

# An Inhibitor of Tobacco Mosaic Virus Produced by *Physarum polycephalum*

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## ABSTRACT

An inhibitory substance produced by *Physarum polycephalum* prevents tobacco mosaic virus (TMV) infection when assayed on beans and tobacco. The 35,000-55,000 mol wt inhibitor has properties of a carbohydrate, and is stable in high temperature and high pH. Foliar application before and after inoculation of TMV prevents TMV infection on beans. Dilution and high temperature treatment of the

virus-inhibitor complex reverses inhibition. Electron micrographs suggest that the inhibitor coats TMV, thus preventing normal coat "stripping".

The inhibitor reduced tobacco ringspot virus infection in *Vigna sinensis* but not that of southern bean mosaic virus in *Phaseolus vulgaris*. Phytopathology 61:636-640.

Virus inhibitors have been known since 1925, when it was found that a substance produced by *Phytolacca decandra* inhibited tobacco mosaic virus (TMV) (4). TMV is also inhibited by polysaccharides, proteins, and tannins isolated from higher plants (1).

Many lower plants also produce virus inhibitors. Among fungi, *Trichothecium roseum*, *Neurospora sitophila*, *Lepiota* sp., and *Amanita* sp. produce the most potent inhibitors (6, 7, 8, 10, 11, 12, 18). *Trichothecium roseum* produces a polysaccharide inhibitor consisting of 45% D-galactose (2). Substances from bacteria and yeasts also inhibit TMV (10, 11, 12, 13, 14, 16, 17). Many microbial inhibitors are high mol wt molecules which are very heat stable (1, 11).

All inhibitors described from microorganisms are effective against TMV. Many fungal and bacterial extracts, however, also inhibit tobacco necrosis, cucumber mosaic, tobacco ringspot, and southern bean mosaic viruses. Most recently, workers showed that a polysaccharide produced by *Phytophthora infestans* inhibits infection of potato by potato virus X (9).

Hypotheses advanced to explain inhibition include alteration of leaf cell metabolism (2), other host-directed effects (8), or virus-directed effects, such as coating that prevents protein stripping or degradation (17).

We present evidence of the chemical nature and possible mode of action of a viral inhibitor from a newly reported source, the myxomycete, *Physarum polycephalum* Schw.

**MATERIALS AND METHODS.**—The TMV isolate was a common strain isolated from tomatoes in Oregon and purified (5) to approx 0.1 g TMV/ml of buffer, then diluted 1:1,000 with distilled water to produce the stock solution.

Local lesion assay hosts for untreated or inhibitor-treated TMV were either *Phaseolus vulgaris* L. 'Scotia' or *Nicotiana tabacum* L. 'Samsun NN', grown in 4-inch pots in peat:sand:loam soil (1:1:2). The terminal bud and all leaves, except the top four or five fully expanded leaves of *N. tabacum*, were removed before inoculation. Inoculations of *N. tabacum* and newly expanded *P. vulgaris* unifoliolate leaves were made by rubbing the leaf surface twice with cheesecloth soaked with test solution after dusting with 600 mesh Carborundum.

**Inhibitor production.**—*Physarum polycephalum* was isolated from a rotting elm log in central Iowa. The culture was maintained in the dark in plastic refrigerator crispers lined with moistened filter paper at  $24 \pm 3$  C by supplying rolled oats as a nutrient source. Extracts were prepared by grinding 20 g of filter paper covered with plasmodium but void of oats in 100 ml of distilled water. The mixture was then strained through cheesecloth and centrifuged at 1,085 g for 15 min, and the pellet discarded. A bacterial flora was normally associated with the myxomycete, so an attempt was made to grow the myxomycete in pure culture. No plasmodial growth occurred on nutrient agar, malt extract agar, cornmeal agar, oatmeal agar, and water agar in which sterile rolled oats were dispersed, or on a specific medium for growing *P. polycephalum* (3). Thus, a small plasmodium, after migrating from one edge of nutrient agar in a petri dish to the other in 24 hr at 22 C, was harvested and was free of bacteria. The plasmodium was harvested with a sterile loop and mixed with 1 ml of distilled water. Absence of bacteria at the harvest site on the original plate 48 hr after harvest, and on plates of subsequent transfer, indicated that the plasmodium was free of bacteria. In each experiment, approx 100  $\mu$ g TMV was mixed with 1 ml of the test solution or water control, and each mixture was kept at room temperature for 1 hr before inoculation.

