Induced Interference by Synthetic Polyanions with the Infection of Tobacco Mosaic Virus

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ABSTRACT

Polycarboxylates, with maleic or acrylic acids as the anionic component of the polymer, were evaluated as to their effect on infection and lesion development of tobacco mosaic virus (TMV). Two effects were observed: inhibition and induced interference. The polyanions tested did not inactivate TMV in vitro, but inhibited infection when present during inoculation. Inhibition ranged between 0 and 75%, depending on the concentration of the polyanion, but no effect on lesion size was observed. Ethylene-maleic anhydride (EMA) 31 was least inhibitory. When copolymers with a maleic acid component were injected intercellularly into leaves of *Nicotiana tabacum* 'Samsun NN', *N. glutinosa*, or *Phaseolus vulgaris* 'Scotia', they induced resistance which developed gradually after application. In the resistant tissue, both lesion number and size decreased significantly. The EMA polymers in particular were potent inducers of interference, irrespective of their molecular weights. Polymers with an acrylic or methacrylic acid component did not induce interference. The esterified vinyl methyl ether-maleic acid, in which the carboxylate groups of maleic acid were partially blocked, was also ineffective as an inducer of interference. Best distinction between inhibition and induced interference was obtained with EMA 31, at a concentration of 0.5 mg/ml, which did not inhibit infection when present during inoculation. Interference, in respect to lesion number, became apparent 24 hr after injection, reaching 75-80% between the 3rd and 15th days. Lesion diameter decreased 50-60%. The development of interference was sensitive to actinomycin D, when applied close in time to the injection of EMA 31. It is suggested that for the development of the polyanion- induced interference, the transcription mechanism of the cell has to operate, and that the respective polyanions activate that part of the genome responsible for localization.

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Previously, it was reported from our laboratory that injection of yeast-RNA into leaves of *Nicotiana tabacum* L. 'Samsun NN' caused a significant reduction in the number of lesions when tobacco mosaic virus (TMV) was inoculated 3 or more days after RNA application (6). These results were confirmed with yeast RNA and with RNA isolated from plants different from the test species (foreign RNA), whereas the resistance response was not elicited by RNA isolated from plants of the test species (1). Similar results were obtained with the synthetic double-stranded RNA, polyinosinic-polycytidilic acid (poly I•poly C) (12). Poly I•poly C was active at microgram quantities, although the reduction in lesion number was not so marked as with yeast RNA. It was suggested that these materials activate a resistance mechanism in the treated plant tissues, because a time interval between their application and the challenge inoculation is necessary. Furthermore, by increasing this time interval (within certain limits),the reduction in lesion number becomes more pronounced.

Foreign RNA, in particular the synthetic double-stranded poly I•poly C, has been reported to induce resistance, mediated through the interferon mechanism, in various animal cells (5, 9). Other anionic polyelectrolytes, such as synthetic carboxylates with a carbon-carbon backbone, are also known to induce resistance to viruses in animal cells, by similar though probably not identical mechanisms (2, 8). We were, therefore, interested to see whether representatives of three groups of synthetic carboxylates induce resistance against viruses in plants; i.e., activate the localizing mechanism before inoculation.

MATERIALS AND METHODS.–Plants of *Nicotiana tabacum* L. 'Samsun NN', *N. glutinosa* L., *Phaseolus vulgaris* L. 'Scotia', and *Cucumis sativus* L. 'Bet Alpha' and 'Elem' were grown in a screened greenhouse, and transferred 2-3 days before use to a greenhouse chamber set at 21 C.

Plants of *N. glutinosa* and Samsun NN tobacco, 4-6 weeks after planting, trimmed to four expanded leaves, and the primary expanded leaves of Scotia beans, 10-14 days after seeding, were used for the competitive inhibition tests. Inhibition was determined by the half-leaf method, whereby a standard inoculum of purified TMV was mixed with the respective material and compared with the control, consisting only of TMV at the same final concentration in water. The two solutions were alternately inoculated on the opposite halves of 16 leaves, and the results analyzed by the "sign test" (10). Percentage of inhibition caused by the material was calculated on the basis of 0% inhibition on the control half-leaves.

