Resistance

Recovery of an Inhibitor of Virus Replication from the Intercellular Fluid of Hypersensitive Tobacco Infected with Tobacco Mosaic Virus and from Uninfected Induced-Resistant Tissue

S. Spiegel, A. Gera, R. Salomon, P. Ahl, S. Harlap, and G. Loebenstein

First, second, third, fifth, and sixth authors: Virus Laboratory, Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel; fourth author: Agricultural Division, CIBA-GEIGY, Basel, Switzerland.

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ABSTRACT

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A compound was obtained from the intercellular fluid of tobacco cultivar Samsun NN infected with tobacco mosaic virus (TMV) which inhibited virus replication when applied to TMV-inoculated protoplasts or leaf disks. Based on the criteria of serology, polyacrylamide gel electrophoresis, and molecular weight estimations, the compound from the intercellular fluid was judged to be identical to the inhibitor of virus replication (IVR) obtained previously from TMV-infected Samsun NN protoplasts. The recovery of IVR is facilitated when leaves are used as a source. A similar compound was obtained from systemic induced-resistant tissue of Samsun NN and cultivar Xanthi-nc. The belief that induced resistance results from an activation of the localizing mechanism is supported by this result.

Additional keywords: induced resistance, inhibitor of virus replication, serology.

Previously, we reported that a substance(s) that is an inhibitor of virus replication (IVR) is released into the medium from tobacco mosaic virus (TMV)-infected protoplasts of a tobacco cultivar in which the infection in the intact plant is localized. IVR inhibited virus replication in protoplasts from both local lesion-responding resistant (cultivar Samsun NN) and systemic-responding susceptible (cultivar Samsun) tobacco plants. IVR was not released from TMV-infected Samsun protoplasts (9). It was suggested that IVR is associated with localization (11). IVR inhibited virus replication in leaf tissue disks and in intact leaves when applied to cut stems or by spray. IVR was partially purified using $ZnAc_2$ precipitation (crude protoplast IVR), and two biologically active principles were obtained with molecular weights of approximately 26,000 (fractionated protoplast IVR-1) and 57,000 (fractionated protoplast IVR-2), as determined by gel filtration. IVR activity is sensitive to the proteolytic enzymes trypsin and chymotrypsin, but not to RNAse, suggesting that IVR is proteinaceous (3). Antisera to fractionated protoplast IVR-1 and IVR-2 are highly cross-reactive (4) which suggests that IVR-2 is a dimer of IVR-1 (9). Polyacrylamide gel electrophoresis (PAGE) of crude protoplast IVR under denaturing conditions identified a 23K protein, which gave a reaction with IVR-1 antiserum. Further purification of IVR yielded a biological active fraction, which contained a single 23K protein (Gera et al, *unpublished*).

Preliminary attempts to recover IVR in reasonable quantities from homogenated leaf tissue were not successful. An objective of the present study was to obtain IVR from the intercellular fluid of TMV-infected leaves of a hypersensitive tobacco. This approach was chosen based on previous findings that IVR is released from infected protoplasts into the incubation medium (9), that pathogenesis-related (PR) proteins accumulate in the intercellular fluid (12), and that the intercellular fluid of TMV-infected Samsun NN leaves has virus-inhibitory activity (14).

The detection of IVR in "induced-resistant" tissue has not been reported previously. Induced resistance is a phenomenon whereby uninoculated parts of hypersensitive hosts become partially and nonspecifically resistant to diverse pathogens (6–8). Virus lesions

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developing after challenge inoculation of the resistant tissue are consistently smaller and usually fewer in number than those formed on previously uninoculated control plants. In Samsun NN plants, induced resistance was found to be closely correlated to reduced virus concentration of the challenge virus (13), which indicates that virus replication is suppressed in the resistant tissue. Induced resistance seems to require an active cellular process, depending on the transcription mechanism from DNA to RNA, because its development is markedly inhibited in the presence of actinomycin D (10). It has been suggested that after initial virus inoculation of hypersensitive plants a substance(s) is produced that induces resistance in uninoculated tissue (8).

Here we report that IVR can be obtained from the intercellular fluid of TMV-infected Samsun NN, compare its identity with IVR obtained from protoplast incubation medium, and show its presence in induced-resistant tissue of tobacco cultivars Samsun NN and Xanthi-nc.

