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Mode of Action and Properties of a Plant Virus Inhibitor in Citrus Extracts

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THE OCCURRENCE in healthy leaves of substances that interfere with mechanical transmission of viruses was discovered many years ago and has been studied in various host-virus combinations (1, 8). Extracts from healthy citrus leaves added to viral inocula interfere with the transmission of citrus variegation virus (CVV), cucumber mosaic virus-necrotic strain (CMV-N), and tobacco mosaic virus (TMV), reducing the number of lesions in cowpea and *Nicotiana glutinosa* L. (2, 5). The experiments with citrus extracts suggested an inhibitory action on the host rather than on the virus particles, but did not disclose the chemical nature and properties of the inhibitor. In this paper some results dealing with the characterization of the inhibitor are summarized.

Procedures and Results

INHIBITOR SOLUTIONS.—Young but entirely expanded leaves of the citrus species and varieties listed in Table 1 were used in a preliminary screening for inhibitor activity. Only lemon leaves were used in further trials. Outdoor and greenhouse-grown seedlings and grafted nucellar trees were used. The fresh or frozen leaves were homogenized in a blender with phosphate buffer 0.02 M pH 7 (1:2 or 1:1; w:v) and squeezed through cheesecloth. The juice was centrifuged at low speed (10,000 *g* for 20 min), and the supernatant was immediately assayed for inhibitor activity or kept frozen at −18–20°C to be used later.

VIRUS SOLUTIONS.—Citrus variegation virus (CVV), citrange stunt virus (CSV), both kindly supplied by Dr. J. M. Wallace, and a local lesion strain of TMV were used. Citrus viruses were multiplied in Lady finger round cowpea, and TMV in Xanthi tobacco. Infected fresh leaves homogenized with phosphate buffer 0.02 M pH 7 (1:1 or 1:2; w:v) and

TABLE 1. RATIO OF INHIBITION OF INFECTION INDUCED IN COWPEA LEAVES BY MIXING EXTRACTS FROM VARIOUS CITRUS CULTIVARS WITH CVV AND CSV

Citrus species	Percentage inhibition	
	CVV[a]	CSV[b]
Sweet orange	79	52
Eureka lemon	99	45
Troyer Citrange	82	52
Trifoliate orange		55
Mexican lime	93	
Volkameriana lemon	99	
Etrog citron	95	
Avana mandarin	76	
Sour orange	80	
Marsh grapefruit	99	
Orlando tangelo	60	

a. Citrus leaves were homogenized with buffer (1:1).
b. Citrus leaves were homogenized with buffer (1:2).

squeezed through cheesecloth was used as inoculum.

ASSAYS FOR INHIBITOR ACTIVITY.—Inocula diluted 1:1 or 1:2 with extracts or with buffer were rubbed on cowpea (CVV and CSV) or tobacco leaves (TMV) previously dusted with Carborundum (600 mesh), an opposite leaf method or a half leaf method being employed. Inoculated plants were kept at a constant temperature (21°C) under 12 hours' illumination. Each diluted inhibitor solution was tested in at least 10 replications—10 leaves or 10 half leaves—for each experiment.

ACTIVITY OF DIFFERENT CITRUS EXTRACTS.—Extracts from various citrus species and varieties were mixed with solutions of CVV or CSV and immediately assayed on cowpea. All tested extracts showed a high inhibitor activity (Table 1).

MODE OF ACTION.—In experiments carried out with CSV, we tried to ascertain whether the inhibitor present in the extract of lemon leaves acts only if mixed with viral inoculum before inoculation. Therefore, the inhibitor was applied a few minutes before inoculation; the leaves were allowed to dry and then inoculated on the same surface. The inhibition rate was the same as that observed when the extract was added to virus before inoculation. On the contrary, no inhibitor activity was detected when the extract was applied to the lower leaf surface and inoculation was done on the upper surface, 0–5 hours later.

Further experiments were performed to investigate a possible activity of the inhibitor towards virus particles. A mixture of virus and extract of lemon leaves was kept in a test tube at room temperature (21°C) for 5 hours and assayed on cowpea at different times. This standing in vitro did not appreciably modify the inhibition rate.

PHENOL-BUFFER EXTRACTION.—Phenol-buffer extracts were prepared by homogenizing 1 g leaf tissue in 1 ml buffer and 1 ml water-saturated phenol. Homogenates were centrifuged as mentioned above; the buffer layers were withdrawn with a hypodermic syringe and washed twice with about 10 volumes of ether. The water phase did not show any appreciable inhibitory activity when tested with CSV and TMV and compared with buffer extract (Table 2,a,b).

