Effect of Detached Leaf Treatment on Tobacco Mosaic Virus Multiplication in Tobacco and Bean Leaves

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ABSTRACT

Effect of host senescence on tobacco mosaic virus (TMV) multiplication and local lesion formation was investigated by using attached and detached tobacco and bean leaves. In the systemic host, detachment of leaves before inoculation caused remarkable decrease in the amount of nucleic acids and dry weight, but not in chlorophyll and soluble protein per dry weight. In leaves detached for 2 days, however, TMV concentration increased 42% over attached leaves, but after longer periods virus concentration was lower than in attached leaves. Detachment of leaves after inoculation promoted chlorophyll degradation, whereas TMV infectivity was slightly increased over that in attached leaves until 4 days after inoculation. TMV concentration was found similar in both the detached and attached leaves 5-6 days after inoculation, despite the fact that chlorophyll content was sharply lowered. In bean leaves, detachment of leaves just after inoculation decreased the number of local lesions by 33%; whereas, diameter of lesion size and TMV infectivity within the lesions increased about 2.3 and 4.3 times over that shown by attached leaves, respectively. It is suggested that moderate leaf senescence associated with partial degradation of nucleic acids and protein closely relates with increases in TMV concentration in tobacco and bean leaves. Phytopathology 61:22-27.

Tobacco mosaic virus (TMV) multiplication may be influenced by age of host tissue or by a certain state of host senescence. Opel (17) indicated that older leaves were more susceptible to tobacco necrosis virus than young leaves. The increase in host susceptibility in local lesion hosts has been proven by the application of different treatments: actinomycin D (11); chloramphenicol (10, 23); high temp (8); and heat (10, 19, 27, 28). Király et al. (10) indicated that host susceptibility to TMV infection was decreased by treatment of leaves with kinetin, but was increased by a certain state of host senescence. Milo & Srivastava (14) could not prove a direct correlation between chlorophyll retention and virus production caused by cytokinins treatment. Hirai et al. (7) stated that application with plant hormones stimulated TMV multiplication in systemic hosts. In a previous paper, Nakagaki et al. (15) reported that TMV multiplied to the same extent in ethylene-treated leaf discs as in untreated ones, although ethylene caused leaf senescence. Since host reaction to plant hormones may be influenced by hormonal level in leaf tissues, experimental induction of senescence in a natural way is desirable when investigating the effect of host senescence on TMV multiplication. This paper describes the effect of detachment of tobacco and bean leaves on TMV multiplication and local lesion formation.

MATERIALS AND METHODS.—*Plant materials and virus.*—Tobacco (*Nicotiana tabacum* L. 'Bright Yellow') and bean (*Phaseolus vulgaris* L. 'Kairyo Otebo') were grown in 18-cm and 10-cm diam pots, respectively. The ordinary strain of TMV was purified using differential centrifugation. Tobacco plants used were trimmed except for 3-4 sufficiently expanded leaves, 20-25 cm long. The upper surface of tobacco leaves was dusted with Carborundum (600 mesh) as an abrasive and rubbed with 0.5 mg/ml of TMV suspended in 1/10 M phosphate buffer at pH 7.0. Inoculated leaves were rinsed with tap water, and half-leaves were obtained by immediately splitting along the midrib. Discs, 12 mm in diam, were punched with a cork-borer from the detached leaves and incubated for a given period in a petri dish containing a moist filter paper. The rest of the half-leaf remained attached to the plant as a control. Discs from both the half-leaves were used for the determinations of TMV concn, chlorophyll content, and dry wt, which were estimated after drying leaf discs at 105 C overnight.

For testing the effect of host senescence prior to inoculation, the previously detached half-leaf and the corresponding opposite half-leaf, which remained attached to the plant, were placed in an incubator for various intervals at 18 C daytime and 13 C night-time temp. After a given time, an intact or whole leaf was removed from the plant, upper and lower surfaces of detached and attached leaves were inoculated in the same manner as stated above, and discs from each half-leaf were punched and incubated for 5 days in a petri dish containing a moist filter paper. These petri dishes were placed in a growth chamber under continuous fluorescent light (ca. 5,000 lux) at about 25 C. Two primary leaves of a bean plant, 12-15 days old in winter, were inoculated with 10 μg/ml of TMV, then detached and the opposite attached half-leaves were incubated in the same manner as stated above.

