Further Studies of an Inhibitor of Virus Replication from Tobacco Mosaic Virus-Infected Protoplasts of a Local Lesion-Responding Tobacco Cultivar

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ABSTRACT

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The inhibitor(s) of virus replication (IVR) released into the medium from tobacco mosaic virus (TMV)-infected protoplasts of *Nicotiana tabacum* L. 'Samsun NN,' in which the infection in the intact plant is localized, was found to inhibit virus replication also in leaf tissue disks, in addition to the previously reported evaluation on isolated protoplasts. IVR inhibited replication of tobacco mosaic, cucumber mosaic (CMV), and potato X viruses in leaf disks from different hosts, indicating that IVR is neither host- nor virus-specific. Inhibition rates, determined by infectivity or by enzyme-linked immunosorbent assay, ranged between 60 and 90%. IVR also inhibited TMV replication in intact leaves when applied to cut stems or by spray. IVR was recovered from intact protoplasts in addition to the incubation medium. IVR did not affect TMV directly and no IVR could be detected in the medium from Samsun NN protoplasts inoculated with CMV. IVR was found to be sensitive to trypsin and chymotrypsin, but not to RNase, and its activity was abolished by incubation at 60 C for 10 min, suggesting that IVR is proteinaceous.

The occurrence of a substance(s) inhibiting virus replication (IVR), which is released into the medium from tobacco mosaic virus (TMV)-infected protoplasts of a cultivar in which the infection in the intact plant is localized, was reported recently (6). IVR inhibited virus replication in protoplasts from both local lesion-responding cultivar Samsun NN and systemic-responding cultivar Samsun *N. tabacum* plants when applied up to 18 hr after inoculation. It was not produced in protoplasts from cultivar Samsun or from uninoculated protoplasts of cultivar Samsun NN. IVR was partially purified by using $ZnAc_2$ precipitation, and yielded two biologically active principles with molecular weights of about 26,000 and 57,000, as determined by gel filtration.

Antiviral activity of IVR was previously evaluated only on isolated tobacco protoplasts. We now present additional data on the inhibitory effect of IVR in plant tissues, its specificity, and chemical nature.

MATERIALS AND METHODS

Growing of *Nicotiana tabacum* L. 'Samsun' and 'Samsun NN' plants and the preparation of protoplasts and IVR by precipitation with $ZnAc_2$ were as described before (6). Control preparations were obtained similarly from sham-inoculated protoplasts. Preparations obtained from 10^6 protoplasts, incubated for 72 hr after inoculation, will be termed as one "unit."

Assay of inhibition of virus multiplication on protoplasts and leaf disks. Assays on protoplasts were done as described before (6).

To assay IVR activity on leaf disks, Samsun tobacco plants were inoculated with a solution containing 2.5 μg of purified TMV per milliliter and kept in the greenhouse. Five hours or later, disks 11 mm in diameter were cut from the inoculated leaves, and floated on IVR and control preparations (partially purified by $ZnAc_2$ precipitation) dissolved in an incubation medium containing 0.2 mM KH_2PO_4, 1 mM KNO_3, 0.1 mM $MgSO_4$, 10 mM $CaCl_2$, 1 mM KI, and 0.01 μM $CuSO_4$ (VIM) (7) (without mannitol) in 5-cm-diameter petri dishes. Disks were incubated at 25 C under continuous illumination. After various times of incubation, two disks from each IVR and control test were washed with distilled water, and homogenized in 2 ml of 0.05 M phosphate buffer, pH 7.5. The homogenate was used to inoculate 12 half-leaves of *Nicotiana glutinosa* L. plants and compared with a standard solution of purified TMV on the opposite half-leaf. In several samples, virus titer was also determined by ELISA (2).

