

The Nature of Virus Inhibition by a Polysaccharide from *Phytophthora infestans*

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ABSTRACT

A polysaccharide isolated from *Phytophthora infestans* completely inhibited local lesion development by potato virus X on *Nicotiana tabacum* 'Samsun' without inhibiting virus multiplication. The inhibitory effect was instantaneous. Dilution or centrifugation of the virus:polysaccharide mixture restored virus infectivity. Maximum inhibition resulted when virus and polysaccharide were applied as a mixture or when the polysaccharide preceded

the virus. The polysaccharide was confined to the epidermal layer of tobacco, and was not translocated from the site of application.

It is suggested that polysaccharide inhibits virus entry by modifying infectible sites on the leaf surface, but the mechanism involved in masking of lesions is not understood. *Phytopathology* 60:1566-1569.

Additional key words: virus inhibitor, mode of inhibitor action, masking of symptom by an inhibitor.

Whether inhibitors act by affecting the viruses or the host plants has been much debated (2, 3, 5, 6). Among the various substances studied as virus inhibitors are two fungal polysaccharides (3, 12) presenting a conflicting nature of action. Takahashi (12) concluded that a polysaccharide from yeast acted on the virus. On the other hand, Bawden & Freeman (3), working on a polysaccharide from *Trichothecium roseum*, suggested that the virus inhibition was caused by a temporary alteration in leaf cell metabolism, in which introduced virus particles failed to multiply and thus resulted in inactivation.

We (7) isolated a polysaccharide from the mycelium of *Phytophthora infestans* (Mont.) d By. that inhibited potato virus X (PVX) in tobacco. The present study was made to see whether the effect of this polysaccharide is on virus or the host plant.

MATERIALS AND METHODS.—*Viruses.*—Six viruses were used: the X⁵ strain of PVX (7); cucumber mosaic virus (CMV), obtained from R. H. E. Bradley, and tobacco mosaic virus (TMV) from R. H. Bagnall of this station; tomato ringspot virus (TRSV), tobacco necrosis virus (TNV), and turnip mosaic virus (TuMV) from R. Stace-Smith, C.D.A. Research Station, Vancouver, British Columbia.

PVX was purified by homogenizing freshly excised infected tobacco (*Nicotiana tabacum* L. 'Samsun') leaves in distilled water (0.5 g/ml), followed by three to five cycles of differential centrifugation (9). The virus exhibited strong anisotropy of flow and had an ultraviolet absorption ratio (260/280 m μ) of 1.51. Purified virus from 100 g infected tissue was dissolved in 10 ml of distilled water and kept frozen until needed. Dilutions for individual tests were made to give 150-250 lesions/half leaf. All other viruses were used as clarified sap. Samsun tobacco plants were used for propagation and as indicator hosts for CMV, TNV, and TRSV. TuMV was propagated in turnip (*Brassica rapa* L.), and Samsun tobacco plants were used for local lesion assay. TMV was propagated in Samsun tobacco, and *Nicotiana glutinosa* L. plants were used as assay hosts. Inoculation experiments were carried out using the half-leaf technique (7) on Samsun tobacco. Per

cent inhibition caused by the polysaccharide was calculated on the basis of 0% inhibition on the control half-leaves.

Inhibitor was obtained from the mycelium of *P. infestans* as reported earlier (7). Labeled polysaccharide was prepared by growing the fungus in culture media containing ¹⁴C glucose. Stock solutions of the polysaccharide were kept frozen, and samples were thawed and diluted as needed.

RESULTS.—*Effect of the polysaccharide on PVX in different host plants, and on other viruses in Nicotiana species.*—The inhibitory effect of the polysaccharide against PVX was tested on the following species: *Capsicum annuum* L., *Datura metel* L., *D. stramonium* L., *D. tatula* L., *Gomphrena globosa* L., *Nicotiana debneyi* Domin, *N. glutinosa* L., *N. rustica* L., *N. tabacum* 'Samsun' and 'White Burley', *Nicandra physalodes* Gaertn., and *Physalis floridana* Rydb. Each species was tested 3 times using six plants each time. Solutions of PVX and polysaccharide were mixed to obtain a 1:20 dilution of PVX and 2,000 ppm concn of the polysaccharide. On local lesion hosts, 12 half-leaves were inoculated with the virus:polysaccharide mixture and the other 12 halves with virus mixed with water as control. In tests on each systemic host, six plants were inoculated with virus:polysaccharide mixture and six with the virus:water as control. The absence of mottling symptoms on leaves inoculated with virus polysaccharide was considered "inhibition".