MATERIALS AND METHODS

Plants. *Nicotiana tabacum* L. 'Samsun NN,' 'Samsun,' and 'Xanthi-nc' were grown in a greenhouse for 5 to 6 wk after transplanting. A complete nutrient solution was supplied three times at weekly intervals, starting 1 wk after transplanting. One or 2 days before use, plants were transferred to a greenhouse or controlled-temperature chamber maintained at 21–22 C. Each plant was trimmed to four to seven expanded leaves.

Inoculation with TMV. Leaves of Samsun NN used to isolate IVR were inoculated with a solution containing purified TMV (1.5 μg/ml) in one of two ways: inoculation of the entire leaf, giving a lesion density of three or four lesions per square centimeter (treatment a); or inoculation in 10–15-mm strips (25–30 mm apart) on both halves of each leaf parallel to the midvein (treatment b). For systemic induced resistance, Samsun NN plants were trimmed to five leaves. The lower three expanded leaves were inoculated and the upper two leaves (resistant) were used for extraction (treatment c). In addition, the basal halves of two lower leaves of Xanthi-nc plants were inoculated with TMV. The distal uninoculated part of these leaves and the upper five uninoculated leaves were used for extraction (treatment d). Plants inoculated with water were used as controls for all experiments.

Extraction of inhibitory intercellular fluid. Intercellular fluid was extracted as described by Parent and Asselin (12) with the following modifications. Whole leaves (for treatment a) or the tissue between the TMV strips and the TMV strips themselves (for treatment b) were collected 6–7 days after inoculation. Upper leaves were collected 7 and 14 days after inoculating the lower leaves (treatment c). Leaves were cut into 4–6 pieces after removal of the midvein. Leaf pieces were infiltrated in vacuo for two or three periods of 30–50 sec each with a large excess of cold (4 C) 0.05 M phosphate buffer, pH 7.0, containing 0.1% 2-mercaptoethanol. Pieces were gently blotted dry, rolled, and placed in centrifuge tubes containing an inner matching part with a pierced bottom. The intercellular fluid was collected by centrifugation at 2,000 *g* for 10 min. The collected fluid was treated with $ZnAc_2$, as described previously for IVR (9). The term crude tissue IVR will refer to $ZnAc_2$-treated intercellular fluid prepared this way. A preparation obtained from 1 g of leaf tissue will be termed 1 "unit." A unit was found to be roughly equivalent to the amount of crude protoplast IVR obtained from 10^6 protoplasts and is equivalent to approximately 10 ng of protein. This amount was estimated from staining reactions following PAGE and by amino acid analysis after high-pressure liquid chromatography (HPLC) (unpublished results obtained in collaboration with Y. Burstein and V. Buchner, Weizmann Institute of Science, Rehovot, Israel).

An inhibition assay of tissue IVR was done on protoplasts and on leaf disks infected with TMV, either by infectivity assays or by enzyme-linked immunosorbent assay (ELISA), as described previously (2,3,9).

Preparation of protoplasts and crude protoplast IVR. Procedures for maintenance of protoplasts and preparation of IVR from incubation medium were as previously described (9).

Serology. Antisera against fractionated protoplast IVR-1, fractionated protoplast IVR-2, and the 23K band obtained from PAGE ("PAGE" antiserum) were prepared in rabbits (4; Gera et al, *unpublished*). Antisera (1 ml) were absorbed with a lyophilized preparation obtained from the incubation medium of 75×10^6 mock-inoculated protoplasts.

Agar-gel-diffusion tests were done in 55-mm petri dishes containing a 4-mm layer (9 ml) of 0.75% agar (Bacto agar), 0.001 M ethylenediaminetetraacetic acid, 0.85% NaCl, and 0.2% sodium azide at pH 7.8. Agar plates were incubated in a moist chamber at 22 C for 15 hr. Those experiments with intercellular fluids from Xanthi-nc were carried out in agar on glass slides. Intercellular fluids were concentrated 25 times by lyophilization, and 20 μl of each were put into each well. Reference solutions consisted of 1 unit of crude protoplast IVR dissolved in 20 μl of water. After diffusion overnight, slides were washed for 24 hr in 0.8% NaCl, rinsed with water, and dried. They then were stained with a 2% Coomassie blue solution, in 25% ethanol and 10% acetic acid. Plant preparations contained 0.04% sodium dodecyl sulphate (SDS) when PAGE antiserum was used.