For evaluation of IVR against cucumber mosaic virus (CMV) and potato virus X (PVX), tobacco cultivar Samsun NN, *Cucumis sativus* L. 'Bet Alpha', and *Capsicum fructescens* L. 'Vinedale' plants were inoculated with CMV and *N. glutinosa* plants with PVX. Plants were kept in the greenhouse, and disks were removed from inoculated leaves and floated on IVR and control preparations as described above. Infectivity of CMV was assayed on *Vigna sinensis* Endl. 'Blackeye' and that of PVX on *Gomphrena globosa* L. plants by comparing IVR and control treatments on opposite half-leaves.

Application of IVR through stems and by spraying. Two developed leaves of Samsun tobacco plants, 4–5 wk after transplanting, were inoculated with TMV (2.5 μg/ml). After 5 hr, stems were cut about 2 cm above the soil, and the plants were placed in vials containing IVR or control preparations (one unit dissolved in 5 ml of distilled water) for 3 hr for the solution to be taken up. Plants were then transferred to flasks with distilled water and kept at 25 C under continuous illumination. After various time intervals, two 11-mm-diameter disks were sampled from the inoculated leaves and assayed as described.

Spray applications of IVR and control preparations (three units in 10 ml of distilled water) were made to the upper side of the leaves of Samsun plants (3 wk after transplanting) 5 hr after inoculation with TMV (2.5 μg/ml) on the lower side. TMV was assayed after various times from both inoculated and uninoculated leaves.

Treatment of IVR with enzymes. Gel-bound trypsin (Trypsin-30, Enzygell, E.C. 2.4.214) and chymotrypsin (Enzygell, E.C. 3.4.21.1) (both from Bohringer, Mannheim, W. Germany) were suspended in 0.05 M phosphate buffer pH 7.5, and RNase (Enzyte Agarose Ribonuclease I, 36-512 Miles Biochemicals) in 0.1 M acetate buffer, pH 6, and washed by centrifugation to remove the nonbound fraction. Enzyme suspensions were then added to IVR (three units

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in 0.5 ml) and control preparations to a final concentration of 100, 100, and 10 $\mu g/ml$, respectively. After incubation for 3 hr at 25 C, enzymes were removed by centrifugation, and IVR activity was assayed on tobacco protoplasts or leaf disks inoculated 5 hr previously with TMV. Protoplasts were assayed 72 hr after inoculation and leaf disks after 48 and 72 hr.

Heat treatment of IVR. IVR (two units) and control preparations were suspended in 0.5 ml of virus incubation medium (VIM) (7), and heated at 40, 50, 60, and 80 C in thin-walled glass tubes for 10 min. Tubes were then cooled quickly, and IVR activity was assayed on TMV-inoculated protoplasts and leaf disks.

Recovery of IVR from protoplasts. TMV-infected Samsun NN and control protoplasts (10^7) were collected at various times after inoculation by centrifugation at 35 g for 6 min. Protoplasts were homogenized in 5 ml of 0.1 M phosphate buffer pH 7.0 for 2.5 min at 5 C, with the aid of a microattachment to an Omni-Mixer. The homogenate was centrifuged at 8,000 g for 10 min, the precipitate discarded, and the supernate centrifuged at 100,000 g for 1.5 hr to remove the virus. No infectivity could be detected in the supernate. IVR was then partially purified from the supernate using precipitation by $ZnAc_2$ as described previously (6), and assayed on Samsun tobacco leaf disks.

RESULTS

Inhibition of virus replication by IVR in leaf disks. IVR inhibited TMV replication in Samsun leaf disks. Floating disks 5 hr after inoculation on a solution containing one unit of IVR reduced virus replication 60–90%, as determined by both local lesion assay and ELISA (Table 1). No difference in virus titers was observed between that in disks floated on control medium ($ZnAc_2$ preparations from uninoculated protoplasts in VIM) and that from disks floated on VIM only. In further experiments, inhibitory activity rose with increasing concentrations of IVR. Thus, 0.5, 1, 3, 5, and 10 units inhibited TMV replication in disks by 54, 56, 67, 75, and 84%, respectively, as determined by local lesion assay 72 hr after inoculation. IVR (one unit) applied to leaf disks 8 hr after inoculation with TMV reduced virus replication by 55–65%, as determined by local lesion assay. In tomato leaf disks one unit of IVR inhibited replication of TMV by 64, 62, and 72%, respectively, when assayed 48, 72, and 96 hr after inoculation with TMV.