HEAT TREATMENT OF EXTRACTS.—Aliquots of buffered extracts were heated in thin-walled glass tubes for 10 min at 75°, 100°, and 121°C (autoclaving). The solutions were then quickly cooled, mixed with solutions of CSV, and tested. The inhibitory activity was largely destroyed by heating at 75°C and completely destroyed at 100° and 121°C (Table 2,c,d,e).

DIALYSIS.—An extract of lemon leaves centrifuged at low speed was dialyzed with mechanical stirring for 36–48 hours at 4°C against 100 volumes of buffer. The contents of the dialysis bag had almost the same inhibitory activity against CVV and TMV as the nondialyzed extract (Table 2,f,h).

ULTRACENTRIFUGATION.—Leaf ex-

tracts were centrifuged at 95,000 *g* for 2 hours in a Spinco model L ultracentrifuge; the supernatant was recovered and assayed against CSV. The inhibitory activity was higher than that induced by extracts centrifuged at low speed (Table 2,f,g). On the contrary the resuspended pellet showed very little inhibitory activity.

clarified extract was fractionated through a Sephadex G-75 (Pharmacia, Uppsala, Sweden) column in a cold room at 4°C. Effective column size was 30 x 400 mm. The elution was performed by applying the same phosphate buffer 0.02 M pH 7 with an elution rate of 60 ml per hour. Samples of 10 ml were collected until the optical density, meas-

TABLE 2. INHIBITORY ACTIVITY OF LEMON LEAF EXTRACTS AFTER DIFFERENT TREATMENTS

Treatment	Percentage inhibition		
	CVV[a]	CSV[b]	TMV[a]
a. None (buffer extract)		42	38
b. Phenol-buffer extract		3	10
c. Heating at 75°C × 10 min		14	
d. Heating at 100°C × 10 min		0	
e. Autoclaving × 10 min		0	
f. Supernatant from 10.000 *g* × 20 min	19	50	24
g. Supernatant from 95.000 *g* × 2 hours		76	
h. Dialysis	18		32
i. Charcoal clarification	34		20
l. 1st Sephadex G75 filtration	14		
m. Rerun of eluate from 1st column	68		
n. Lyphogel concentration	61		

a. Citrus leaves were homogenized with buffer (1:1).
b. Citrus leaves were homogenized with buffer (1:2).

CHARCOAL CLARIFICATION.—Active charcoal for analysis (Merck 2186) was added (0.1 g per ml) to extract centrifuged at low speed and the mixture shaken for a few minutes. Charcoal was removed by low-speed centrifugation, and the supernatant passed through filter paper to remove residues. The treatment was effective in eliminating brown pigments, giving a clear extract that had an increased inhibitory activity against CVV and almost the same activity against TMV in comparison with centrifuged extracts (Table 2,f,i).

SEPHADEX FILTRATION.—Charcoal-

ured at 254 nm and 280 nm, had reached a constant low level. Optical density of the eluted fractions revealed 2 distinct peaks. Inhibitory activity appeared to be associated with the first peak and was slightly lower than that of centrifuged extract (Table 2,f,l) as a result of dilution during filtration. Large amounts of pigments were visible in the following fractions. The fraction showing the highest absorbance among those of the first peak was treated with charcoal and rerun through another Sephadex G75 column (12 x 60 mm) with an elution rate of 50 ml per hour. Samples of 5 ml were

collected. The optical densities revealed 2 peaks (Fig. 1). Inhibitor activity was again associated with the first peak. Assays of fractions 2, 3, 4, and 5 revealed a good concordance between optical density and percentage inhibition (Fig. 1). Fraction 4 of the second column was 5 times as active as fraction 6 of the first column (Table 2,l,m).

ABSORPTION SPECTRUM.—The absorption spectrum of the fraction collected after the second Sephadex filtration is typical of a nucleoprotein, having a minimum at about 245 nm and a maximum at about 262 nm.

NUCLEIC ACID EXTRACTION.—In order to ascertain whether the inhibitory activity is related to nucleic acid or to protein, the phenol-emulsion method was used to remove protein from the filtered extract. No inhibitory activity was detected when the water phase was tested with TMV. The orcinol test was positive, giving evidence for the presence of ribonucleic acid.