Bacterial contaminants growing with the plasmodium also were tested for the production of inhibitory substances. Ten bacterial isolates from the myxomycete cultures were streaked on nutrient agar. Individual colonies were then transferred to nutrient broth cultures and incubated 48 hr, and samples from these cultures were tested for inhibitory properties by adding approx 100  $\mu$ g of TMV to 1 ml of bacterial culture filtrate.

**RESULTS.**—The procedure to free cultures of *P. polycephalum* of bacteria was successful. Ten of 30 plasmodial isolates were free of bacterial contamination after the first transfer, and the remainder of the isolates were free of contamination after a subsequent transfer.

Four of the 10 bacterial contaminants isolated from plasmodia growing on oatmeal cultures, then grown in pure culture, reduced infection of TMV by 92% (avg).

No attempt was made to identify the bacterial isolates.

The extract prepared from plasmodia reduced TMV infection in all tests on both tobacco and bean. In five tests, the average number of lesions on half-leaves of tobacco was 22.8 for the water control and 0.2 for the test solution, while on beans the average was 38.0 and 0, respectively.

*Inhibition of other viruses.*—The *P. polycephalum* extract was mixed with partly purified preparations of southern bean mosaic virus (SBMV) or tobacco ring-spot virus (TRSV), and assayed on respective local lesion hosts, *P. vulgaris* 'Scotia' and *Vigna sinensis* L., to determine inhibition of infectivity of these viruses.

The infectivity of SBMV in the presence of plasmodial extract resulted in an average of 17.5 lesions on test leaves as compared with 17.0 lesions for SBMV: water controls. TRSV in the presence of the plasmodial extract produced 4.4 lesions (avg) on test leaves as compared with 11.8 lesions for controls.

*Application of inhibitor.*—The inhibitor was sprayed on the upper surface of bean leaves with an atomizer until the entire leaf surface was moist. Any excess moisture was allowed to run off the leaf surface before inoculation. Applications were made at intervals of 1, 15, 30 min, and 1 hr before and after inoculation with TMV. One untreated plant/pot was inoculated with a TMV:water control.

Foliar application of the plasmodial extract before inoculation with TMV prevented infection. No lesions developed at any time interval when inhibitor was applied before inoculation, compared with 57.4 lesions on control leaves. Application of the extract after inoculation was successful in reducing infection up to 30 min after inoculation (Table 1).

*Physical properties of inhibitor.*—The plasmodial extract was diluted with distilled water in a 10-fold dilution series. TMV was added to each solution and assayed on *N. tabacum*. The virus inhibitor is either very potent or produced in large quantity by the myxomycete. The dilution end point is about 1:10,000 (Table 2).

The effect of temperature was determined by heating

TABLE 1. The effect of time of application of *Physarum polycephalum* extract on unifoliolate bean leaves before or after tobacco mosaic virus (TMV) inoculation

Time of inhibitor application	Avg. no. lesions/leaf	
	Test	% Reduction of TMV infection
1 Hr before	0 <sup>a</sup>	100.0
30 Min before	0	100.0
15 Min before	0	100.0
1 Min before	0	100.0
1 Min after	13.0	77.4
15 Min after	16.2	71.8
30 Min after	27.1	52.8
1 Hr after	56.4	1.7

<sup>a</sup> Average no. lesions/leaf on control plants was 57.4. This figure was calculated by averaging the total number of lesions produced on one control plant for each treatment time (i.e., total 8 plants).