The virus was not inhibited on the local lesion hosts *C. annuum*, *G. globosa*, *N. rustica*, and *Datura* species, or on the systemically infected hosts *Nicandra physalodes* and *P. floridana*; however, local lesion production was completely inhibited on *N. debneyi*, *N. glutinosa*, and both varieties of *N. tabacum*.

We also tested the inhibitory effect of the polysaccharide on CMV, TNV, TRSV, and TuMV in Samsun tobacco, and TMV in *N. glutinosa*. All viruses except PVX required polysaccharide concn of more than 1,000 ppm for complete inhibition (Table 1). For further studies on the nature of inhibition, PVX and Samsun tobacco virus-host system was used.

Effect on inhibition of diluting a virus:polysaccharide

TABLE 1. Inhibition of several viruses in *Nicotiana* species by the polysaccharide from *Phytophthora infestans*^a

Viruses	% Inhibition			
	Concn of polysaccharides (ppm)			
	100	500	1,000	5,000
Potato virus X	99	99	100	100
Cucumber mosaic (CMV)	89	86	95	100
Tobacco mosaic (TMV)	86	99	99	98
Tobacco necrosis (TNV)	87	86	82	99
Tomato ringspot (TRSV)	87	90	97	100
Turnip mosaic (TuMV)	72	80	98	100

^a CMV, TNV, TRSV, and TuMV were assayed on *Nicotiana tabacum* 'Samsun' and TMV on *N. glutinosa* plants.

mixture.—Restoration of infectivity by dilution of non-infectious virus:inhibitor mixtures has been accepted as evidence that the effect of an inhibitor is on the host rather than on the virus (4, 5, 14). In our own work we studied the nature of the inhibiting effect of the polysaccharide by maintaining the same virus:polysaccharide ratio over a range of dilution and measuring the change in inhibition. Polysaccharide and PVX were mixed to provide a solution containing 500 ppm of polysaccharide and a virus dilution of 1:20. Then, using distilled water, twofold dilutions of the virus:polysaccharide and virus:water mixtures were made until virus dilution was about 1:2,560. Six half-leaves of tobacco were inoculated with each dilution of virus:polysaccharide, and opposite halves were inoculated with corresponding dilutions of virus:water. Data from the tests showed (Table 2) that the per cent inhibition decreased with dilution of virus inhibitor mixture, indicating that the effect of the polysaccharide is on the host and not on the virus.

Relationship between the order of application and inhibition.—Tests were made to determine whether the max effect of the polysaccharide occurred when it was applied before, after, or with the virus. A 1:10 dilution of virus and 1,000 ppm concn of polysaccharide was used in these tests. In the first test, half-leaves of tobacco were rubbed with polysaccharide and the opposite halves with water, then both halves were inoculated with virus immediately and after intervals of 5,

TABLE 2. Comparison of numbers of local lesion produced by serial dilutions of potato virus X and a mixture of potato virus X and polysaccharide from *Phytophthora infestans*