Polyacrylamide gel electrophoresis. Ten units of crude protoplast IVR or crude tissue IVR was concentrated to 8 μl, mixed with 4 μl of 0.06 M tris-HCl buffer, pH 6.8, containing 3% SDS, 5% 2-mercaptoethanol, 10% glycerol, and a few grains of bromophenol blue (disruption buffer), and boiled for 2 min. Twelve microliters of the SDS-disrupted IVR then was applied to 5–15% gradient polyacrylamide minigels (10×7.5 cm, 0.45 mm thick) containing 0.1% SDS and separated by electrophoresis using a Bio-Rad Mini Protean II apparatus (Bio-Rad Laboratories, Richmond, CA).

The following marker proteins (Bio-Rad Laboratories) were used: lysozyme (14,400 daltons [Da]), soybean trypsin inhibitor (21,500 Da), carbonic anhydrase (31,000 Da), ovalbumin (45,000 Da), bovine serum albumin (66,200 Da), and phosphorylase B (92,500 Da). The gels were stained with Coomassie blue (1). Extract from the medium of sham-inoculated protoplasts and plant extract from sham-inoculated tissue were prepared similarly.

RESULTS

Extraction of crude tissue IVR from TMV-infected Samsun NN leaves. Intercellular fluid obtained 6–7 days after inoculation of Samsun NN leaves was assayed on TMV-infected Samsun NN protoplasts. Effects of increasing amounts of crude tissue IVR on TMV titers in protoplasts, determined by infectivity assay and ELISA, are summarized in Table 1. Crude tissue IVR from the intercellular fluid of TMV-inoculated Samsun NN leaves consistently inhibited virus replication in protoplasts, and the inhibition was dose responsive. Comparable preparations obtained from the intercellular fluid of Samsun leaves 3 and 7 days after inoculation with TMV were not inhibitory when assayed in the protoplast system.

When crude tissue IVR (3 units) was applied to Samsun leaf disks inoculated with TMV (3), inhibition rates of 60 and 61% were obtained when infectivity was assayed 3 and 4 days after inoculation, respectively.

Partial purification and molecular weight estimation of tissue IVR from TMV-infected Samsun NN leaves. Lyophilized crude tissue IVR obtained from 100 g of TMV-inoculated Samsun NN leaves was dissolved in 1.0 ml of 0.1 M phosphate buffer and passed through a 33×2.3 cm Sephadex G-75 column. One-milliliter fractions were eluted with the same buffer and collected. A preparation from the same amount of sham-inoculated leaves was passed through the same column and collected in a similar manner. The fractions were tested in the protoplast virus-inhibition assay. Activity was detected in two sets of fractions (72 and 73% inhibition, respectively, average of three experiments) expected to contain proteins with molecular weights of 26,000 (fractionated tissue IVR-1) and 57,000 (fractionated tissue IVR-2). These properties are similar to those previously reported for fractionated IVR-1 and fractionated IVR-2 from protoplasts (9).

Serological relationship between tissue IVR and protoplast IVR. The two partially purified active fractions (fractionated tissue IVR-1 and IVR-2), obtained from the Sephadex G-75 column, were compared with fractionated protoplast IVR-1 and IVR-2 in agar-gel-diffusion tests, using antisera against fractionated protoplast IVR-1 and fractionated protoplast IVR-2. Five units of each fraction, dissolved in 0.2 ml of 0.1 M phosphate buffer, pH 7, were applied to each well. Clear precipitation lines were observed between fractionated tissue IVR-1 and the antisera to fractionated protoplast IVR-1 (Fig. 1a) and fractionated protoplast IVR-2 (Fig. 1b). These lines fused completely without spur formation, with the precipitation lines obtained between fractionated protoplast IVR-1 and fractionated protoplast IVR-2 and their respective antisera. Similarly, clear precipitation lines were obtained between fractionated tissue IVR-2 and the antisera against fractionated protoplast IVR-1 (Fig. 1a) and fractionated protoplast IVR-2 (Fig. 1b), indicating that the fractions are serologically similar. Clear precipitation lines also were obtained between the PAGE antiserum and crude tissue IVR (15 units) from the intercellular fluid, fusing without spurs with the precipitation line obtained between crude protoplast IVR (15 units) and this antiserum (Fig. 1c).