TABLE 2. Effect of dilution on inhibitory properties of plasmodial extract of *Physarum polycephalum* on tobacco mosaic virus (TMV) infection of beans

Dilution	Avg. no. lesions/leaf	
	Test	% Reduction of TMV infection
1:1	0	100.0
1:10	0.3	98.2
1:100	6.8	53.8
1:1,000	8.7	49.4
1:10,000	14.2	4.1
1:100,000	18.2	4.2

samples of extract 10 min each at 40, 60, 80, and 100 C. The solutions were cooled, and about 100 µg/ml of TMV was added to each sample and assayed on *P. vulgaris*. When *P. polycephalum* extract was heated to 40 and 60 C, no lesions were produced when TMV was mixed with these treated extracts, as compared with 31.5 and 34.2 lesions (average) for the respective controls. The virus mixed with extract treated at 80 and 100 C produced only one or two lesions (average) on test leaves, as compared with 36.1 and 34.7 lesions (average), respectively, on controls. There was 95% or greater reduction of infection in every test.

The influence of pH on inhibitor activity was tested by adjusting two 10-ml samples of extract to pH 4.0 or pH 10.0 with concentrated HCl or NaOH, respectively. The samples remained at room temperature for 10 min, and were centrifuged at 3,020 g for 10 min. The pH of the supernatant was then readjusted to pH 7.0, and about 100 µg/ml of TMV were then added to each sample and assayed. When the inhibitor was treated with concentrated acid and base, TMV mixed with extract treated at pH 4.0 produced 12.1 lesions (average) as compared with the 31.5 lesions on control leaves. TMV added to extract treated at pH 10.0 produced 1.0 lesions (average), while controls produced 32.1 lesions (average). Apparently, high pH had little effect on the inhibitor, while treatment at pH 4.0 considerably reduced the inhibitor's effectiveness.

Plasmodial extract was tested for its inhibitory properties after storage at -20, 4, and 23 C. The extract stored at 23 C was tested at intervals of 1, 2, 4, 7, 10, and 14 days. The extracts stored at -20 and 4 C were tested only at the end of 1 month. The inhibitory properties of the plasmodial extract prepared from filter paper cultures also were not altered by storage at -20 or 4 C after 1 month. An average of 38.0 lesions was produced by TMV:water controls; no lesions were produced by test solutions. TMV treated with extracts stored at room temperature produced 1.8 lesions (average) on leaves tested after 1, 2, and 4 days as compared with 15.0 lesions for controls. After 4 days, samples were overgrown with fungal and bacterial contaminants, so further measurements would have been inconclusive.

The speed of inhibition was also determined. To determine the speed of inhibitory effect, 100 µg/ml of TMV were added to plasmodial extract at intervals of 10 sec, 1, 5, 10, 15, 30 min, or 1 hr at 23 C, rapidly

shaken, and assayed. Infection of bean plants by TMV was prevented upon immediate exposure of the virus to the plasmodial extract. No infection occurred at any time interval tested. No local lesions formed on any test leaves after adding TMV to extract at intervals of 10 sec, 5, 10, 15, 30, or 60 min. TMV:water controls produced 40.1 lesions (average).

The lower limit of the mol wt of the inhibitor was estimated by dialysis. Twenty ml of plasmodial extract were boiled 20 min, centrifuged at 12,000 *g* for 15 min, and placed in a dialysis membrane in 1,000 volumes of demineralized distilled water, which was replaced 5 times at 4-hr intervals. After 24 hr, the contents of the membrane were tested for inhibition of TMV infection of tobacco. The plasmodial extract inhibited TMV infection after 24-hr dialysis. TMV mixed with dialyzed extract produced 10.0 lesions/leaf (average), compared with 21.0 lesions/leaf (average) for the control, while TMV mixed with untreated extract produced 8.0 lesions/leaf (average), as compared to 24.8 for the control. Therefore, the mol wt must be 12,000 or greater.

