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Interference between Two Satellite Viruses of Tobacco Necrosis Virus

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SUMMARY

The tobacco necrosis satellite viruses SV_1 and SV_2 interfere with one another's replication, and the larger the dose of the interfering satellite the greater the degree of interference produced. The amount of interference also depends on the strain of tobacco necrosis virus (TNV) used as helper. Suppression of SV_2 by SV_1 is greater than that of SV_1 by SV_2, although SV_2 is the more infective. SV_c differs serologically from SV_1 and SV_2 no more than these two differ from each other, but it needs a different strain of TNV for replication. Nevertheless, there is no interference between SV_c and either SV_1 or SV_2. The interference between SV_1 and SV_2 takes place in the first 2 h after inoculation. Satellite viruses inoculated 3 days after TNV do not interfere with one another provided the TNV strain is one that aids their multiplication. The results suggest that SV_1 and SV_2 compete for an early metabolite.

INTRODUCTION

Satellite viruses SV_1, SV_2 and SV_c are serologically related, but when antisera are sufficiently diluted the 3 serotypes react only with their own antiserum and therefore concentrations of each can be estimated serologically in mixed infections. Each serotype multiplies only when inoculated together with tobacco necrosis virus (TNV), but the TNV strains that aid replication of SV_1 and SV_2 do not aid SV_c and vice versa (Uyemoto, Grogan & Wakeman, 1968; Kassanis & Phillips, 1970). SV inhibits replication of TNV and the amount of inhibition increases with increasing concentration of SV in the mixed inoculum (Kassanis, 1962). We now find that SV_1 and SV_2 interfere not only with TNV but also with one another, provided one is at a greater concentration in the inoculum than the other. By contrast, SV_1 and SV_2 do not interfere with SV_c although it has the same degree of serological relationship with SV_1 and SV_2 as these two serotypes have with one another.

METHODS

Virus inoculum. The various isolates were propagated and purified as previously described (Kassanis & Phillips, 1970).

Plants. To study the interference, the viruses were inoculated in *Nicotiana clevelandii* Gray and unless otherwise mentioned the inoculation was made using carborundum. The leaves were detached 1 day after inoculation and placed in enamelled dishes, the bottoms of which were covered with four layers of wet tissue paper. The dishes were enclosed in polythene bags and kept in the glasshouse, but covered with a sheet of muslin.

Estimation of concentration. Five days after inoculation, sap was extracted from inoculated leaves, clarified by centrifuging at 9000 *g*, heated for 10 min at 50 °C and clarified

again. Twofold serial dilutions of the clarified sap were mixed in narrow tubes with 1/40 dilutions of antisera to serotypes of SV (the titres of the antisera were 1/320). At this dilution the antisera of the three serotypes reacted only with their own antigens. Final readings of precipitation titres were made after 8 h incubation at 40 °C.

RNA extraction. The nucleic acid was extracted from a purified preparation of virus in 0·06 M-phosphate buffer pH 7 with an equal volume of water-saturated phenol. Traces of phenol were removed by shaking with ether.

RESULTS

Interference between SV_1 and SV_2

SV_2 was slightly more infective than SV_1 because it caused infection at higher dilutions than SV_1 (Table 1). When mixtures of SV_1 and SV_2 were inoculated with the two viruses at the same concentration over a wide range of concentrations each multiplied as well as it did when inoculated alone. At very low concentrations, SV_2 multiplied better or was the only one to multiply, because the inoculum of SV_2 was more infective than that of SV_1 (Table 1). However, when mixtures of the two serotypes were inoculated with one serotype in excess, multiplication of the satellite virus which was in minority was strongly inhibited. Table 2 shows that the amount of inhibition depended both on the degree by which the two inocula were unbalanced and on the strain of TNV used to aid the multiplication of the satellite virus. Inhibition of the minor SV was much greater with TNV A and TNV CT than

Table 1. *Serological titres of SV_1 and SV_2 when inoculated at different concentrations separately and in mixtures*

Inoculum (μg/ml)	Separately		In mixtures	
	SV_1	SV_2	SV_1	SV_2
125†	—	—	32	8
30	—	—	32	32
3	32*	32	64	32
0·25	16	16	16	16
0·025	8	16	16	32
0·0025	4	4	2	4
0·00025	0	2	0	2

* Reciprocal of the serological dilution end-point.
† Final concentrations of SV_1 and SV_2, each after mixing in equal volumes with TNV A; the final concentration of TNV A was 3 μg/ml.

