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Section III. Plant viruses

Resistance to tobacco necrosis virus induced by salicylate in detached tobacco leaves

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Sodium salicylate reduced both the size and viral antigen content of non-self-limiting necrotic lesions produced by tobacco necrosis virus (TNV) in detached tobacco leaves during the partly localized reaction to virus. The antiviral effect of salicylate occurred at concentrations close to the limits of toxicity, and depended on the timing of administration. Both viral antigen accumulation and lesion size were strongly inhibited by a continuous supply of salicylate before or just after virus infection. Salicylate treatment did not prevent TNV accumulation when given after the establishment of infection or when arrested 24 h after TNV inoculation. Both procedures, however, did limit lesion enlargement. These results constitute a limit for the use of salicylate as a chemotherapeutic agent but do not exclude its use in limiting the pathogenetic effects of the virus. The salicylate treatments induced the formation of four pathogenesis-related proteins (PRs). The PRs formation was stimulated during the first 8 h of treatment and persisted for some time after the salicylate supply was discontinued. No correlation was found between the presence of PRs and the reduction of TNV accumulation: low salicylate concentrations (0.25 mM) inducing the formation of the PRs did not induce resistance against the multiplication and/or cell-to-cell spread of TNV.

tobacco necrosis virus; salicylate; chemotherapy; pathogenesis-related protein

Introduction

The infection of *Nicotania tabacum* L. cv White Burley with tobacco necrosis virus (TNV) results in a hypersensitive reaction characterized by the development of non-self-limiting necrotic lesions confined to the inoculated leaves and the acquisition of a systemic acquired resistance against further TNV infection. Four pathogenesis-related proteins (PRs) appear in inoculated and uninoculated leaves, but they are not associated with resistance against TNV multiplication [21].

PRs have been shown to be induced in several tobacco cultivars by administration of salicylates, which also induce resistance against tobacco mosaic virus (TMV) infection [1,27,28], but a direct correlation between the appearance of PRs and the inhibition of TMV multiplication or spread following salicylate treatments of tobacco leaves has not been demonstrated.

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In this paper, we present results showing the development of resistance against TNV infection in detached tobacco leaves after salicylate administration. The partly localized reaction induced by TNV in White Burley tobacco leaves is particularly useful in distinguishing between resistance against viral multiplication, resulting in the accumulation of less viral antigen, and resistance against cell-to-cell spread of the virus, resulting in the formation of smaller lesions.

Materials and methods

Plant materials and virus

N. tabacum cv White Burley plants were grown from seed in a greenhouse. In order to minimize variability due to leaf and plant age, we used plants and leaves whose age was determined by the plastochron index (PI) and leaf plastochron index (LPI) [25]. Plants having PI7 (5 leaves well developed) were transferred to a climatic chamber and preconditioned for 24 h at 22°C, 90 $\mu E \cdot m^{-2} \cdot s^{-1}$ of continuous Philips TL 55 fluorescent light, and 65% relative humidity. The leaves having LPI3 (i.e. serial number 4) were detached under distilled water and their petioles immersed in the salicylate solutions to be tested. The leaves were then kept under the environmental conditions described above.

TNV was multiplied and partially purified as already described [21]. The inoculation procedure was performed by immersing the 1.2 mm tip of a glass rod in the TNV suspension supplemented with 3% celite. Seven to 10 well-defined lesions per leaf were produced by rubbing the tip on the leaf lamina (standard inoculation). Preliminary experiments had confirmed that with this procedure the lesions developed homogeneously.

Salicylate treatment

The detached leaves were treated with sodium salicylate (Merck, Darmstadt, W. Germany) dissolved in sterile distilled water. The maximum tolerated concentration was 0.75 mM but, although no severe symptoms of toxicity were apparent, leaves treated with this concentration usually lost chlorophyll more rapidly than control leaves. Higher concentrations were toxic and produced chlorosis, necrosis and leaf wilting, depending on concentration.