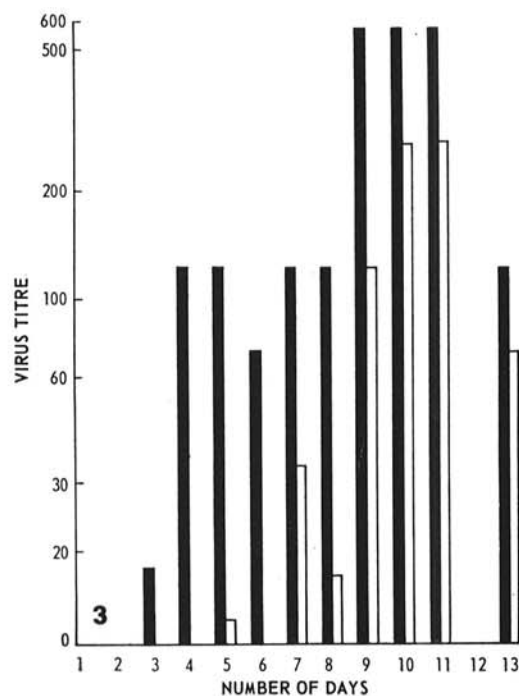
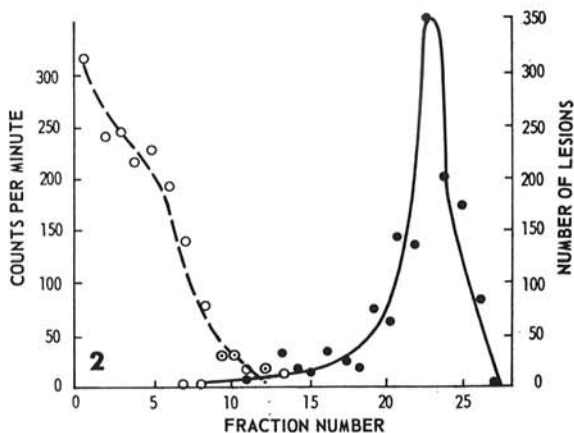
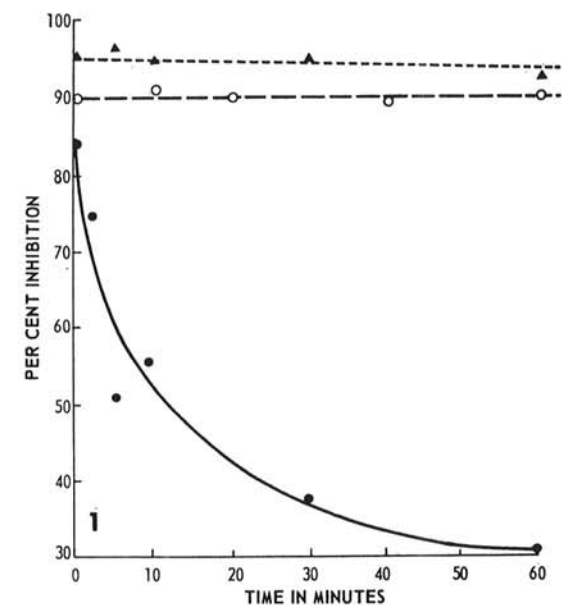
Virus dilutions	Poly-saccharide concn (ppm)	No. lesions on 12 half-leaves of Samsun tobacco		
		Virus + poly-saccharide	Virus + water	% Inhibition
1:20	500	599	2,467	75.0
1:40	250	734	1,754	59.0
1:80	125	818	1,340	39.0
1:160	62	871	1,017	14.0
1:320	31	859	911	6.0
1:640	15	887	918	3.0
1:1,280	7	739	782	5.0
1:2,560	3	627	643	2.0

10, 30, and 60 min. In the second test, both halves of the leaves were inoculated with virus. Then one-half of each leaf was rubbed with the polysaccharide and the other half with water immediately and after intervals of 3, 5, 30, and 60 min. In the third test, the polysaccharide and virus were mixed to provide the same final concn of virus and polysaccharide as was used in the first and second tests. Half-leaves were then inoculated with the mixture immediately and 10, 20, 40, and 60 min after mixing. As a control, virus diluted with water was inoculated to the opposite half of each leaf.

The results of the three tests (Fig. 1) indicated that there was no apparent decrease in inhibition when the polysaccharide was applied before the virus, or when virus and polysaccharide were mixed together. The inhibition decreased from 84% immediately after inoculation to 32% after 60 min, when the virus was applied before the polysaccharide.