IVR also was active against other viruses in several hosts when tested in leaf disks. IVR inhibited CMV in leaf disks of Samsun NN tobacco and Bet-Alpha cucumbers. Inhibition rates, by one unit of IVR, ranged between 60 and 80% when assayed up to 120 hr after inoculation (Table 1). Similar results were obtained with leaf disks of Vinedale bell pepper, which showed inhibition rates of 63, 69, and 64% when assayed 48, 72, and 96 hr, respectively, after inoculation with CMV.

IVR also reduced replication of PVX in leaf disks of *N. glutinosa* with inhibition rates (by one unit of IVR) between 60 and 70% (Table 1).

Inhibition of TMV replication by IVR applied through cut stems and by spray. Application of IVR through cut stems of Samsun plants 2 hr before or 5 hr after inoculation reduced extractable TMV by about 80 and 70%, respectively (Fig. 1). Spraying intact plants with IVR 2 hr before or 5 hr after inoculation and assayed 7 days after inoculation gave inhibition rates of about 78 and 56%, respectively, when inoculated leaves were assayed. In systemically infected leaves, one spray gave inhibition rates of 81 and 43%, respectively (Fig. 2). Assays 8 days after inoculation generally gave lower rates of inhibition, perhaps due to degradation and/or dilution of IVR in the plant.

Inactivation of IVR by enzymes. Incubation of IVR-containing solution with trypsin or chymotrypsin either inactivated IVR completely or markedly reduced its activity in assays on infected protoplasts or leaf disks. When assayed on TMV-infected protoplasts, IVR reduced virus multiplication (measured by local lesion assay 72 hr after inoculation) by 68%, while trypsin- and chymotrypsin-treated IVR gave 5 and 8% inhibition, respectively, compared with the control preparation treated similarly with the enzymes. No differences in virus titers were observed between protoplasts treated with control preparations incubated with the enzymes and those incubated without the enzmyes. Trypsin and chymotrypsin inactivated by heating the enzyme for 15 min at 100 C did not reduce IVR activity. Similar results were obtained when IVR was assayed on TMV-infected leaf disks. Thus, incubation with trypsin or chymotrypsin reduced inhibitory activity of IVR

TABLE 1. Effect of an inhibitor of virus replication (IVR) on replication of several viruses when applied to leaf tissue disks of different hosts[a]