*Inhibition reversal.*—The effects of heat, dilution, and pH on inhibition reversal were determined. Samples of the inhibitor-TMV complex of 5 ml each were heated to 60, 70, 80, 90, and 100 C for 10 min, after sitting for 1 hr at room temperature. The samples were immediately cooled in cold running tap water and assayed. Treating the virus-inhibitor complex at 90 and 100 C reduced infectivity of TMV sufficiently for the reversal of inhibition at these temperatures to be inconclusive. Treatment of the complex at 60, 70, and 80 C did reverse inhibition (Table 3).

One ml of a 1:100 dilution of TMV stock solution was added to 9 ml of plasmodial extract prepared from agar plates. This test solution was immediately diluted with distilled water in a 10-fold series to 1:10,000, and each dilution was assayed on beans. Inhibition of TMV by *P. polycephalum* extract can be reversed successfully by diluting the virus-inhibitor complex with distilled water (Table 4). At a 1:1 dilution, there is evidently some inhibitor-free or infective virus in the solution, as indicated by an average of 1.4 lesions. At 1:100 and 1:1,000 dilutions, however, the inhibition becomes reversed. At 1:10,000, the number of lesions is so small that interpretation is difficult.

The pH of a solution containing the inhibitor-virus

TABLE 3. The effect of temperature on the reversal of inhibitory effects of *Physarum polycephalum* extract on tobacco mosaic virus (TMV) infection after reaction with TMV as assayed on beans

Temperature, C	Avg no. lesions/leaf	
	Test	% Reduction of TMV infection
60	15.0	9.0
70	16.5	54.0
80	1.8	96.5
90	0	
100	0	

TABLE 4. The effect of dilution of *Physarum polycephalum* extract on the reversal of its inhibitory effects on tobacco mosaic virus (TMV) infection as assayed on beans

Dilution	Dilution of virus	Avg no. lesions/leaf		
		Test	Control	% Reduction of TMV infection
1:1	10 <sup>-3</sup>	1.38	170.90	99.2
1:10	10 <sup>-4</sup>	6.50	13.00	50.0
1:100	10 <sup>-5</sup>	3.00	3.13	4.2
1:1,000	10 <sup>-6</sup>	1.00	1.88	46.9

complex was adjusted to 5.0 or 9.0 with concentrated HCl or NaOH, set 1 hr at 23 C, and assayed on beans. Virus controls were treated in an identical manner. Treating the virus-inhibitor complex with concentrated HCl or NaOH did not reverse inhibitory effects. At pH 5.0, the average number of lesions for test leaves was 0.8 compared with 33.5 for controls. At pH 9.0, the average number of lesions was 0.3 lesions as compared with 40.5 for controls.

*Identification of inhibitor.*—To prepare the inhibitor for identification, the plasmodial extract was boiled in a hot-water bath for 20 min. The extract was then centrifuged at 12,000 *g* for 15 min, and the pellet discarded. Twenty-five ml of the supernatant was then applied to a column of G-75 Sephadex as described in the Sephadex manual (15). The column, 22 mm in diam and 25 cm in length, was filled with 65 ml of Sephadex, which allowed a flow rate of 0.6 ml/min. The column was eluted with distilled water, 1-ml fractions were collected, and every fifth sample was tested. The inhibitory properties of each sample were rated + for some inhibition of TMV infection and ++ for prevention of infection. The peak of inhibitor concentration was the midpoint among samples rated ++. Four fractions were chosen at random from the inhibitor peak and tested for the presence of peptides with the biuret test, and for carbohydrates with the Molisch and anthrone tests.

Elution of the inhibitor at the 20-ml sample suggests that its mol wt ranges between 35,000 and 55,000. The biuret test was negative for peptides. Both the Molisch and anthrone tests for carbohydrates were positive. Twenty ml of the sixty-three 1-ml fractions demonstrated some inhibitory properties, indicating that either the inhibitor is produced in large quantities by the myxomycete or that there is more than one inhibitory compound.