At different time intervals before, during and after salicylate application, the leaves were inoculated to assay the resistance against TNV. Detached leaves that had been immersed in distilled water were included as controls.

Determination of induced resistance

The induction to resistance to TNV was assessed in single, complete lesions by (1) calculating the percent reduction in lesion area and (2) determining the amount of TNV antigen by the ELISA.

ELISAs were performed according to Clark and Adams [6] with minor modifications. An antiserum against TNV having a dilution end-point of 1/512 in the agar gel double diffusion test, and showing no reaction with healthy tobacco sap, was used. Antibodies were purified with Protein-A-Sepharose (Pharmacia Fine Chemicals), according to manufacturer's instructions. Single lesions, whose areas were determined, were taken from different leaves, ground in a mortar with phosphate buffer saline, pH 7.2, containing 0.05% Tween 20 (PBS-Tw). The homogenate was diluted as necessary with the same buffer. The quantitative assays were performed in plates (Dynatech Microtiter M 129 A) in comparison with twofold dilutions of a standard purified suspension of known concentration. The TNV was stored at -20°C in an equal volume of glycerol. Each sample was assayed in three wells. Peripheral wells were not used, to avoid the plate edge effect.

Protein extraction and electrophoresis

At different time intervals after salicylate administration, treated and control leaves were harvested and the midrib discarded. Soluble proteins were extracted at 4°C by grinding 1 g of fresh leaf tissue with 1 or 2 ml citrate–phosphate buffer, pH 2.8 [2]. The homogenates were centrifuged at 105 000 × *g* for 60 min and the supernatants were immediately subjected to electrophoresis. Aliquots of 100 µl were applied to the gels. Electrophoresis, gel staining and destaining were carried out as previously described [19].

Results

Uptake of salicylate by the detached leaves

Studies on bean leaves have shown that salicylates affect the transpiration rate by inducing stomatal closure [14,15]. Since transpiration influences water and solute uptake, a preliminary experiment was done to ascertain the influence of the salicylate treatment on the uptake of fluid in the tobacco leaves.

The petioles of four series of detached leaves were immersed in calibrated vials containing 25 ml of 0, 0.25, 0.5 and 1 mM salicylate solutions, respectively. The volume of the solutions was restored daily to 25 ml and the difference considered as uptake by the leaves. After 24 h, the volume of water taken up by leaves was significantly greater in water than in salicylate solution (Table 1). The differences were no longer maintained during the subsequent days, and, starting from the second day, no significant difference was noted among the four series of treatments. The amount of salicylate taken up daily by the leaves decreased linearly with the time (Fig. 1).

Antiviral effects of salicylate

Tobacco leaves were inoculated with TNV, detached just after inoculation and their petioles immersed for 6 days in salicylate solutions of different concentrations,

TABLE 1

Fluid uptake by detached tobacco leaves during the first 24 h of administration.

Treatment (mM of salicylate in immersion fluid)	Uptake[a] (ml·g^{-1} fresh wt)
0	4 (0.06)
0.25	3 (0.07)
0.5	2.5 (0.08)
1.0	1.9 (0.06)

[a] Each value is the average of 5 independent determinations. SD in brackets. All results were significantly different from the control ($P \leqslant 0.001$).

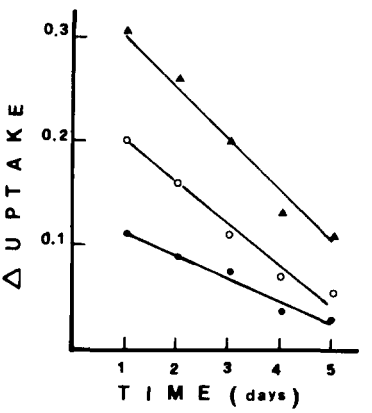

Fig. 1. Rate of sodium salicylate uptake (△ = uptake) expressed as mg taken up daily/g fresh wt of tobacco leaves. Petioles were immersed in salicylate solutions at concentrations of ● = 0.25 mM; ○ = 0.5 mM; ▲ = 1 mM.

including distilled water as control. At the end of this 6-day immersion treatment, the areas of single lesions were measured and their viral antigen content assayed (Table 2). No resistance was induced by low salicylate concentrations (0.25 mM or less); higher concentrations reduced both viral antigen content and lesion size: 0.75 mM salicylate treatment gave the best results. The effects were not greater following 1 mM salicylate treatment, which caused evident symptoms of toxicity.