Determination of TMV content.—The amt of TMV produced in leaf discs was measured by the nucleoprotein method of Taniguchi (26), and by a bioassay which consisted of inoculating a leaf homogenate to *P. vulgaris* L. 'Kairyo Otebo' and counting the number of local lesions produced (TMV infectivity). Relative values were calculated by the following equation:

$$\frac{\text{Number of lesions on A or D leaves} \times \text{dilution level}}{\text{Number of lesions produced by TMV (10 μg/ml)}} \div$$

dry wt in A or D, where "A" is attached leaves and

"D" is detached leaves. Lesion size was measured 3 days after inoculation by using an ocular micrometer.

Determination of the amt of total nucleic acids and soluble protein.—The amt of total nucleic acids in leaf discs was determined by a modified method that combined those of Schneider (22) and Ogur & Rosen (16). Twenty leaf discs were homogenized in a mortar with 1 ml of 1/10 M phosphate buffer at pH 7.0 and then with 5 ml of cold 10% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 *g* for 15 min. To the precipitate, 5 ml of cold 5% TCA was added and centrifuged at 3,000 rpm. The precipitate was washed twice with cold 95% ethanol, then twice with ethanol-ether (3:1) for 5 min at 60 C, followed by 15 min centrifugation at 3,000 rpm. To the precipitate, 5 ml of cold 0.2 N perchloric acid (PCA) was added and centrifuged at 3,000 rpm. The precipitate was washed with 5 ml of 1 N PCA for 20 min at 70 C and then 5 ml of 1 N PCA. Optical density at 260 mμ of the resulting supernatant was measured by a spectrophotometer. For soluble protein determination, the method of Atkin et al. (2) was used. Twenty leaf discs were homogenized in a mortar with 4 ml of 50 mM Tris[tris(hydroxymethyl) amino methane]-HCl buffer at pH 7.6, containing 5 mM cysteine hydrochloride. The homogenate was centrifuged at 1,500 *g* for 15 min, then at 30,000 *g* for 30 min. The resulting supernatant was dialyzed twice against 2 liters of the extraction buffer over a 48-hr period. The dialyzed extract was centrifuged again at 30,000 *g* for 30 min. Three ml of the supernatant were mixed with cold TCA to a final concn of 5%, followed by 15 min centrifugation at 10,000 *g*. To the precipitate, 4 ml of 5% TCA was added and again centrifuged at 3,000 rpm. The precipitate was washed twice with 95% ethanol, then with ethanol-ether (3:1). After drying in air, it was thoroughly dissolved in 3 ml of 1 N NaOH. Soluble protein content was determined by the Folin phenol procedure (12) using bovine serum albumin as the control.

Determination of chlorophyll content.—Chlorophyll content in leaf discs was determined by the method of Arnon (1).

RESULTS.—*Effect of detachment of leaves before inoculation on the amounts of chlorophyll, nucleic acids, soluble protein, and TMV concn.*—Amounts of nucleic acids, soluble protein, and chlorophyll in the leaves attached and detached were compared before inoculation. Figure 1-A shows the ratios of the values per dry wt of detached leaves to those of attached ones. A remarkable decrease in the ratio of nucleic acids was found when leaves detached 2 days before inoculation, and this decrease was even sharper when intervals between detachment and inoculation were increased. The ratio of soluble protein, however, increased in leaves detached 2 days before inoculation; during longer incubation periods it decreased to the same level as that of attached leaves. The ratio of chlorophyll in detached leaves to attached leaves seemingly increased, because dry wt per unit leaf disc greatly decreased in detached leaves. Figure 1-B shows that TMV concn increased 42% when leaves were detached 2 days before inoculation, but sharply decreased after longer incubation periods.