Table 2. *Competition between SV_1 and SV_2 when inoculated in mixtures in the presence of different strains of TNV*

Inoculum (μg/ml)		Strains of TNV*							
		TNV S		TNV B		TNV CT		TNV A	
SV_1	SV_2	SV_1	SV_2	SV_1	SV_2	SV_1	SV_2	SV_1	SV_2
3	1	64†	32	64	64	—	—	—	—
9	1	64	4	64	8	32	1	32	1
27	1	64	1	64	8	32	0	16	0
1	9	64	64	32	64	16	32	8	32
1	27	16	64	16	64	8	32	4	32

* TNV at 10 μg/ml was mixed with equal volumes of SV_1 and SV_2 at concentrations shown in the table.
† Reciprocal of serological dilution end-point.

with TNVB, while TNVs gave intermediate results. TNVA was used in all subsequent experiments. Table 2 shows also that SV_2 was inhibited by SV_1 much more than the other way around regardless of the strain of TNV used.

In other experiments when the inoculum contained 25 times as much of one satellite virus as the other, inhibition occurred at different virus concentrations; e.g. when 25, 5 and 1 μg/ml of SV_1 was inoculated together with 1, 0·2 and 0·04 μg/ml of SV_2 respectively, the latter virus was completely inhibited in all three mixtures. Complete inhibition of SV_2 by SV_1 occurred over a wide range of TNV concentrations.

RNA inocula

Inhibition of one serotype by the other occurred also when their RNAs were inoculated in mixtures. When the RNA from 0·1 mg/ml of each of the two serotypes were mixed at a weight of 25 to 1, the serological titres of SV_1 and SV_2 were 16 and 0 when SV_1 was in excess and 1 and 16 when SV_2 was in excess.

Plant protection test

Strains forming local lesions usually cause fewer lesions when inoculated in mixtures with other strains that do not cause local lesions than when inoculated singly; the number of lesions decreases further as more of the non-lesion forming strain is added. It is usually assumed that this is because the strains compete for infectible sites (Kassanis, 1963).

Table 3. *Protection test between the* NI118 *and* TYPE *strains of TMV*

Treatment*	Average no. of lesions	% of control
NI118 0·1 μg/ml+TMV 2·5 μg/ml	48	47
NI118 0·1 μg/ml+buffer	102	100
NI118 0·1 μg/ml+dil. 1/10 TMV RNA ex 3·4 mg/ml of virus	83	81
NI118 RNA ex 0·14 mg/ml +TMV RNA ex 3·4 mg/ml	112	41
NI118 RNA ex 0·14 mg/ml+buffer	271	100
NI118 RNA ex 0·14 mg/ml+TMV 2·5 μg/ml	230	81

* The two strains were mixed in equal volumes; all dilutions were made in 0·06 M-phosphate buffer pH 7.

Experiments were made to test whether interference occurred between strains of tobacco mosaic virus (TMV) when one was 25 times more concentrated than the other. The two strains of TMV used were NI118, which forms lesions, and the TYPE strain which does not. The test plant was *Nicotiana tabacum* L. type White Burley cv. Judy's Pride. Inocula were prepared by mixing whole virus or RNA, or whole virus from one strain with RNA from the other, and all contained 25 times more of the non-lesion forming than the lesion forming strain. When mixtures of whole virus or mixtures of RNA were inoculated, lesion numbers were halved, but they were only decreased by 20% when RNA and whole virus were mixed (Table 3). The interference as measured by lesion production between the two strains of TMV was much less than that between the two satellite viruses when one almost completely inhibited the multiplication of the other. However, the comparison was not entirely valid because we compared the number of infections by TMV with final concentration of satellite viruses. Nevertheless, the fact that we failed to detect SV serologically suggested that a very few sites were infected if it was assumed that the inhibition was at the sites of infection.