Disks assayed (hr after inoculation)	Infectivity[b] in leaf disks floated on: IVR	Control medium[c]	VIM[e]	Inhibition infectivity[g] (%)	Virus yield[d] in leaf disks floated on: IVR	Control medium[c]	VIM[e]	Reduction of virus yield (%)
TMV in Samsun tobacco								
24	7.4 ± 4.2	36.2 ± 9.1	36.2	80	0.06 ± 0.05	0.96 ± 0.42	0.67	94
48	57.2 ± 5.7	234 ± 84	240.7	76	0.46 ± 0.34	2.3 ± 0.26	3.0	80
72	387.5 ± 65.9[f]	1,660 ± 103.9[f]	1,261[f]	77	5.85 ± 1.25	43.35 ± 10.45	31.5	87
96	1,997 ± 403[f]	14,000 ± 869[f]	16,300[f]	86	66.0 ± 8.85	367.5 ± 31.8	430	82
120	6,660 ± 56.7[f]	19,075 ± 1,873[f]	18,300[f]	65	153.5 ± 3.5	497.5 ± 53.0	480	69
CMW in Samsun NN tobacco								
48	36.7 ± 12.9	111.7 ± 28.5	88	67	0.7 ± 0.1	2.1 ± 0.7	1.75	67
72	257 ± 41.01[f]	958 ± 174[f]	807[f]	73	4.4 ± 0.95	18.0 ± 5.3	16.0	76
96	1,034 ± 120.4[f]	4,690 ± 208[f]	4,200[f]	78	30.5 ± 4.3	88.3 ± 9.4	80	65
120	1,110 ± 42.4[f]	5,065 ± 226	6,000[f]	78	27.5 ± 3.5	97.5 ± 5.3	115	72
CMV in Cucumber								
48	14.4 ± 0.75	65.2 ± 17.8	55.5	78	0.28 ± 0.03	1.33 ± 0.39	1.13	79
72	166 ± 26.2[f]	411 ± 51.6[f]	445[f]	60	3.37 ± 0.88	8.0 ± 1.41	8.5	58
96	696 ± 82.5[f]	2,008 ± 15.9[f]	2,166[f]	65	11.3 ± 2.4	35 ± 3.07	39.5	68
120	1,355 ± 59.4[f]	4,280 ± 305[f]	4,340[f]	68	27.5 ± 3.5	80 ± 14.4	84.0	66
PVX in *N. glutinosa*								
48	32 ± 15.3	100 ± 15.8	112	68				
72	98 ± 13.0	331 ± 85.8	471	70				
96	1,176 ± 33.2[f]	3,331 ± 292.8[f]	3,620[f]	65				

[a] Average of three to five experiments when IVR (one unit) was applied to leaf tissue disks 5 hr after inoculation.

[b] Average number of local lesions ± standard error on one half-leaf of *N. glutinosa* for TMV, one leaf of *Vigna sinensis* for CMV, and one leaf of *Gomphrena globosa* for PVX.

[c] $ZnAc_2$ preparation from medium in which uninoculated protoplasts were suspended.

[d] Average yield (μg/disk) ± standard error, as determined by ELISA.

[e] Virus incubation medium (7) without mannitol.

[f] Homogenate was diluted 10-fold or 100-fold. The average number of lesions on 12 half-leaves (for TMV) and 12 leaves (for CMV and PVX) induced by the appropriate dilution was multiplied by the dilution factor.

[g] IVR relative to control medium.

from 80 to 5 and 2%, respectively, while that of IVR incubated with heat-inactivated enzymes remained 79 and 69%, respectively.

RNase did not affect the activity of IVR when assayed on protoplasts or on leaf disks. Inhibitory activities of IVR treated with RNase, inactivated RNase (by adding 1 mM Aurintricarboxylic acid (ATA), and nontreated IVR were 74, 76, and 83%, respectively, when assayed on leaf disks 48 hr after inoculation. Similar results were obtained when IVR activity was assayed on protoplasts.

Inactivation of IVR by heat. IVR was inactivated by heating at 60 C for 10 min. Heating the IVR preparation for 10 min at 40, 50, 60, and 80 C resulted in inhibition rates of 70, 72, 6, and 0%, respectively, compared with 68% from the nonheated preparation, when assayed on TMV-infected leaf disks.

Evaluation of IVR on TMV in vitro and on leaf disks with mature infections. Mixing IVR (three units) with purified TMV (2.5 μg/ml) and incubating the mixture for 1 hr did not affect the infectivity of TMV recovered by ultracentrifugation.

IVR also had no effect on mature infections of TMV and CMV. When disks from Samsun tobacco leaves, infected for 10 days with TMV, were floated for 48 hr on IVR (three units), control preparations, and VIM (without mannitol), and assayed on *N. glutinosa*, the average number of lesions per half-leaf was 371, 363, and 387, respectively. Assay of infectivity after 120 hr of floating on the test solution resulted in 398, 373, and 392 lesions, respectively. Similar results were obtained with disks from cucumber leaves, infected for 10 days with CMV. The average number of lesions per cowpea leaf was 68, 65, and 79, respectively, when disks were floated for 48 hr on IVR, control preparation, or VIM. Assay of infectivity after 120 hr of floating resulted in 109, 102, and 81 lesions, respectively.