PAGE of crude tissue IVR from TMV-infected Samsun NN leaves. A specific band corresponding to a 23K protein was observed regularly in PAGE of crude tissue IVR preparations (Fig. 2, lane 4) and was at the same position as the specific band obtained from crude protoplast IVR (Fig. 2, lane 2).

Extraction of crude tissue IVR from induced-resistant tissue. Preparations purified from the intercellular fluid of induced-resistant tissue were tested for inhibitory activity on TMV-infected protoplasts. Extracts were made from TMV-strips, in between strips, and from systemic induced-resistant leaves 7 days after the inducing inoculation, and they were compared with the respective controls. Results, which are averages from two or three experiments, are summarized in Table 2. Extracts from induced-resistant tissue gave inhibition rates between 46 and 63%; extracts from tissue with local induced resistance between TMV strips had a higher inhibition rate than those from upper leaves with systemic induced resistance. Inhibition rates of 44% were observed when extracts (2 units) from tissue between strips were tested on TMV-infected Samsun leaf disks (averages from two experiments).

Release of IVR from induced-resistant tissue protoplasts. Protoplasts were obtained from the tissue between TMV strips ("resistant protoplasts") 7 days after the inducing inoculation and incubated for 72 hr. The incubation medium then was collected and evaluated in the protoplast virus inhibition assay, as described previously (9). For controls, protoplasts were obtained from tissue between sham-inoculated strips and treated similarly. In addition, resistant protoplasts and protoplasts from tissue between sham-inoculated strips were inoculated with TMV and evaluated for inhibitory activity. Results, which are averages from three or four experiments, are summarized in Table 3.

Protoplasts from the resistant tissue between TMV strips released an inhibitory substance into the incubation medium. Inoculation of these "resistant" protoplasts increased amount of inhibitor in the medium, when compared with that from uninoculated resistant protoplasts and with that from inoculated protoplasts obtained from sham-inoculated control strips.

Serology of tissue IVR from induced-resistant tissue. Crude

TABLE 1. Effect of crude tissue inhibitor of virus replication (IVR) from tobacco cultivar Samsun NN leaves inoculated with tobacco mosaic virus (TMV) on virus replication in Samsun NN protoplasts [a]

Amount of inhibitor added (units)	Infectivity[b] in protoplasts incubated in: Tissue IVR	Sham[c]	Percent inhibition	Virus yield[d] in protoplasts incubated in: Tissue IVR	Sham	Percent reduction of virus yield
0.5	32.6 ± 3.16	52.6 ± 2.36	38	0.68 ± 0.21	1.2 ± 0.29	43
1.0	28.5 ± 7.36	55.4 ± 12.37	49	0.45 ± 0.14	1.25 ± 0.29	64
3.0	17.3 ± 1.67	54.9 ± 12.81	68	0.33 ± 0.05	1.25 ± 0.35	74
5.0	15.9 ± 0.55	56.4 ± 7.46	72	0.28 ± 0.06	1.8 ± 0.17	84
10.0	12.7 ± 0.70	59.3 ± 8.49	79	0.21 ± 0.04	1.77 ± 0.25	88
Control[e]		55.1 ± 1.60			1.72 ± 0.23	

[a] As determined by local lesion assay and enzyme-linked immunosorbent assay (ELISA). Averages from two experiments.
[b] Average number of local lesions and standard error per 10^6 protoplasts on one half-leaf of *Nicotiana glutinosa* L. calibrated to a standard TMV solution (1.5 μg/ml), which yielded about 70 lesions per half leaf.
[c] Protoplasts incubated in medium with a $ZnAc_2$ preparation from sham-inoculated plants.
[d] Average virus yield (μg/10^6 protoplasts) and standard error, as determined by ELISA.
[e] TMV-inoculated protoplasts in incubation medium with no additions.