When the leaves were pretreated for 24 h in 0.75 mM salicylate solution, inoculated and then kept 5 days in the solution, high levels of resistance were induced (Table 3). However, these levels were not different from those shown in Table 2, suggesting that pre-inoculation treatments do not offer particular advantages when compared with treatment just after inoculation.

Keeping detached, inoculated leaves in salicylate solution for 24 h and then transferring them to distilled water had a variable effect on viral antigen content (Table 4) suggesting that complete resistance to TNV infection is acquired only following

TABLE 2

Effect of continuous salicylate treatment, started after virus inoculation, on TNV infection in detached tobacco leaves[a]

Treatment (mM of salicylate in immersion fluid)	Antigen amount		Lesion area	
	ng · mm^{-2}	Reduction (%)	(mm^2)	Reduction (%)
0	64.3 (19.0)		48.2 (4.8)	
0.25	60.2 (17.8)	6.4	43.3 (6.5)	10.1
0	71.3 (23.4)		60.0 (5.4)	
0.25	68.9 (23.1)	3.4	56.3 (3.8)	6.1
0	54.8 (17.8)		83.2 (9.0)	
0.25	56.2 (15.7)	-2.6	78.9 (4.8)	5.2
0	56.7 (18.5)		40.8 (4.5)	
0.5	41.7 (11.3)	26.5*	22.3 (6.7)	45.3**
0	75.7 (9.6)		27.6 (8.7)	
0.5	48.8 (13.8)	35.5**	18.1 (4.4)	34.4*
0	72.5 (13.4)		52.4 (14.2)	
0.5	57.8 (9.5)	20.3*	13.3 (2.1)	74.6**
0	94.0 (35.6)		121.9 (28.9)	
0.75	2.2 (0.7)	97.7**	12.9 (4.5)	89.4**
0	69.0 (27.4)		77.9 (18.5)	
0.75	3.3 (1.3)	95.2**	22.3 (5.5)	71.4**
0	52.4 (21.5)		106.7 (32.8)	
0.75	6.2 (4.6)	88.2**	8.4 (2.4)	92.1**
0	142.1 (32.0)		46.6 (7.7)	
1.0	23.2 (18.1)	83.7**	9.1 (3.7)	80.5**
0	79.0 (40.0)		49.1 (6.3)	
1.0	7.2 (5.8)	90.9**	5.6 (1.4)	88.6**

[a] Detached leaves were inoculated with virus and immersed for 6 days in salicylate solutions or distilled water. Single lesions were then measured and tested for TNV-antigen content. Each value is the average for 7 lesions (SD in brackets). Significant difference (Student's *t*-test) between control and treated leaves at $P=0.05$ (*) or $P=0.01$ (**).

prolonged salicylate supply. The erratic results were not apparently due to differences in leaf age, environmental conditions or the absolute size of the lesions produced in different batches of leaves. On the contrary, lesion area was significantly reduced by this treatment (Table 4), indicating that resistance against cell-to-cell spread of TNV was induced.