AMINO ACID ANALYSIS.—Aliquots of fraction 4 were hydrolyzed with HCl

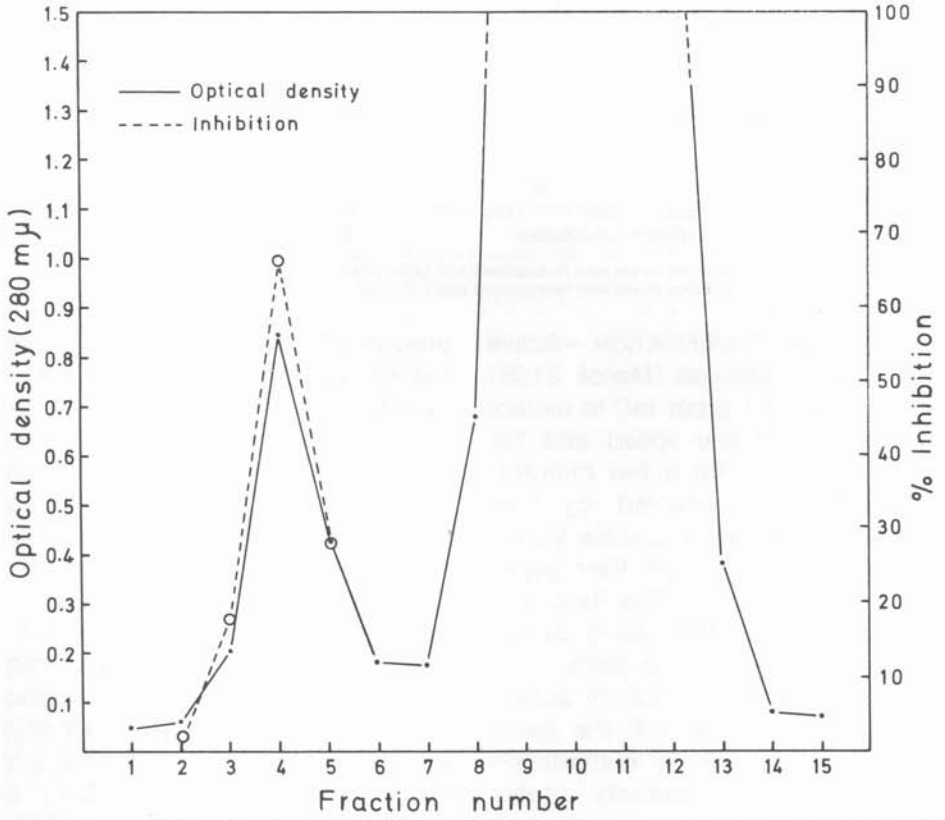

FIGURE 1. *Optical density at 280 mμ and percentage inhibition of 5 ml fractions eluted through a Sephadex G-75 column at an elution rate of 50 ml per hour.*

and analyzed by two-dimensional paper chromatography. Phenol (80 per cent in water) was used as the first solvent and n-butanol:acetic acid:water (4:1:5) as the second. Spraying the chromatograms with ninhydrin (0.4 per cent in n-butanol) revealed the presence of aspartic and glutamic acids, glycine, glutamine, alanine, tyrosine, valine, leucine(s), phenylalanine, and other amino acids.

MOLECULAR WEIGHT DETERMINATIONS.—Attempts to estimate the molecular weight of the inhibitor were made on the basis of the exclusion limit of Lyphogel (20,000), a polyacrylamide hydrogel available from the Gelman Instrument Company, and Sephadex G75 (50,000). Lyphogel pellets were added to filtered inhibitor and left in a refrigerator for at least 5 hours. When the pellets were removed, the concentrated fluid (1/10 the original volume) showed a high inhibitory activity against CVV (Table 2,n). Therefore, the inhibitor was not absorbed by the pellets. In other experiments, a mixture of inhibitor and egg albumin (m.w. 45,000) solution was eluted through a Sephadex G75 column (12 x 60 mm). Both inhibitor and egg albumin were recovered in the same fractions, suggesting a very close molecular weight.

Discussion

The results suggest that the inhibitor isolated is a nucleoprotein. Additional studies are necessary to determine whether the inhibitor acts only as intact nucleoprotein or whether the protein fraction alone has an inhibitory activity.

As suggested for other inhibitors of plant virus infections (3, 4, 7), the substance responsible for the inhibitory activity of lemon leaf extracts seems to act on the host rather than on the virus particles. Not yet clear is how the inhibitor modifies the host and by what mechanism it interferes with virus infection.

Stahmann and Gothoskar (6), investigating the action of natural and synthetic polyelectrolytes on TMV, suggested the possibility that the mechanism of virus inhibitors can be best explained on the basis of an ionic combination of the inhibitor with either the virus particles or the virus-receptor sites of the plant cells. Either combination may block or prevent the initial binding of the virus by the host cell. A similar action of various virus inhibitors was reported by other authors, who suggested the possible role of ξ-amino groups of proteinaceous inhibitors, but only a few gave sufficient support to their hypothesis (5). Our preliminary amino acid analysis did not reveal any ξ-amino group in the extracts we worked with, which could suggest a similar behavior. On the other hand, the results discussed do not exclude the possibility that the inhibitor in some way interferes with the biosynthesis of virus after its cell penetration.

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