Recovery of IVR from the inoculated protoplasts. IVR could be obtained from intact protoplasts and not only from the medium in which the infected protoplasts were suspended. IVR obtained from intact protoplasts reduced TMV multiplication in leaf disks by 78–85% compared with the controls. Control preparations obtained from noninfected protoplasts did not affect virus multiplication when compared with that in leaf disks floated on VIM.

Absence of IVR from the medium of Samsun NN protoplasts infected with CMV. CMV systemically infects Samsun NN plants with no production of necrosis. Attempts to elicit and recover IVR from Samsun NN protoplasts inoculated with CMV-6 (5) and incubated for 72 hr were unsuccessful.

DISCUSSION

In our previous work (6) it was shown that protoplasts from a cultivar, in which the infection in the intact plant is localized, release into the incubation medium a substance(s) that inhibits TMV replication (IVR) in infected protoplasts from Samsun NN or from Samsun leaves. In the present work, we found that IVR inhibited TMV replication also in leaf tissue disks of tobacco and tomato and in intact tobacco plants—all being infected systemically. Inhibition rates ranged between 60 and 90% when virus yield was assayed either by infectivity or by ELISA. These rates are still far from being of practical value, although it should be considered that only one spraying, with relatively low concentrations of IVR (two to three units), was given. In addition, no data are yet available on the quantity of IVR taken up by the leaf tissue and the amount reaching the site of action inside the cell, or possible ways of increasing both.

IVR inhibited replication of TMV, CMV, and PVX in different host tissues, indicating that it is neither virus- nor host-specific.

IVR was found to be thermolabile and sensitive to proteolytic enzymes, but not to ribonuclease, suggesting that IVR is proteinaceous.

No conclusion can be drawn at present as to the relation of IVR to the antiviral factor (AVF) obtained by Sela and co-workers (1,8).