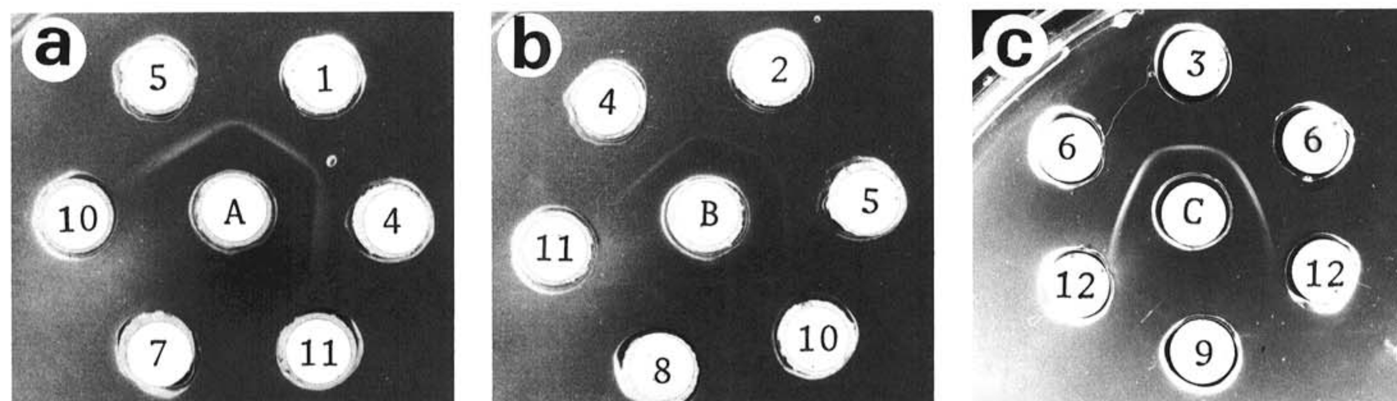

Fig. 1. Agar-gel double-diffusion serology of tissue inhibitor of virus replication (IVR) from the intercellular fluid of tobacco cultivar Samsun NN leaves with antiserum against: **A,** fractionated protoplast IVR-1, **B,** fractionated protoplast IVR-2, and **C,** the 23K protein band (polyacrylamide gel electrophoresis antiserum), compared with fractionated IVR-1, fractionated IVR-2, and crude IVR from protoplasts. 1, fractionated protoplast IVR-1; 2, fractionated protoplast IVR-2; 3, crude protoplast IVR; 4–6, fractionated tissue IVR-1, IVR-2, and crude, respectively, from leaves; 7–12, control preparations for 1, 2, 3, 4, 5, and 6, respectively.

preparations (20 units) obtained from the intercellular fluid from two systemic induced-resistant upper leaves 7 days after inoculation of three lower leaves gave a positive reaction with PAGE antiserum in agar-gel-diffusion tests (Fig. 3). Clear precipitation lines were obtained, which fused without spurs with the precipitation line obtained between crude protoplast IVR and the antiserum. Similar results (not shown) were obtained when the intercellular fluid was recovered 14 days after the inducing inoculation and when tested against fractionated protoplast IVR-1 antiserum.

The intercellular fluid from the resistant tissue of Xanthi-nc also was tested for the presence of IVR in agar-gel-diffusion tests. Clear precipitation lines with fractionated protoplast IVR-1 antiserum were obtained (Fig. 4). IVR was detected in the intercellular fluid from the distal parts of inoculated leaves 7 days after inoculation. It was not detected in the intercellular fluid from upper leaves before 14 days after the inducing inoculation.

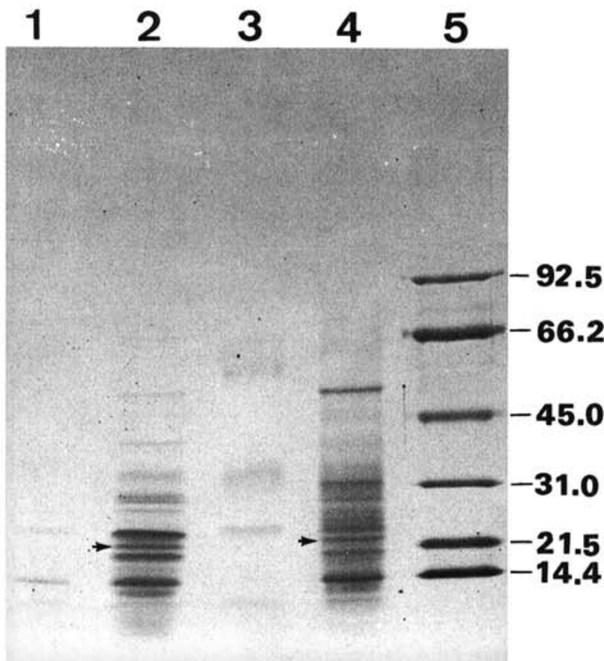

Fig. 2. Polyacrylamide gel electrophoresis of inhibitor of virus replication (IVR) after disruption in sodium dodecyl sulphate. Lane 2, crude IVR after $ZnAc_2$ (10 units from protoplasts); lane 1, control (from protoplasts); lane 4, crude tissue IVR after $ZnAc_2$ (10 units); lane 3, control from plants; and lane 5, molecular weight standards (from top to bottom) phosphorylase B (92.5 kilodaltons [kDa]), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). IVR band indicated by arrow.