Analogous patterns emerged from experiments performed to test the effect of salicylate on well-established infections (Table 5). Inoculated leaves were detached 3 days after inoculation (i.e. about 24–30 h after the appearance of the lesions), and their petioles immersed in 0.75 mM salicylate for 6 days. The TNV antigen content, relative

TABLE 3

Effect of a 24 h pretreatment with salicylate (0.75 mM) on TNV infection in detached tobacco leaves[a]

Treatment	Antigen amount		Lesion area	
	($ng \cdot mm^{-2}$)	Reduction (%)	(mm^2)	Reduction (%)
Water	41.0 (21.0)		57.8 (7.8)	
Salicylate	4.1 (1.0)	90.0*	11.6 (1.7)	79.9*
Water	54.1 (17.0)		56.7 (6.8)	
Salicylate	3.6 (1.7)	93.3*	10.4 (2.1)	81.7*
Water	40.0 (13.5)		79.0 (7.8)	
Salicylate	3.9 (1.8)	90.3*	7.7 (1.4)	90.3*

[a] Single lesions were measured and tested for TNV-antigen content 5 days after virus inoculation. Each value is the average for 7 lesions (SD in brackets). Significant difference (Student's t-test) between control and treated leaves at $P = 0.01$ (*).

TABLE 4

Effect of a 24 h salicylate treatment (0.75 mM), started after virus inoculation on TNV infection in detached tobacco leaves[a]

Treatment	Antigen amount[b]		Lesion area[b]	
	($ng \cdot mm^{-2}$)	Reduction (%)[b]	(mm^2)	Reduction (%)
Water	135.0 (20.9)		60.1 (9.0)	
Salicylate	76.2 (24.1)	43.6*	18.9 (2.0)	68.5*
Water	134.1 (63.0)		86.4 (12.6)	
Salicylate	135.9 (36.2)	-1.3	29.4 (5.8)	66.0*
Water	116.9 (13.8)		58.3 (9.1)	
Salicylate	126.1 (36.8)	-7.9	26.7 (5.1)	54.2*
Water	90.2 (24.9)		63.9 (12.8)	
Salicylate	113.3 (24.0)	-25.6	25.3 (6.1)	60.4*
Water	139.0 (44.0)		61.0 (11.5)	
Salicylate	187.4 (36.3)	-34.8	28.6 (6.7)	53.1*

[a] Leaves were inoculated while still attached, detached and their petioles immersed for 24 h in salicylate solution or distilled water; they were then transferred to water for 5 days. Single lesions were then measured and tested for TNV-antigen content. Each value is the average for 7 lesions (Standard deviations in brackets). Significant difference (Student's t-test) between control and treated leaves at $P = 0.01$ (*).

[b] Negative values indicate an increased susceptibility to TNV of the treated leaves.

TABLE 5

Effect of continuous 0.75 mM salicylate treatment, started 3 days after establishment of TNV infection in detached tobacco leaves[a]

Treatment	Antigen amount[b]		Lesion area[b]	
	($ng \cdot mm^{-2}$)	Reduction (%)[b]	(mm^2)	Reduction (%)
Water	122.8 (51.0)		68.2 (18.0)	
Salicylate	98.1 (28.7)	20.1	32.9 (10.5)	51.8*
Water	127.0 (36.3)		122.1 (39.7)	
Salicylate	118.3 (59.4)	6.9	45.3 (18.3)	62.9*
Water	125.2 (26.7)		125.0 (22.6)	
Salicylate	131.1 (44.3)	-4.7	49.2 (11.5)	60.6*
Water	92.9 (23.4)		71.8 (27.2)	
Salicylate	110.0 (23.0)	-18.4	38.9 (12.3)	45.8*
Water	126.0 (30.1)		75.0 (9.4)	
Salicylate	185.9 (91.9)	-47.5	44.1 (11.7)	41.2*

[a] Leaves were inoculated while still attached, detached 3 days later and their petioles immersed in the salicylate solution or distilled water for 5 days. Single lesions, were then measured and tested for TNV antigen content. Each value is the average for 7 lesions (SD in brackets). Significant difference (Student's t-test) between control and treated leaves at $P = 0.01$ (*).

[b] Negative values indicate an increased susceptibility to TNV of the treated leaves.

to control, was on the average not reduced whereas enlargement of the lesions was significantly inhibited.