TMV infectivity and chlorophyll content in leaves detached after inoculation.—Figure 2-A shows time course changes in TMV infectivity and chlorophyll content during the 4 days after inoculation, both in leaves which remained attached and those which were detached after inoculation. In attached leaves, chlorophyll degradation was retarded, whereas in detached leaves, chlorophyll degradation was increased as shown by the linear decrease of chlorophyll content (Fig. 2-A). TMV infectivity, shown by the number of local lesions produced on bean leaves, was detected 2 days after inoculation in both kinds of leaves. The rate of infectivity was always slightly higher in detached leaves than in attached leaves during the 4 days after inoculation. TMV infectivity on the basis of chlorophyll content, as shown in Fig. 2-B, was 74% higher in detached leaves as compared with attached leaves. Table 1 presents chlorophyll content and TMV concn in detached and attached leaves 5-6 days after inoculation. Dry wt of leaves slightly decreased, and chlorophyll content remarkably decreased in detached leaves, whereas TMV multiplied almost to the same extent in both kinds of leaves.

Effect of detachment of leaves on local lesion formation and TMV infectivity in bean leaves.—Table 2 compares the number of local lesions and TMV infectivity in bean leaves attached and detached after inoculation. The number of local lesions and chlorophyll content decreased about 33% and 18% in detached leaves, respectively. The diam of lesion size was larger, about 2.3 times in detached leaves as compared with attached leaves (Fig. 3). TMV infectivity, which is shown by the number of lesions produced on bean leaves, was about 3.5 times higher than that of attached leaves. TMV infectivity per unit lesion size was calculated, and the ratio of detached leaves to attached leaves was obtained by the following equation:

$$\frac{\text{TMV infectivity in D}}{\pi r^2 \text{ (lesion size) in D}} \div \frac{\text{TMV infectivity in A}}{\pi r^2 \text{ (lesion size) in A}},$$

where "A" is attached leaves and "D" is detached leaves. These ratios were 3.9, 3.5, 3.1, —, 6.3, —, and 4.6 in Experiments 1 to 7 in Table 2, respectively, and these values showed the same tendency in case of TMV infectivity per dry wt in Table 2. Therefore, TMV infectivity per unit lesion size was about 4.3 times higher in detached leaves than in attached leaves.

DISCUSSION.—During the incubation of detached tobacco half-leaves, the amt of total nucleic acids and dry wt decreased, except that of soluble protein and chlorophyll. The chlorophyll content slightly increased, especially in the later incubation stages, probably as expressed unit per dry wt (Fig. 1-A). TMV concn remarkably increased over attached leaves in leaves detached for 2 days before inoculation, then inoculated; however, it decreased in those detached for 3-5 days. Concomitant with the decrease in TMV concn, both the nucleic acids and soluble protein levels lowered. Fig. 2-B, which involves leaves detached after inoculation,