To test if the polyanions directly inactivate TMV, a suspension of the respective material (2 mg/ml) and TMV were incubated for different times, at room temperature. The virus was then recovered by differential ultracentrifugation, dissolved in water, and inoculated on 16 half-leaves of *N. glutinosa*. The opposite control halves were inoculated with TMV which had been suspended in water and was treated similarly.

For most induced interference studies, Samsun NN tobacco plants were trimmed to two or three lower expanded leaves 3 days before use. The respective polyanion, or sterile double-distilled water as a control, was injected intercellularly into the opposite halves of six tobacco leaves, on two or three plants, as described previously (7). In additional control plants, sterile water was injected into one side of the leaves, and the opposite sides remained uninjected. In beans, the solutions were injected into the opposite primary leaves. Several hours later, the leaves were washed carefully with tap water. After varying intervals, the whole leaves of Samsun NN tobacco were inoculated with purified TMV (1-5 μg/ml). Bean seedlings were kept in the dark for 24 hr before inoculation with TMV (50-100 μg/ml). Lesions were counted after 4-7 days, and the interference percentage induced by the polyanion was calculated. The results were analyzed by the "sign test". Lesion size was determined by measuring two diameters, at right angles to each other, under a stereoscopic microscope equipped with an ocular micrometer. All the lesions (about 20) on two or three 15-mm discs, cut at random from each half-leaf, were measured, and the mean diameter was calculated. Those experiments in which water injections alone affected lesion number or size by more than 15% were not included in evaluating the results obtained with the polyanion.

Polyanions, except VME/MA and VME/MA_C were synthesized and characterized at Monsanto Co., St. Louis, Mo., and donated to us. VME/MA and VME/MA_C were products of GAF Corp. (General Analysis and Films, New York, N.Y.). Data on the materials used, structure or the polymer and molecular weights, are summarized in Table 1. No modifications of the compounds were made in our laboratory, and symbols for the polymers refer to the manufacturer's designations. The polyanions were dissolved in sterile, double-distilled hot water at 50-60 C, except VME/MA for which the temperature had to be raised to 80 C. After cooling, the solutions were adjusted to pH 6.5 with 1 M NaOH. Fresh solutions were prepared for each series of experiments.

Actinomycin D (Lyovac, Cosmogen, Merck, Sharp & Dohme) was injected into both halves of Samsun NN tobacco leaves. Control plants were injected similarly with sterile, double-distilled water, or with mannitol, at concentrations equal to those in the actinomycin D preparations. Additional controls were kept without actinomycin D or water injections.

RESULTS.—The polyanions did not inactivate TMV in vitro, even when incubated for 5-24 hr. In several cases, the recovered infectivity even exceeded that of the control.

Inhibition.—When the polyanions were mixed with TMV and inoculated on Samsun NN tobacco, *N. glutinosa*, or Scotia beans, varying degrees of inhibition were observed. Only preliminary experiments were done with S/MA, IB/MA, and OD/MA because they proved to be poor solutes and/or lethal to the plants. Results, expressed as percentage of inhibition on NN tobacco, and averages from 2-4 experiments, are summarized in Table 2. With EMA 31, 4 mg/ml were necessary to obtain a 75% inhibition. No effect on lesion size was observed, and no inhibition became evident when the polyanions were sprayed or rubbed on the leaves 2-3 hr after TMV inoculation.

Induced interference.—When half-leaves of Samsun NN tobacco were injected with the respective polyanion and challenge-inoculated 1 or 4 days later, a time-dependent reduction in lesion number was observed, especially with EMA 31 (Table 3). Succinic acid, a component of the EMA polymer, did not induce interference. With EMA's 1, 11, 21, VME/MA, and VME/MA_C, a significant reduction in lesion number was already observed after 24 hr. This seems to have been caused, in part, by competitive inhibition, as these materials were effective even at low concentrations (Table 2). However, they also induced an interference mechanism, as the degree of interference (lesion number) increased with time and because lesion size was reduced significantly, whereas no effect on size was noted in the competitive inhibition tests. Injecting EMA 1 4-5 hr before the challenge did not affect lesion number, although lesion size was reduced by 49%. A time-dependent reduction in lesion number was also observed when EMA 31 was injected into half-leaves of *N. glutinosa*, or into primary leaves of Scotia beans. In *N. glutinosa*, interference (lesion number) increased from 16 to 59% when challenged after 1 or 4 days, respectively; and in Scotia beans, from 19 to 67% when challenged after 2 or 4 days, respectively.