TABLE 2. Effect of crude tissue inhibitor of virus replication (IVR)[a] from induced-resistant leaves of tobacco cultivar Samsun NN on tobacco mosaic virus (TMV) replication in Samsun NN protoplasts

Mode of induced resistance	Infectivity[b]	Percent inhibition
Strips, TMV[c]	12.2 ± 0.48	61
Strips, sham[c]	31.4 ± 2.81	
Between strips[c]	12.3 ± 1.4	63
Between strips, sham[c]	33.5 ± 3.62	
Control[d]	30.4 ± 2.19	
Upper leaves	20.5 ± 4.6	46
Control upper leaves[e]	38.0 ± 4.8	

[a]Two "units" of respective extract was added to 10^6 protoplasts.
[b]Average number of local lesions and standard error per 10^6 protoplasts on one half-leaf of *Nicotiana glutinosa* L. calibrated to a standard TMV solution (1.5 μg/ml), which yielded about 70 lesions per half leaf.
[c]Averages from three experiments; intercellular fluid sampled 7 days after the inducing inoculation.
[d]TMV-inoculated protoplasts in incubation medium with no additions.
[e]Averages from two experiments; intercellular fluid sampled 7 days after the inducing inoculation.

TABLE 3. Effect of inhibitor of virus replication (IVR) from protoplasts of induced-resistant tissue on tobacco mosaic virus (TMV) replication in tobacco cultivar Samsun NN protoplasts

Incubation medium[a] from protoplasts from:	Infectivity from test protoplasts[b]	Percent inhibition[c]
1. Between TMV strips, uninoculated	22.9 ± 4.45	44
2. Between TMV strips, inoculated	12.0 ± 4.90	70
3. Between control strips, uninoculated	40.6 ± 4.61	
4. Between control strips, inoculated	17.3 ± 6.66	57
Control protoplasts[d]	37.6 ± 2.19	

[a]Incubation medium (10 ml) from 10^6 protoplasts (1 unit). Protoplasts obtained 7 days after the inducing inoculation.
[b]Average number of local lesions and standard error from three to four experiments. Inoculum prepared from 10^6 protoplasts, suspended in respective incubation medium 4–5 hr after inoculation with TMV, and applied to one half-leaf of *Nicotiana glutinosa* L. Lesion counts were calibrated to a standard TMV solution (1.5 μg/ml), which yielded about 70 lesions per half leaf.
[c]Compared with incubation medium from protoplasts between control uninoculated strips (treatment No. 3).
[d]TMV-inoculated protoplasts in incubation medium with no additions.

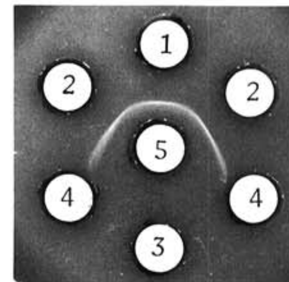

Fig. 3. Agar-gel double-diffusion serology of crude tissue inhibitor of virus replication (IVR) from the intercellular fluid of induced-resistant tissue from the upper two uninoculated leaves of tobacco cultivar Samsun NN plants, 7 days after inoculating three lower leaves with polyacrylamide gel electrophoresis (PAGE) antiserum. 1, crude protoplast IVR; 2, crude tissue IVR from induced-resistant leaves; 3, mock protoplast IVR; 4, intercellular fluid from healthy plant; 5, PAGE antiserum.