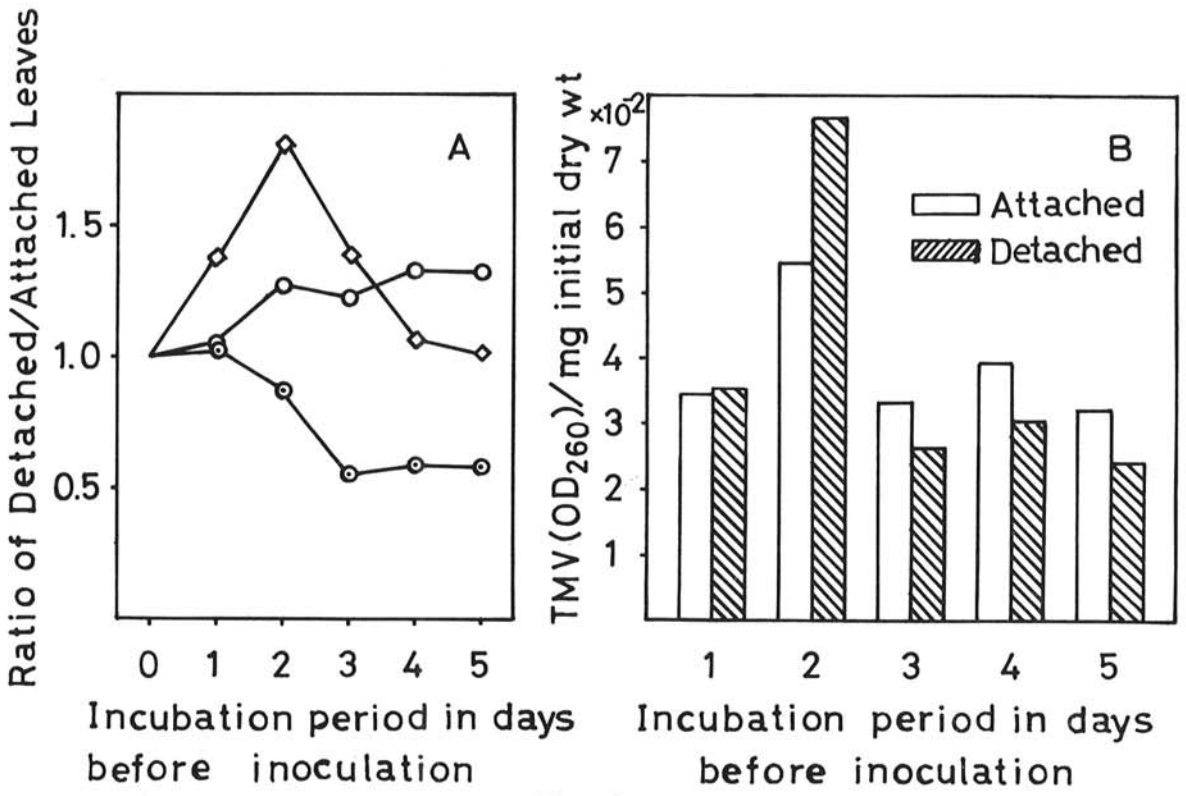

Fig.1

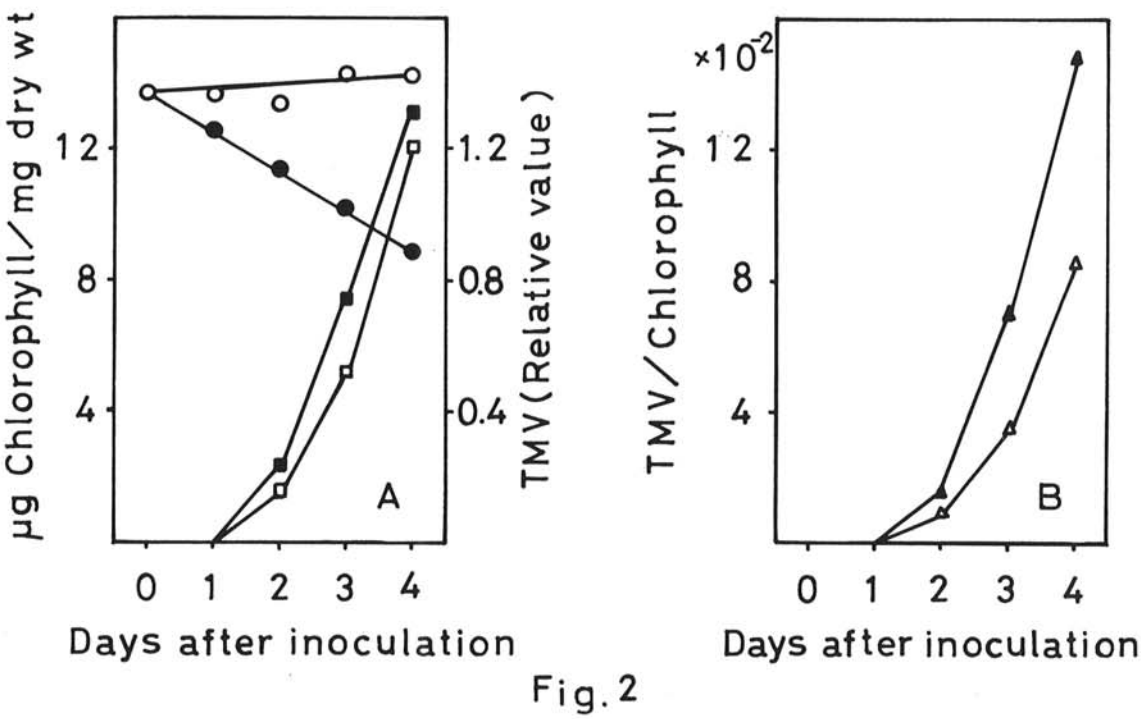

Fig. 2

Fig. 1-2. 1) A) The ratio of total nucleic acids, soluble protein, and chlorophyll content per dry wt of detached tobacco leaves to attached leaves that were treated before inoculation with tobacco mosaic virus (TMV). ○ = Chlorophyll