Interference tests with SV_c

Serologically SV_c differs from SV_1 and SV_2 as much as SV_1 differs from SV_2. If the inhibition of one satellite by another results from competition for infectible sites then it should occur with any pair of these serotypes. The strains of TNV that aid multiplication of SV_c differ from those that aid SV_1 and SV_2. Therefore interference between SV_c and SV_1 or SV_2 was tested using two strains of TNV in one inoculum (TNV AC36 is the helper of SV_c). Table 4 shows that there was no interference between SV_c and either of the other two satellite viruses. It seems that interference occurs only between satellite viruses that have the same helper virus, probably because both satellite viruses compete for the same metabolite produced during the multiplication of the helper virus. The fact that the inhibition depended much on the strain of helper virus used (Table 2) supports this explanation.

Table 4. *Inoculations of mixtures of SV_1+SV_c or SV_2+SV_c with their respective TNV helper*

Treatment*	SV_1	SV_2	SV_c
SV_1 25 μg/ml+SV_c 25 μg/ml	16†	—	8
SV_1 1 μg/ml+SV_c 25 μg/ml	16	—	16
SV_1 25 μg/ml+SV_c 1 μg/ml	16	—	16
SV_2 25 μg/ml+SV_c 25 μg/ml	—	8	8
SV_2 1 μg/ml+SV_c 25 μg/ml	—	16	16
SV_2 25 μg/ml+SV_c 1 μg/ml	—	16	16

* TNV A and TNV AC36 each at 10 μg/ml were added to all the mixtures of satellite viruses, the four viruses being mixed in equal volumes, therefore the final concentration is a quarter of that given.

† Reciprocal of serological dilution end-point. No controls were run for each of the satellite virus and its helper because constantly the serological titres varied between 1/16 and 1/32.

Inoculation of one satellite virus before the other

In experiments not presented here, there was almost complete inhibition when the serotype in excess was inoculated before the one in minority. However, when the serotype in minority was inoculated first, inhibition occurred only when the interval between the two inoculations was less than 2 h (Table 5). Table 5 shows results with SV_1 in excess but there were comparable results when SV_2 was in excess; these suggest that the interference occurs during the early stages of infection. TNV was incorporated in both first and second inocula. When TNV was mixed only with the SV in minority and inoculated first, multiplication of this SV was not inhibited even when the SV in excess was inoculated immediately afterwards. By contrast, when TNV was mixed only with the SV in excess and inoculated second, multiplication of the SV in minority was inhibited even with a long interval between the two inoculations.

Table 5. *Inhibition of the multiplication of SV_2 by subsequent inoculations of SV_1*

Interval between inoculations	SV_1	SV_2	SV_1/SV_2
Immediately	32*	4	8
30 min	32	4	8
1 h	32	8	4
2 h	32	16	2
4 h	32	16	2
8 h	32	32	1

* The reciprocal of serological dilution end-point. SV_2 0·5 μg/ml+TNV A 10 μg/ml mixed in equal volumes was inoculated at different intervals before inoculating SV_1 25 μg/ml+TNV A 10 μg/ml mixed in equal volumes.

Inoculating the satellite viruses after TNV

If satellite viruses compete for a metabolite provided by the helper then inoculating TNV some time before the satellite viruses might increase this metabolite and so decrease the inhibition. A mixture of the two satellite viruses with SV_1 in excess, was inoculated immediately or 1, 2 or 3 days after inoculating with TNV A. The same mixture was also inoculated three days after inoculating with TNV AC36, which is not a helper for SV_1 or SV_2. In the last instance TNV A was inoculated together with the satellite mixture to help their multiplication. Table 6 shows that, as the interval between the two inoculations increased, the degree of inhibition of SV_2 by SV_1 decreased and there was no inhibition when the interval was 3 days. By contrast, there was complete inhibition when TNV AC36 was used, meaning that this helper virus does not provide the metabolite.

Table 6. *Inhibition of SV_2 by SV_1 in mixed inoculations when the mixture is inoculated at different intervals after TNV A or TNV AC36*

Strain of TNV	Interval	SV_1	SV_2	SV_1/SV_2
TNV A	Immediately	64*	0	∞
TNV A	1 day	32	2	16
TNV A	2 days	16	4	4
TNV A	3 days	16	16	1
TNV AC36	3 days	8	0	∞

* Reciprocal of serological dilution end-point. The TNV strain was inoculated at 45 μg/ml without carborundum and at different intervals the mixture of SV_1 25 μg/ml + SV_2 0·5 μg/ml with carborundum; when, however, TNV AC36 was used in the first inoculation then TNV A 10 μg/ml was included in the mixture of the two satellite viruses.