*Electron microscopy of virus-inhibitor complex.*—The virus-inhibitor complex was set at 23 C for 1 hr and atomized onto Formvar-coated grids. After drying, they were shadowed with Platinum-Palladium (80:20) at a 20 degree angle. Another sample was negatively stained with phosphotungstic acid (pH 6.4), atomized onto Formvar grids, and observed directly. All observations and electron micrographs were made with a Hitachi HU-11C electron microscope.

Electron micrographs show that the virus particles treated with inhibitor remained intact, which suggests that digestion or stripping of the protein coat does not

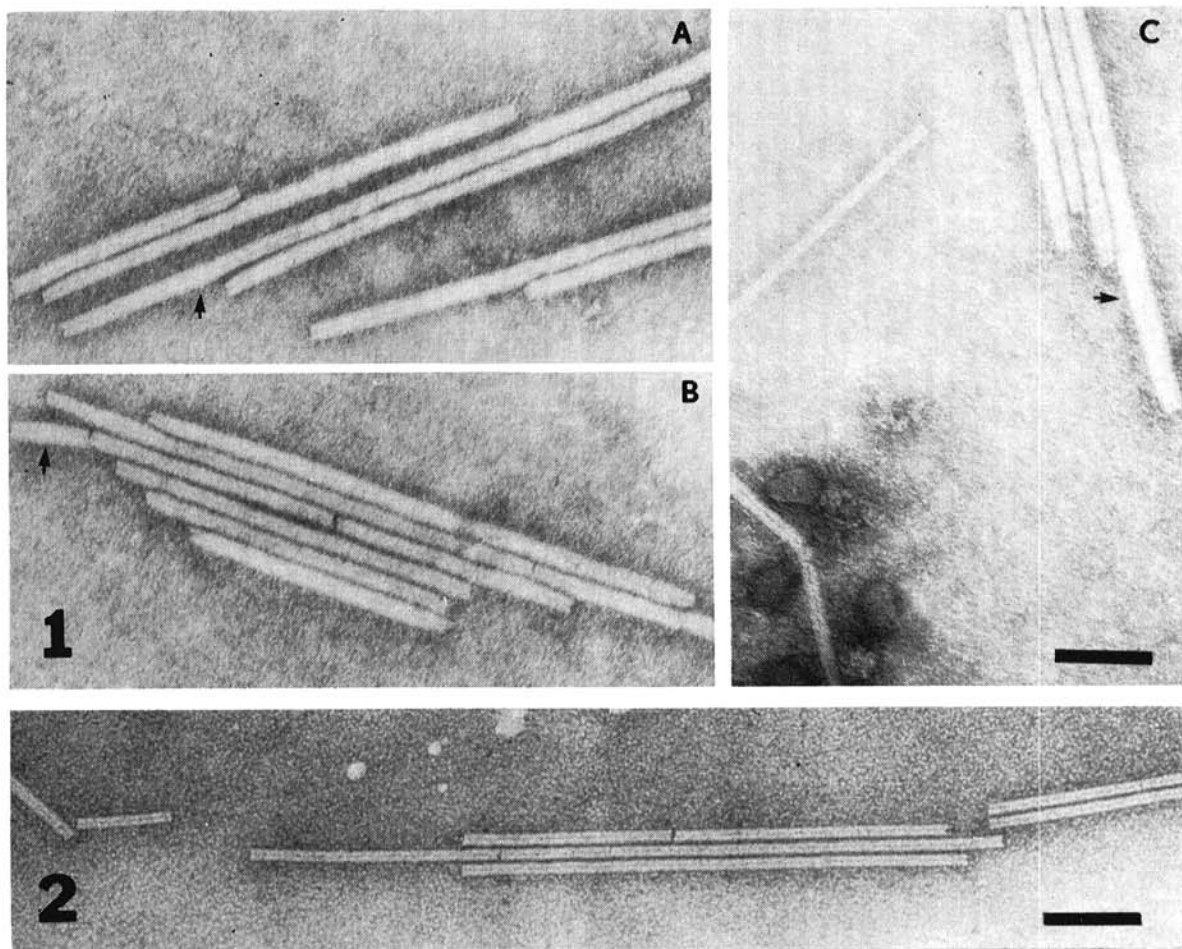


Fig. 1-2. 1, A, B, C) Purified tobacco mosaic virus mixed with an inhibitory extract prepared from *Physarum polycephalum* plasmodium and negatively stained with 2% phosphotungstic acid, pH 6.9. Arrows indicate portions of virus particles which seem to be affected by the inhibitor. Note individual virus particles at left (1-C) which do not seem to be affected by the inhibitor like those which are "stacked" or aggregated. Bar represents 100 nm. 2) Purified tobacco mosaic virus diluted 1:1,000 with distilled water and negatively stained with 2% phosphotungstic acid, pH 6.9. Bar represents 100 nm.

explain inhibition. Comparison of the treated virus particles (Fig. 1) with untreated particles (Fig. 2) suggests that the inhibitor may coat the particles (Fig. 2). The uneven stacking and the rough edges of individual, treated particles supports this explanation.

DISCUSSION.—Experimental factors causing inhibition and inhibition reversal, and interpretation of the electron micrographs, suggest that the inhibition mechanism does not involve the host plant, but instead, acts directly on the virus particle. The experiment on rapidity of interaction demonstrated that the inhibition begins upon immediate contact between plasmodial extract and virus. This inhibition, which is 100% effective, can be reversed by dilution and treatment at high temperature. This reversal can be explained only in terms of an effect on the virus because, as was previously shown, dilution and temperature have little or no effect on the inhibitor itself. If the inhibition was a host involvement, the unaltered inhibitor would still function.

Further evidence for virus involvement is demon-