TABLE 1. Tobacco mosaic virus (TMV) concn and chlorophyll content in inoculated leaves attached and detached 5-6 days after inoculation[a]

Exp. no.	Treatment[b]	Dry wt mg/10 discs	Chlorophyll content μg/mg dry wt	Chlorophyll content D/A	TMV concn OD_{260}/mg dry wt	TMV concn OD_{260}/mg D/A
1.	Attached (A)	24.8	17.15	0.59	2.09^{10-2}	0.93
	Detached (D)	23.3	10.12		1.95	
2.	A	30.0	9.05	0.12	3.51	0.88
	D	29.2	1.08		3.08	
3.	A	33.8	9.88	0.34	2.52	1.13
	D	27.6	3.38		2.84	
4.	A	22.5	11.89	0.30	5.03	0.99
	D	22.3	3.53		4.99	
5.	A	23.7	17.07	0.70	1.76	1.13
	D	22.8	11.92		1.99	

[a] Averages of 6-7 leaves consisted of two plants.
[b] Detached leaves were removed immediately after inoculation. Attached leaves remained on plant during incubation periods.

TABLE 2. Effect of detached leaf treatment after inoculation on local lesion formation and tobacco mosaic virus (TMV) infectivity in bean leaves[a]

Exp. no.	Treatment[b]	No. lesions/10 half-leaves	D/A	Lesion[c] size, diam mm	Chloro-phyll content %	TMV[d] infectivity	D/A
1.	Attached (A)	5442	0.65[e]	0.39 ± 0.12	100	1535	3.6
	Detached (D)	3582		0.90 ± 0.23	74.0	5404	
2.	A	3085	0.65[e]	0.41 ± 0.13	100	654	3.6
	D	2030		0.92 ± 0.07	87.6	2336	
3.	A	1249	0.79[e]	0.31 ± 0.09	100	2120	3.9
	D	989		0.61 ± 0.11	85.9	8270	
4.	A	2008	0.54[e]	0.37 ± 0.08	100		
	D	1076		0.87 ± 0.14	80.9		
5.	A	1335	0.71	0.41 ± 0.11	100	1878	2.8
	D	952		1.22 ± 0.27	83.9	5202	
6.	A	795	0.66				
	D	523					
7.	A	1230	0.71[e]	0.31 ± 0.09	100	570	3.2
	D	871		0.55 ± 0.15	90.2	1800	

[a] Three days after inoculation with 10 μg/ml of TMV.
[b] Detached leaves were removed immediately after inoculation and attached leaves remain on plant during 3 days.
[c] Two hundred lesions produced on 10 half-leaves were measured in one experiment.
[d] Two half-leaves were homogenized in 2 to 5 volumes of 1/100 M phosphate buffer at pH 7.0, and TMV infectivity was assayed on 10 half-leaves of *Phaseolus vulgaris* L. Kairyo Otebo. Figures were the sum of lesion number of 2 half-leaves, repeated 5 times using 10 half-leaves/assay.
[e] Significant at level of .01.

indicates that TMV infectivity was always higher in detached leaves than in attached leaves. These two results suggest that TMV multiplication increases in leaves which show a moderate senescence, but decreases in leaves showing an extreme senescence.

The increase in TMV concn in detached leaves may be caused by the supply of substrates for TMV synthesis which are produced by the degradation of host components. Reddi (20, 21) and Gate (4) reported that breakdown products of host RNA and protein were used for the synthesis of viral RNA and protein. But, as shown in Fig. 1-A, when the ratio of total nucleic acids reached 0.5, i.e., half the level of that in intact leaves, TMV multiplication is restricted. This fact

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content; ⊙ = total nucleic acids; ◇ = soluble protein. TMV concn 5 days after inoculation. Average of 2 independent experiments, each of which consisted of four assays using 10 or 20 leaf discs each. **2) A)** Time course changes in chlorophyll content and TMV infectivity in attached and detached tobacco leaves treated after inoculation. TMV infectivity was assayed by inoculating leaf homogenate to leaves of *Phaseolus vulgaris* L. Kairyo Otebo. ○ = Chlorophyll content in attached leaves; ● = chlorophyll content in detached leaves; □ = TMV content in attached leaves; ■ = TMV content in detached leaves. Averages of three independent experiments, each of which consisted of four assays. **B)** Changes in TMV conc per chlorophyll in attached and detached leaves during the incubation following inoculation. △ = Attached leaves; ▲ = detached leaves.