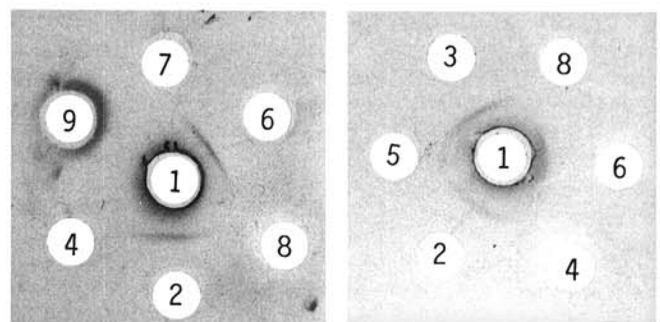

Fig. 4. Agar-gel double-diffusion serology of plant inhibitor of virus replication (IVR) from the intercellular fluid of induced-resistant tissue of tobacco cultivar Xanthi-nc leaves with antiserum against fractionated protoplast IVR-1. 1, fractionated protoplast IVR-1 antiserum; 2 and 3, intercellular fluid from distal part of leaf 7 and 14 days, respectively, after inducing inoculation; 4 and 5, intercellular fluid from upper leaves 7 and 14 days, respectively, after inducing inoculation; 6, crude protoplast IVR; 7, mock protoplast IVR; 8, intercellular fluid from healthy plant; 9, extraction buffer.

DISCUSSION

IVR-like compounds were obtained from the intercellular fluid of Samsun NN leaves infected with TMV and from induced-resistant tissue. Based on serology (using antisera to protoplast IVR), PAGE, and molecular weight estimations by molecular sieving, the compounds from leaf intercellular fluids are indistinguishable from IVR-1 and IVR-2 obtained from TMV-infected Samsun NN protoplasts. The serological data indicate that tissue IVR-1 and IVR-2 obtained from the intercellular fluid have identical serological determinants. This strengthens our previous suggestion (3) that protoplast IVR-2 is a dimer of protoplast IVR-1. The observation that IVR is released from cells into the intercellular fluid of leaf tissue parallels our previous finding that IVR is released from protoplasts into the incubation medium (9). The yield of tissue IVR per cell obtained from the intercellular fluid of leaf tissue was in the same range as that obtained from protoplasts. From 100 g of leaf tissue, which is estimated to contain $1-5 \times 10^8$ mesophyll cells, about 100 units of tissue IVR was obtained (equivalent to approximately 1 μg of protein). This is similar to the amount of IVR obtained from 10^8 protoplasts. The procedure for obtaining intercellular fluid is much simpler than the one for preparing protoplasts, which greatly facilitates the recovery of IVR.

Some discrepancies between the molecular weight estimations based on PAGE under denaturing conditions and on gel filtration were noticed. Using SDS-PAGE, a molecular weight of about 23,000 was estimated for IVR-1, from both the intercellular fluid and protoplast media, whereas by gel filtration a value of 26,000 was obtained. Similar observations have been made with other proteins (5). Based on molecular weight estimates, IVR differs from the biological active fractions obtained by Wieringa-Brants and Dekker from the intercellular fluid of tobacco plants with systemic acquired resistance (15).

IVR was detected by serology in induced-resistant tissue as soon as 7 days after the inducing inoculation, in either the distal half of Xanthi-nc leaves or the upper two leaves of Samsun NN plants. In the upper leaves of Xanthi-nc plants, IVR was detected 14 days, but not as soon as 7 days, after the inducing inoculation. In these plants, the basal parts of only two leaves were inoculated and intercellular fluid was extracted from five upper uninoculated leaves. The relatively smaller amount of tissue inoculated may be related to the longer time interval required for the development of IVR in the upper leaves.

The intercellular fluid obtained from the induced-resistant tissue between TMV strips had a higher inhibitory activity than that recovered from upper resistant leaves (Table 2). This parallels observations that the intensity of local induced resistance in the leaf tissue between TMV strips is significantly higher than systemic induced resistance in upper leaves (13).

The presence of IVR in induced-resistant tissue may explain the induced-resistance phenomenon, strengthening the suggestion that in induced-resistant tissue the localizing mechanism is activated before the challenge inoculation (8). The presence of IVR may affect virus replication immediately after challenge inoculation. In noninduced tissue of a hypersensitive plant, because the host genome has first to be activated, IVR production becomes evident only 30–36 hr after inoculation. Whether IVR is transported from the primary infected tissue, or whether there is a signal that moves from the inoculated tissue and activates IVR production in the tissue to become resistant is not yet known and will require further studies.

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