Effects of salicylate on PRs

Soluble proteins extracted from salicylate-treated leaves showed important qualitative differences from those of water-treated controls. Four new protein bands were apparent after 48 h (or more) of continuous administration of 0.25 mM (Fig. 2) or more concentrated salicylate solutions. The electrophoretic mobilities of the PRs were identical to those of the PRs accumulated during the hypersensitive reaction to TNV (Fig. 3).

A 24-h supply of 0.75 mM salicylate induced the appearance of the PR-1a and traces of the other three PRs. Eight hours of 0.75 mM salicylate administration (minimum inductive period), followed by post-inductive periods during which the leaves were kept in water, were sufficient to stimulate the formation of the PRs. PR-1a appeared after 16 h of the post-inductive period in water, while 40 h were necessary for the appearance of the other proteins. Their amounts increased with the length of the post-inductive period (Fig. 4).

Comparable treatments with water or 0.75 mM NaCl solution did not induce any PR.

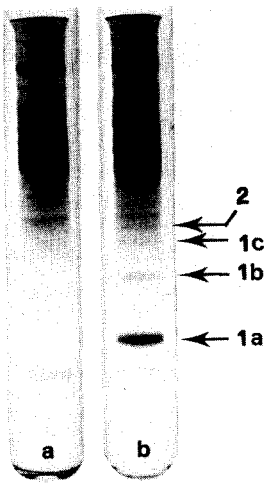

Fig. 2. Electrophoresis in 10% polyacrylamide gels of soluble proteins extracted at pH 2.8 from tobacco leaves treated for 48 h with (a) water and (b) 0.25 mM Na salycilate. The new bands (PRs) are indicated by arrows.

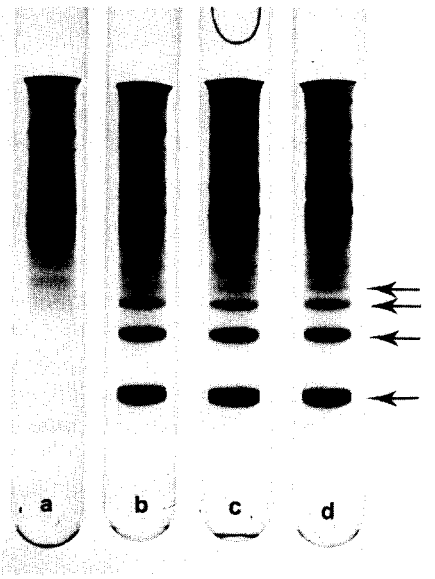

Fig. 3. Electrophoresis in 10% polyacrylamide gels of soluble proteins extracted at pH 2.8 from tobacco leaves submitted to different treatments. (a) 4 days in water; (b) 4 days in 0.75 mM salicylate solution; (c) 5 days after inoculation with TNV; (d) coelectrophoresis of the extracts from (b) and (c).

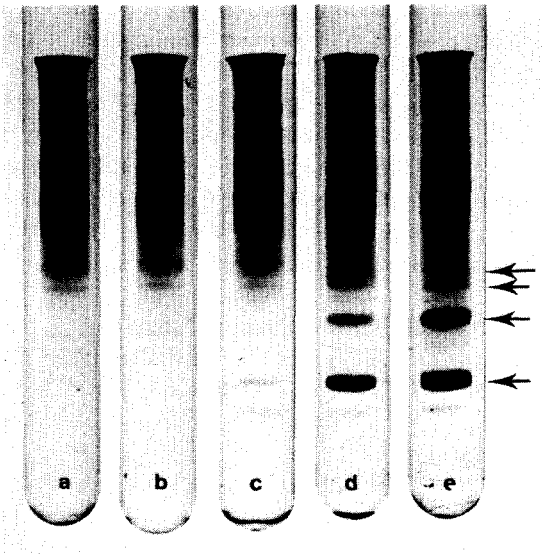

Fig. 4. Electrophoresis in 10% polyacrylamide gels of soluble proteins extracted at pH 2.8 from tobacco leaves kept: (a) 8 h in water; (b) 8 h in 0.75 mM salicylate; (c) 8 h in 0.75 mM salicylate + 16 h in water; (d) 8 h in 0.75 mM salicylate + 40 h in water; (e) 8 h in 0.75 mM salicylate + 5 days in water. Each gel was loaded with an amount of protein equivalent to 75 mg fresh weight of leaf. Note the progressive accumulation of the PRs (arrows) with the post-inductive time periods in water.