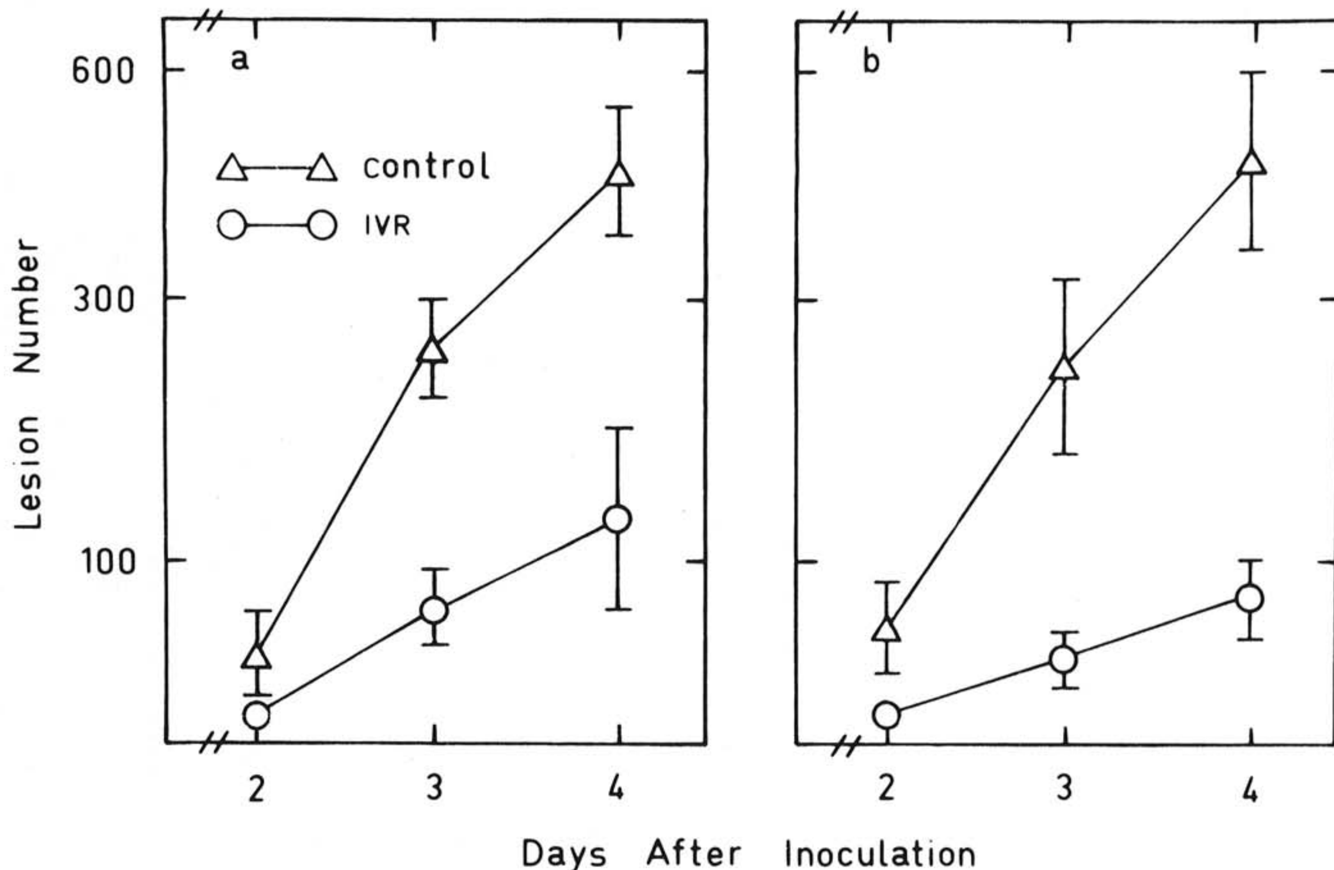

Fig. 1. TMV replication in cultivar Samsun tobacco plants when an inhibitor of virus replication (IVR, one unit) and control preparations were applied through cut stems **a,** 2 hr before and **b,** 5 hr after inoculation. Average number of local lesions ± standard error on one half-leaf of *Nicotiana glutinosa* from four experiments.

IVR and AVF seem to differ in several respects, although both seem to be associated with the *N* gene in tobacco. Thus, AVF was resistant, while IVR was sensitive to trypsin digestion. Furthermore, AVF has been tested mainly as an inhibitor of infection applied together with the virus or at relatively short times after inoculation (15 min and 2 hr), while IVR was found to be effective when applied up to 18 hr after inoculation of protoplasts, and up to 8 hr after when applied to leaf disks. Thus, IVR is truly an inhibitor of virus replication (4).

Several properties of IVR resembled those of interferon (3). Both interferon and IVR are released into the medium from infected cells, and both inhibit virus replication in infected cells suspended in such media. Both are proteinaceous and neither is virus-specific. For example, elicitation by one virus releases active substances that also inhibit viruses that are not related to the inducer. Thus far, the main difference found between interferon and IVR is that the former is tissue-specific, while IVR obtained from tobacco cells was active also in nonrelated hosts, such as cucumber and pepper.

Further work on the mode of action of IVR is required to show whether it affects virus replication directly or indirectly by changing some host component. Nevertheless, the finding that IVR significantly also inhibits virus replication when applied to systemically infected intact tissues may open new approaches for control of virus diseases, in addition to a better understanding of the virus-localizing mechanism in plants.