When the unbalanced mixture of the two satellite viruses was inoculated before TNV A there was complete inhibition even when the inoculation of TNV A was delayed for 3 days, suggesting that the satellite viruses could wait in the cell until the helper virus was introduced. In this case interference was the same as if the satellites and the helper were inoculated together.

TNV inactivated by u.v. irradiation

Both TNV and SV are equally susceptible to u.v. irradiation, so it was not possible to inactivate TNV *in vivo* at different times after inoculation to find out when the metabolite might be produced. However, it seemed possible that inoculating TNV which had been irradiated with u.v. light might produce any metabolite needed by SV without TNV multiplying. TNV solutions were irradiated at 254 nm wavelength for 90, 120, 150 and 180 sec to leave between 1 % and 0·01 % of the original infectivity, and then inoculated together with SV. The serological titre of SV was directly related to the amount of TNV that survived irradiation. Using TNV irradiated for 150 sec the serological titre of SV was 1/2, but if the few small lesions produced were removed, sap from the rest of the leaf tissue did not react serologically with SV antiserum. The results mean that helper virus that has been inactivated by irradiation does not produce the metabolite.

DISCUSSION

The interference system described here is unique among plant viruses in that it is between three viruses. First, replication of TNV is inhibited by its satellite viruses (Kassanis, 1962). This inhibition occurred in all the experiments reported here (data not presented), when the concentration of one of the satellite viruses was 25 μg/ml. At this concentration the

serological titre of TNV is reduced to 1/4 or less of what it is when TNV is inoculated alone. This interference has not been explained and it is relevant that it should be discussed here. Kassanis (1962) showed that in the presence of SV, TNV produces fewer and smaller lesions, and the decrease in the number of lesions has been shown to depend on the concentration of SV and the strain of TNV (Babos & Kassanis, 1963). An SV concentration that halved the number of TNV lesions decreased the TNV concentration below the level that can be detected serologically, although TNV reached a serological titre of 1/32 when multiplying on its own. The reduction in the number of lesions therefore represents only part of the inhibition of TNV replication. A more likely explanation is that the two viruses compete for a metabolite that is probably coded by TNV, as the SV RNA has only two cistrons one of which is coding for its structural protein (Rees, Short & Kassanis, 1970).

Similarly, in the interference between SV_1 and SV_2 the evidence suggests that the two compete for a metabolite produced by TNV. The degree of interference depended on the relative concentration of the satellite viruses and also on the strain of TNV. Interference occurred only when the satellite in excess was inoculated with, or within 2 h after the satellite in minority. When the two satellite viruses were inoculated before TNV there was inhibition even when the inoculation of TNV was postponed for 3 days, but when TNV was inoculated 3 days before the two satellite viruses there was no inhibition provided the TNV strain used was a helper. The last result is evidence that the inhibition results from competition for a metabolite which is able to accumulate when TNV is inoculated before the other two satellite viruses.

Interference between strains of plant viruses is common and is usually attributed to competition for available sites of infection (Kassanis, 1963). We have argued against this explanation for the inhibition of TNV by SV. Also, when two strains of TMV inoculated as mixtures at concentrations that caused inhibition between SV_1 and SV_2, the decreased number of lesions was small and could not explain the complete inhibition between the two satellite viruses. In addition, there was no interference between SV_1 or SV_2 and SV_c although the three serotypes have the same degree of serological relationship (Kassanis & Phillips, 1970). One would expect all three satellite viruses to interfere with one another if interference resulted from competition for a site of infection. SV_c needs the help of a different TNV strain from that which helps SV_1 and SV_2 and it is likely that the metabolites produced by the two TNV strains are different in their specificity.

Very similar interference phenomena have been described between the adenoviruses of man and monkeys and the adeno-associated satellite viruses. Like TNV, the replication of adenovirus is inhibited by its satellite virus, and the degree of inhibition is related to the concentration of the adeno-satellite virus (Parks *et al.* 1968). There are several serotypes of adeno-satellite viruses and it has been shown that some interfere with one another's replication and the larger the dose of the interfering satellite the greater the degree of interference produced (Torikai & Mayor, 1969). The critical period for interference lasted for 8 to 12 h instead of the 1 to 2 h with TNV and its satellite viruses. The two systems were examined with very different experimental procedures and this might account, at least in part, for this difference in time.

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