No interference was induced by injecting EMA 11, EMA 31, PAA, PMAA, VME/MA, or VME/MA_{es}, at 0.5 mg/ml, into cucumber cotyledons, a starch lesion host for TMV. No decreases in infectivity titers were observed when the polyanions were injected 1, 2, or 4 days before inoculation with TMV; infectivity was assayed 5 days after inoculation. Likewise, no decrease in virus titers was observed in two systemic hosts: *N. tabacum* 'Samsun' infected with TMV, and cucumber infected with cucumber mosaic virus (CMV). With the latter, both a susceptible cultivar (Bet Alpha) and a resistant one (Elem) were used. Cucumber cotyledons were injected with the above-mentioned polyanions (0.5 mg/ml) 1, 2, or 4 days before inoculation. Bet Alpha cotyledons were assayed 4-5 days after inoculation; and Elem cotyledons, 7-8 days after. Occasionally, a certain increase in CMV titer was noted in the resistant cultivar after polyanion treatment. Tobacco leaves were injected with EMA 31 (0.5 and 1.0 mg/ml) 4 days before inoculation, and assayed from 2 until 10 days after inoculation with TMV.

Because the time-dependent interference response was most pronounced with EMA 31, which did not inhibit TMV at 0.5 mg/ml, further studies were done, mainly with this material.

Development of induced interference after application of EMA 31.—The effects on lesion number and size after injecting EMA 31 into half-leaves of Samsun NN tobacco are summarized in Table 4. No visible damage to the plants was apparent even 15 days after injection. A time interval of 24 hr between applica-

TABLE 1. Data on structure and molecular weight of polyanions

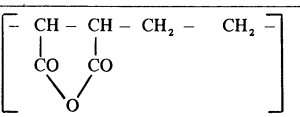
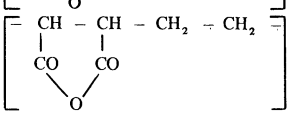
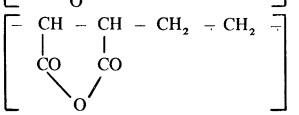
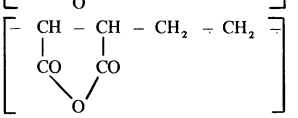
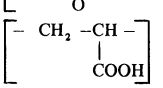
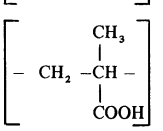
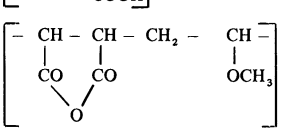
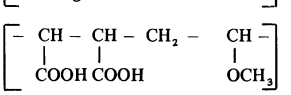
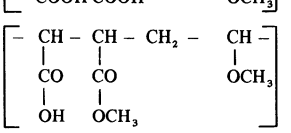
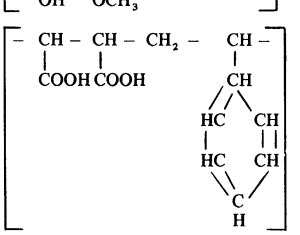
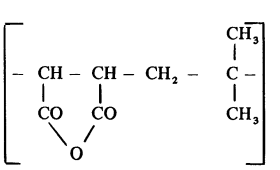
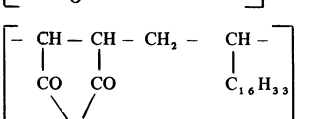
Polyanion	Designation	Repeating unit	Molecular weight
Ethylene/maleic anhydride	EMA 1		ca. 800
	EMA 11		2- 3,000
	EMA 21		20-40,000
	EMA 31		60-90,000
Polyacrylic acid	PAA		60-70,000
Polymethacrylic acid	PMAA		60-70,000
Vinyl methyl ether/maleic anhydride (Product of GAF Corp., Gantrez 169)	VME/MA		200,000
Vinyl methyl ether/maleic acid (Product of GAF Corp., Gantrez HyH)	VME/MA_c		200,000
VME/MA 0.5 methylester	VME/MA_{es}		200,000
Styrene/maleic anhydride	S/MA		60-70,000
Isobutylene/maleic anhydride	IB/MA		over 100,000
α – olefine octadecene/maleic anhydride	OD/MA		3- 4,000