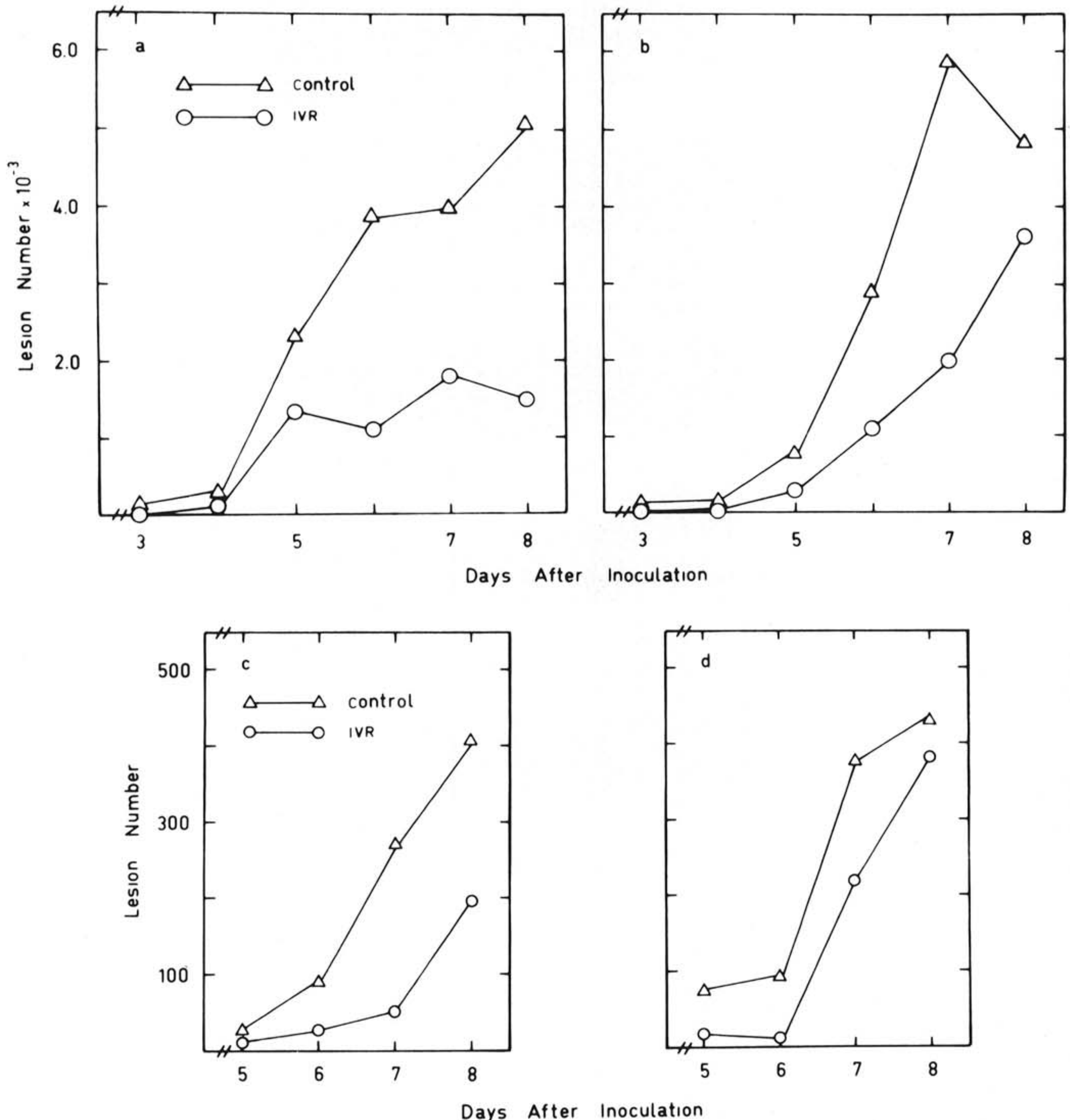

Fig. 2. TMV replication in Samsun tobacco plants sprayed once with an inhibitor of virus replication (IVR, three units) and control preparation 2 hr before inoculation when **a,** inoculated and **b,** systemically infected leaves were assayed; and virus replication in plants sprayed with IVR and control preparation 5 hr after inoculation when **c,** inoculated and **d,** systemically infected leaves were assayed. Average number of local lesions on one half-leaf of *Nicotiana glutinosa* from two experiments.

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