The possibility of an association between the polysaccharide and the virus.—The possibility of binding of polysaccharide and virus was investigated using ¹⁴C-labeled polysaccharide and density-gradient centrifugation. The density gradient tubes were prepared by layering 4, 7, 7, and 7 ml, respectively, of 5, 10, 20, and 30% sucrose. One ml of virus-labeled polysaccharide mixture (1:20 diluted virus and 1,000 ppm polysaccharide) was layered on top of the gradient tube and centrifuged for 3 hr at 23,000 ppm in a Spinco SW 25.1 rotor. The gradient tubes were fractionated with an ISCO density-gradient fractionator (Instrumentation Specialties Co., Lincoln, Neb.). An aliquot of each fraction was used to inoculate *G. globosa* leaves for infectivity assays, and the remainder of each fraction was dried in a planchet for radioactivity counts. Figure 2 shows that the polysaccharide and virus were completely separated, and hence no permanent binding had occurred.

Effect of location of the polysaccharide on virus inhibition.—Experiments were carried out to determine if the polysaccharide, which readily dissolved in water, is absorbed and translocated through the leaf. Labeled polysaccharide (250 count/min) was applied to an area approximately 2.5 cm long on the mid-vein at the tip of the leaves. After 2 weeks, the area to which the polysaccharide had been applied and adjacent areas along the mid-vein were cut from the leaf and tested for radioactivity. The only radioactive area was at the site of application (200 count/min). In a second test, a drop of labeled polysaccharide was applied to a leaf, and the surface under the drop was pricked several times with a needle. Two weeks later, the area under the drop and several concentric rings of the leaf tissue beyond the applied area were cut out with a cork-borer. Again the only radioactive area was the area under the drop (188 count/min). In a third test, labeled polysaccharide was applied to the underside of the leaf and the upperside was inoculated with virus. As a control, the underside of leaves were rubbed with water and the upperside inoculated with virus. No apparent difference in the number of lesions was observed between



polysaccharide and water-treated leaves. After 2 weeks, the epidermis was stripped from the polysaccharide-treated area of the leaves, and the remaining tissue from this area was removed for radioactivity measurement. Only the epidermal layer was radioactive (213 count/min).

Since the polysaccharide remained in the epidermal layer and was not translocated, the site of application and inhibition must be coincident. Further evidence of this was obtained when the polysaccharide was injected into the leaf and the epidermis was inoculated with virus. Using a hypodermic syringe, the polysaccharide solution (1,000 ppm) was injected (8) into one-half of a tobacco leaf, and water was injected into the opposite half. One-half ml of fluid was introduced in each half. Leaves were inoculated with virus 6 hr and 1, 2, and 4 days after injection. A similar number of lesions formed on both halves of the leaf, thus again showing that when the polysaccharide is not present on the epidermis, lesion formation is not affected.

Masking of the local lesions in tobacco.—The X⁵ strain of PVX produces local lesions in Samsun and White Burley tobacco and spreads systemically to new growth. When the polysaccharide, at a concn of 2,000 ppm, was mixed with the virus (1:20 dilution) and applied to test leaves, no lesions were formed, but when sap from these leaves was inoculated on other test plants, lesions were produced. This suggested that either the lesions were the result of residual virus remaining on the leaf after inoculations, or that the polysaccharide was masking lesion formation.

To test the first possibility, inoculated leaves were washed with water in an attempt to remove the virus left on the leaf surface. Sixteen leaves were inoculated with a virus:polysaccharide mixture on one-half and virus:water mixture on the other. Each half-leaf was washed thoroughly with 2 ml of water at intervals beginning at 1 hr after inoculation. Test plants were then inoculated with each washing and observed for lesion production. Plants inoculated with the first washing always produced lesions, but no lesions were produced on plants inoculated with subsequent washings.

The possibility of the virus being translocated from one-half the leaf to the other half was investigated by inoculating half-leaves with virus:polysaccharide or virus:water mixtures and rubbing the opposite halves with water. Each half-leaf was thoroughly washed and the sap from each half was tested separately 1, 2, 4, and 6 days after inoculation. After 4 days, virus was recovered from the virus:polysaccharide or virus:water treated halves, but no virus was recovered from the water-rubbed halves up to 6 days.