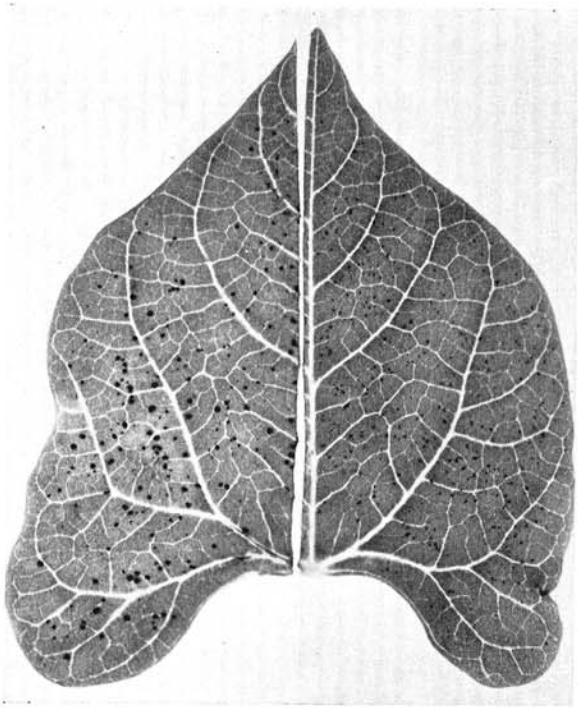

Fig. 3. Necrotic local lesions produced on attached (right) and detached (left) leaves. Photographed 3 days after inoculation.

seems to reflect the lowering of activities concerned with virus synthesis; for instance, ribosome activity and other protein-synthesizing mechanism.

The amt of soluble protein increased in leaves detached for 2 days before inoculation and then decreased during longer incubation period. This means that soluble proteins in leaves released from cell components during early incubation period may be degraded during further incubation. It is of interest that TMV concn is always parallel to changes in soluble protein content in leaves. This suggests that more TMV is synthesized in leaves having high content of soluble protein than in those of low content. Hayashi & Hirai (5) demonstrated that soluble protein was, in part, used for TMV synthesis during early infection stages.

Chlorophyll degradation seems to have no direct relationship to TMV multiplication. In Fig. 2, TMV multiplied slightly more in detached leaves in which chlorophyll content per dry wt was extremely decreased. Much evidence has accumulated on the close relationship between chlorophyll degradation and host senescence associated with decreases in nucleic acid and protein metabolism in leaves (2, 18, 25). It appears, therefore, that host senescence connected with virus synthesis is not directly related to chloroplast components, since the ordinary strain of TMV was multiplied within cytoplasm but not within chloroplasts (3, 6, 9, 24).

In local lesion hosts, lesion number decreased but lesion size and TMV infectivity within the lesions increased in detached leaves as compared with leaves which remained attached (Table 2). Matthews (13) stated that the number of local lesions produced on localized host leaves which were placed in darkness after inoculation was smaller than those produced on leaves in light. Thus, decrease in lesion number may reflect a decline in host susceptibility to TMV infection in detached leaves immediately after inoculation. The increase in size of lesion involves a high rate of TMV infectivity within the lesions, and these two phenomena were stimulated in detached leaves. Since detached leaves were incubated in a moist chamber, the effect of water content in leaf tissues or of humidity on the lesion size cannot be excluded; however, stimulation of TMV multiplication in local lesion hosts was also reported by actinomycin D (11), chloramphenicol (10, 23), high temp treatment (8), and heat treatment (10, 19, 27, 28). It has been suggested that degradation of host RNA and protein caused the increase of TMV concn. Therefore, increase of TMV concn in the leaves showing a moderate senescence can be commonly understood in local lesion hosts as well as in systemic hosts. The state of host senescence for increasing TMV concn seems not to exist in extreme degradation of host components, but exists in the moderate degradation by which leaves are able to supply substrates for TMV synthesis.

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