TABLE 2. Inhibition of tobacco mosaic virus (TMV) lesion number on Samson NN tobacco by polyanions

Polyanion	Inhibition %[a] Concentration of polyanion (mg/ml) 0.5	1	2
EMA 1	12	43[c]	61[c]
EMA 11	35[c]	60[c]	72[c]
EMA 21	27[b]	58[c]	75[c]
EMA 31	0	16	28[b]
PAA	25	33[b]	56[c]
PMAA	11	32[b]	48[c]
VME/MA	31[b]	34[b]	39[c]
VME/MA_c	31[c]	47[c]	62[c]
VME/MA_{es}	42[c]	48[c]	

[a]Averages from two to four experiments, TMV at a final concentration of 5 μg/ml. Number of lesions on control half-leaves averaged 137.
[b]Significant at 5% level.
[c]Significant at 1% level.

TABLE 3. Induced interference to tobacco mosaic virus after injecting polyanions into half-leaves of NN tobacco[a]

Polyanion 0.5 mg/ml	Days between injection of polyanion and inoculation: 1 Interference % Lesion No.[b]	1 Size[c]	4 Interference % Lesion No.[b]	4 Size[c]
EMA 1	67[d]	44	80[d]	45
EMA 11	38[e]	41	79[d]	69
EMA 21	51[d]		82[d]	
EMA 31	28	41	88[d]	43
PAA	7.3	9	5	9
PMAA	−7	12	9.3	1.7
VME/MA	53[d]	41	74[d]	51
VME/MA_c	47[d]	53	79[d]	41
VME/MA_{es}	27	27	10	−2
Succinic acid (2 mg/ml)	8		−14	

[a]Averages from two to three experiments.
[b]Number of lesions on control half-leaves averaged 224.
[c]Lesions were measured 4 days after inoculation. Water-injected controls, 0% = 1.85 mm.
[d]Significant at 1% level.
[e]Significant at 5% level.

tion of the polyanion and TMV challenge was necessary for the development of interference, as expressed by a reduction in lesion numbers. However, effect on size became apparent even when EMA 31 was injected 3-4 hr before inoculation. This is not inconceivable, as effects on size may be noticed only after

TABLE 4. Induced interference development after injection of EMA 31 (0.5 mg/ml) into half-leaves of Samson NN tobacco

Time between EMA 31 injection and inoculation with TMV[a]	Interference % Lesion No.[b]	Lesion size[c]
3-4 h	0	21
10 h	0	–
1 day	31[d]	47
2 days	47[e]	65
3 days	78[e]	57
4 days	81[e]	59
5 days	85[e]	57
7 days	71[e]	–
10 days	86[e]	–
15 days	75[e]	–

[a]TMV (tobacco mosaic virus) at a final concentration of 5 μg/ml.
[b]Average from three to four experiments, 0% interference in water-injected controls = 179 lesions/half-leaf.
[c]Average from two to three experiments, lesions measured 5 days after inoculation, 0% interference in water-injected controls = 2.45 mm.
[d]Significant at 5% level.
[e]Significant at 1% level.

lesion appearance; i.e., about 48 hr after inoculation.

Lesion size was correlated with extractable infectivity. One hundred lesions were sampled from EMA 31 injected half-leaves and compared to a similar number from water-injected half-leaves. Each sample was homogenized in 3 ml water and assayed for infectivity on 16 half-leaves of *N. glutinosa*. In three experiments, relative infectivity, recovered from lesions collected from EMA 31-treated tissues, averaged 33 as compared with 100 for the control samples. No infectivity was recovered from tissues between lesions.

Reducing the concentration of EMA 31 to 100 μg/ml lowered interference to 53% when challenged after 4 days. With 50 μg/ml, no significant reduction in lesion numbers was observed. Interference increased to 78-88% when concentrations of 0.5-2 mg/ml were used.

Injecting EMA 31 into the basal parts of Samsun NN tobacco leaves did not induce systemic resistance in the upper, nontreated tissue.