Discussion

It has previously been demonstrated that salicylates decrease lesion number and size in different host-virus systems [1,20,27,28]. The present study confirms that salicylates can reduce lesion size and viral antigen content in tobacco leaves responding to TNV infection with non-self-limiting necrotic local lesions. The antiviral properties of salicylates are not strictly confined to plant viruses, because in at least two animal systems, they have been described to inhibit viral infection [13,23].

The antiviral effect of salicylates was apparent by the inhibition of TNV accumulation (presumably by blocking its replication or assembly) and /or lesion enlargement (presumably by blocking cell-to-cell spread of the virus).

TNV accumulation was strongly inhibited by continuously supplying salicylate, starting before or just after virus infection. If the administration was interrupted 24 h after infection, the effect on TNV accumulation was not seen. Ben-Tal and Cleland [5] have reported that salicylic acid is rapidly converted in the cells to a bound form, apparently sequestered within the vacuole. This finding might explain the observation that TNV accumulation is efficiently countered only by continuous salicylate treatment. A persisting resistance against TMV accumulation has recently been described [27] in tobacco plants treated by a salicylate spray 7 days before virus inoculation. It is possible that salicylate delivered by this method is taken up very slowly by the leaf cells, mimicking a continuous supply of the drug.

Treatment with salicylate for 24 h induced an antiviral state limiting the cell-to-cell spread of TNV. This finding may be of interest as a possible use of salicylates in the prophylaxis of viral infections in plants.

Salicylate failed to inhibit TNV accumulation when supplied after the establishment of infection, but did inhibit lesion enlargement. This result could constitute a severe limit for the potential use of the drug as a chemotherapeutic agent, although its use as a symptomatic agent limiting the pathogenetic effects of viral infections can not be excluded. The properties of salicylate are reminiscent of those of methyl benzimidazol-2-yl-carbamate, which also inhibits symptom development but not viral replication, when administered after infection [26].

The mechanism of antiviral action of the salicylates in plants is unknown. It has been speculated that PRs, which appear during the induction of resistance [1,27,28] are involved. A direct causal relation between their presence and the reduction of TNV antigen content is not supported by our experiments: treatment at a dose of 0.25 mM failed to induce resistance against TNV but did induce the formation of PRs.

The production of the PRs in stressed leaves is considered the result of activation of silent mRNAs present in unstressed leaves [9]. Salicylate might stimulate conversion of the untranslatable form of mRNAs to a translatable state, this is consistent with the observation that the stimulus persists even after salicylate application is discontinued, resulting in a progressive accumulation of PRs.

In plants, salicylate causes important physiological changes in membrane permeability [11,12,22], ion uptake [10,12] and stomatal closure [14,15]. Salicylic acid is also capable of inducing short-day flowering of long-day plants [7] and this action has tentatively been associated with its chelating properties [17]. The effect on flowering is not inductive and ceases almost immediately when salicylate is removed [8]. Some of these effects of salicylates are also seen in animals. For example, they affect membrane permeability of molluscan neurons [3,16]. The strong complexes formed with Mn and Fe [18] might explain the inhibitory effect on enzymes like RNA polymerase or the uncoupling action on oxidative phosphorylation reactions preventing ATP production [24].

In plants, one or more of the above reported effects may be involved in the induction both of stress and viral resistance. Stress induction, evidenced by increased electrolyte leakage, ethylene evolution, peroxidase activity increase, etc., has been described as a common feature among all agents inciting plant resistance to pathogens [4]. At present, however, nothing is known of the biochemical basis common to the stress condition induced by salicylates and interference with multiplication and spread of viruses.

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