strated in the electron micrographs. The untreated purified TMV preparations, both shadowed and stained, show virus particles with very smooth and straight outlines and a regular and symmetrical "stacking" or aggregating pattern. The treated TMV preparations show particles with a rough and bumpy outline, and the "stacking" is very irregular. No broken particles or damaged protein coats were found, which eliminates digestion as the means of inhibition. All the evidence indicates that the virus particles may be coated by the inhibitor and that, in some way, this coating inhibits normal protein coat "stripping" in the host cell. Because inhibition reversal is relatively easy to perform, the coating probably involves only weak chemical bonding.

We are not able to rule out completely the possibility that the inhibitor is causing an alteration of protein-protein or protein-RNA bonds which allow the swollen appearance.

Our results compare favorably with studies involving inhibitors produced by other microorganisms. Like

others, the inhibitor produced by *P. polycephalum* is carbohydrate in nature (1, 2, 7, 9, 16). Evidence indicates that the carbohydrate is a polysaccharide, but further characterization must be done for positive identification. Like others, the substance produced by *P. polycephalum* is also highly stable, and prevents infection by foliar application before and after inoculation (13, 14).

Two disadvantages of the culture technique should be recognized as possibly affecting the experimental results. The plasmodial culture technique does not insure pure plasmodia because bacteria or bacterial products may be harvested with the plasmodia. Also, the quantity of inhibitor varies in individual samples because the dilution of the plasmodium in extract preparations was not quantitated.

The inhibitor is thermostable. There is evidently a direct relationship between the time required to cool the sample before inoculation and the amount of reversal of inhibition. Apparently, a cooler solution provides more time for the separated inhibitor and TMV to recombine. Temperatures up to 100 C, however, do not significantly alter the inhibitory property of plasmodial extracts of *P. polycephalum* on TMV.

Our study presents strong evidence that the inhibitor alters TMV directly and interferes with normal replication in the host cell. This is in disagreement with evidence which indicates that mechanisms of inhibition of other inhibitory compounds involve the host rather than the virus.

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