Sensitivity of EMA 31-induced interference to actinomycin D.—The degree of interference was generally, though not always, reduced when actinomycin D (8-10 μg/ml) was applied close in time to the injection of the polyanion. Thus, injection of the antibiotic 24 hr before, together with, or 2-3 hr after, EMA 31 application,reduced interference to 38, 42, or 23%, respectively, as compared with 75% in the controls, the challenges inoculated 4 days after the inducing injection. These data are averages from 2-4 experiments for each treatment, including those where no significant effects of the antibiotic were noted. Two injections of the antibiotic (8 μg/ml)—1 and 2, or 1 and 3 days after EMA 31 application,

reduced interference to an average of 10% compared with 70% in the controls, when the challenges were inoculated 6-7 days after the inducing injection. When actinomycin D was given 4 days and the challenge inoculation 7 days after the inducing injection, no reduction in interference was observed.

DISCUSSION.—The above-tested polycarboxylates affect infection by TMV in two ways: they inhibit infection when present at the time of inoculation; and they induce resistance in the plant at various time intervals after application.

Inhibition during inoculation was most marked with VME/MA_{es} and least pronounced with EMA 31. With the latter, concentrations of 2 mg/ml or more were needed to obtain a significant degree of inhibition. Apparently, these polyanions compete with TMV during inoculation for cellular acceptor sites, in a way similar to that reported for polyglutamic or polyacrylic acid (11) and yeast-RNA (6).

Injection of EMA 1, 11, 21 and 31, VME/MA, and VME/MA_c into the leaves induced local interference, which differs from competitive inhibition. A time interval was needed and lesion size, as well as number, was reduced. In addition, the concentrations of the polyanion which induce resistance were generally lower than those required for inhibition. Especially with EMA 31, no inhibition was observed at a concentration of 0.5 mg/ml that potently induced interference.

The development of interference induced by EMA 31 was found to be sensitive to actinomycin D when the latter was applied close in time to the injection of the polyanion. The antibiotic was ineffective when given 4 days after the inducer. This suggests that for the development of interference, the transcription mechanism of the cell from DNA to RNA has to operate. Once the response is established, after 4 days, it is insensitive to the antibiotic. The fact that interference was not induced in two systemic hosts also strengthens the suggestion that the respective polyanions activate in Samsun NN tobacco, *N. glutinosa*, and Scotia beans that part of the genome responsible for localization, perhaps by a derepression process.

No interference was induced in cucumber cotyledons by any of the polycarboxylates tested. Further experiments are needed to explain why a starch lesion host responds to polyanions differently from a necrotic local lesion host. It could be speculated that differences in membrane permeability, or polyanion-caused release of compounds from the membrane, are involved.

Relating chemical structure of the polyanions to their capability to induce interference, it seems that those polyanions with maleic acid as the anionic component, but not acrylic acid, were effective. The EMA series seem to be potent inducers, irrespective of their molecular weights. However, the best distinction between the inhibitory and induced effect is observed with EMA 31. With this material, the time dependence of the induced effect is also most evident. The low-molecular weight EMA 1 induced strong interference already 24 hr after application at a concentration that did not inhibit TMV. A time interval of 4-5 hr between application and challenge was not sufficient. It is possible that due to its small size, EMA 1 is able to reach the site of action faster than the larger-sized EMA's. Alternatively, a different mechanism may be involved. Succinic acid, the monomeric equivalent to the EMA maleic acid polymer moiety, was found to be inactive.

VME/MA, anhydride and acid form, which in water are identical, are both inhibitors and inducers of interference. However, when the anionic component of the polymer is partially blocked-EMA/MA_{es}, the inducing, though not the inhibitory, capacity is almost completely abolished.

Polycarboxylates of the kind tested are known to induce resistance to viruses in animal cells. It is generally accepted that such antiviral activity is mediated through an interferon mechanism (2). Certain properties and structures of the polyanions necessary for the induction of interferon have been determined (2, 8). Among them, a molecular weight above 3,000 for MA-containing polymers is required, as well as a certain density of anionic charges. However, the possibility has also been proposed that the polycarboxylates combine electrostatically with virus or cellular receptors, thereby inhibiting virus attachment or release (3, 4).

There seems to be a certain analogy between interference induced by polyanions, yeast-RNA and poly I·poly C in plants, and interference, mediated through interferon, in animal tissues. However, conclusions about similar mechanisms in plants cannot be drawn before obtaining more data on the mode of action and association with a chemically defined substance developing in the plant.

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