In the third series of tests, virus multiplication was measured by a serological technique. Twenty-four Sam-

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Fig. 1-3. Inhibition, separation, and concn of potato virus X as affected by the polysaccharide from *Phytophthora infestans*. 1) Polysaccharide was inoculated at various intervals of time before, after, or with virus inoculation; △△△ = polysaccharide, then virus; ○○○ = virus:polysaccharide mixture; ●●● = virus, then polysaccharide. 2) Radioactivity counts and number of local lesions produced from the different fractions of density-gradient containing virus:polysaccharide mixture; ○○○ = radioactivity of fractions; ●●● = number of lesions produced on *Gomphrena globosa* by different fractions. 3) Comparative virus titers from leaves inoculated with virus alone (solid bar) and virus:polysaccharide-inoculated leaves (open bar).

sun tobacco plants were inoculated with virus:polysaccharide and virus:water mixtures using the half-leaf technique. Lesions appeared on the virus controls after 3 to 4 days, while no lesions appeared on the virus:polysaccharide leaves during a 13-day observation period. Four half-leaves were removed daily from the inoculated plants, adjusted to equal wt, and ground separately. The extracted sap was heated for 10 min at 45 C, cooled to 10 C, and then centrifuged at 3,000 rpm for 20 min. The supernatant was used to make serial twofold dilutions from $\frac{1}{4}$ to $\frac{1}{1,024}$ with 0.85% NaCl, and these dilutions were serologically tested for the presence of virus by the tube precipitin procedure as outlined by Bagnall et al. (1). Three separate tests yielded essentially similar results, and the results of one test are presented in Fig. 3.

The virus was detected in the virus-inoculated leaves on the 3rd day but not until the 5th day in the virus:polysaccharide-treated leaf. The polysaccharide did not completely prevent virus entry into the leaf, and the virus having entered, multiplied and on the 10th day reached a concn one-half that present in the virus control leaf. Although the virus:polysaccharide-treated leaf contained a high concn of virus, lesion expression was completely masked.

DISCUSSION.—Although the criteria used to determine whether the effect of an inhibitor is on the virus or the host plant are not well defined, restoration of infectivity by dilution, separation of the virus from the virus-inhibitor mixture by centrifugation, and specificity of the inhibitory effect to certain host species are generally accepted as proof that the effect is on the host (3, 4, 5, 6, 11, 14). Our experiments with polysaccharide and PVX indicate that the host is affected. For instance, a virus:polysaccharide mixture diluted 20 times caused 75% inhibition, and there was a consistent decrease as the mixture was further diluted, resulting in 14% inhibition at a dilution of 160 times and practically no inhibition at a dilution of 2,560 times (Table 2). The complete separation of virus from the virus:polysaccharide mixture by density-gradient centrifugation (Fig. 2) showed that no permanent binding occurred between virus and polysaccharide. The effect of the polysaccharide on six genera of plants tested resulted in inhibition in only *Nicotiana* species, supporting the above evidence that the effect is on the host. Again, of the four *Nicotiana* species tested, inhibition was confined to only three, and five other viruses were also inhibited in *Nicotiana* species by the polysaccharide.

Some regions in the epidermis formed by abrasion are regarded as the site of virus infection (10). Suggestions have been made that inhibitors present at the time of inoculation compete with virus for these sites (11, 13, 14). The data we obtained using labeled polysaccharide supports this hypothesis. It was found that the polysaccharide did not translocate from the site of application on the epidermal layer, and inhibition did not occur if the polysaccharide was applied remote

from virus or injected intercellularly. Thus, it appears that a requisite to inhibition is that the polysaccharide and virus must occupy the same site. The hypothesis of competitive inhibition is further strengthened by the rapid decrease in inhibition when the virus is inoculated at different times preceding the polysaccharide application, while no change in inhibition occurs when the order of application is reversed (Fig. 1).

Although local lesion formation was completely inhibited by the polysaccharide, serological tests demonstrated that some virus particles do penetrate and multiply in the leaves (Fig. 3) without forming lesions. This masking of lesion expression by a virus inhibitor in tobacco leaves has not been reported by other workers, and appears to be an unique property of the polysaccharide from *P. infestans*. Further work on this phenomenon is now in progress, and a number of compounds that have previously been reported to be "virus inhibitors" are being tested to determine if they are also "symptom suppressors".

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