loss antigenicity by irradiation for more than 18 hours. From the results reported by Shikata, the inactivated TMV particles thus treated might become very short and denaturated particles having no interference activity. On heat treatment, Fukushi and Shikata (1953) also reported that TMV particles heated at 90°C. for 2 to 15 minutes increased in number of spherical particles and became clumped. Hidaka and Kiriya (1953) reported that TMV heated at 85°C. for 10 minutes increased in number of 20 to 40 m μ particles and TMV heated at 90° or 95°C. for 10 minutes did not show rod-shaped particles. In the present experiments it was considered that TMV heated at 90° and 95°C. lost completely its morphological character, and lost the interfering activity, but TMV heated at 85°C. still kept the particular form and also the interfering activity.

The mechanism of interference is not definitely known and further experiments are necessary to be carried out along this line.

SUMMARY

In this paper experiments are reported concerning the antigenicity and interference with the infectivity of tobacco mosaic virus (TMV) inactivated by ultraviolet radiation (intensity of radiation: 2160 μ W/cm²) and high temperature treatment. TMV was inactivated by relatively low doses of ultraviolet radiation (30 min. at 10 cm distance), but showed no remarkable change in antigenic properties, increased irradiation (18 hours at the same distance) being necessary in order to completely destroy its antigenicity. The inactivated TMV interfered with the infectivity of TMV, as demonstrated by comparison of the number of local lesions produced on the leaves of *Nicotiana glutinosa* inoculated with the mixture of inactivated and active virus solution, with those produced by active virus and buffer solution.

The infectivity of TMV solution decreased by 30 to 70 percent when the inactivated TMV solution of 100 to 10,000 times its quantity was added to it. Higher concentrations of inactivated TMV results in greater decrease in the infectivity of TMV. Similar interference was recognized in the plants reinoculated with active TMV one day after inoculation of the inactivated TMV, but such was not the case when the active TMV was inoculated more than 3 days later. Antagonistic reactions were found to occur between the active and the inactivated but still antigenic TMV, whereas the completely denaturated TMV did not interfere with the

former. TMV subjected to high temperature (90° and 95°C., for 10 min.) lost both its infectivity and antigenicity, and had no interfering activity. But the TMV incompletely inactivated by heating at 85°C., for 10 minutes showed slight interference with the activity of TMV.

要 約

TMV に紫外線照射および加熱処理を行ない、感染性および抗原性を調べ、不活化 TMV の活性 TMV の感染性に対する干渉作用について実験を行なった。

(1) TMV の感染性は紫外線照射 ($64 \times 10^3 \mu\text{W}/\text{cm}^2$) によつて喪失したが、抗原性をも消失させるにはさらに大量の照射が必要であつた。活性 TMV に多量の照射不活化 TMV を混じて *N. glutinosa* に接種すると生ずる壊死斑点数が減少した。すなわち照射不活化 TMV は活性 TMV の感染性に干渉し、この干渉作用は照射不活化 TMV の量を増加しあるいは濃度を高めるにつれて増大した。

(2) 照射不活化 TMV を予め *N. glutinosa* の葉面に接種し、1 日後に活性 TMV を接種した場合には照射不活化ウイルスの干渉作用が認められたが、3 日以上の間隔をおいた場合には明かな干渉作用が認められなかつた。

(3) 以上の干渉作用はいづれも抗原性を有する照射不活化 TMV によつて認められたが抗原性を喪失した不活化 TMV では認められなかつた。

(4) 加熱処理では TMV の感染性と抗原性とほぼ同時に消失した。抗原性が認められなかつた 90° および 95°C., 10 分処理の不活化 TMV は干渉能を示さなかつたが、僅かに感染性と抗原性とを有していた 85°C., 10 分処理 TMV ではそれが認められた。

REFERENCES

- 1) Bawden, F. C. (1950): Plant viruses and virus diseases. 3rd ed. 248, Chronica Botanica, Waltham.
 - 2) Bawden, F. C. and Kleczkowski, A. (1953): J. Gen. Microbiol. 8, 145-156.
 - 3) Fukushi, T. and Shikata, E. (1953): Virus 3, 208.
 - 4) Hidaka, J. and Kiriya, K. (1953): Virus 3, 39-50.
 - 5) Hirayama, Y. (1951): Forch. Pfl. Kr. Kyoto 4, 35-45.
 - 6) Miyamoto, Y. (1954): Virus 4, 256-263.
 - 7) Shikata, E. (1955): Jubilee Publication in Commemoration of Sixtieth Birthdays of Prof. Y. Tochinnai and T. Fukushi. Sapporo, Japan, 344-346.
 - 8) Stanley, W. N. (1936): Science 83, 626-627.
 - 9) Thomson, A. D. (1960): Nature 187 (4739), 761-762.
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