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Comparison of the effects of salicylic acid and ethephon with virus-induced hypersensitivity and acquired resistance in tobacco

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Abstract

The induction of a hypersensitive reaction in 'Samsun NN' tobacco by tobacco mosaic virus (TMV) at 20 °C leads to the development of both localized and systemic acquired resistance, and is associated with the appearance of pathogenesis-related proteins (PR's) and large increases in peroxidase activity and ethylene production. Salicylic acid (SA) induced a similar resistance in treated plant parts and occasionally also in untreated upper leaves of plants of which three lower leaves had been injected. SA also induced the same four PR's, but these were confined to the treated leaves. Thus, the connection between the presence of PR's and the reduction of TMV multiplication and spread may not be direct.

In contrast to TMV, SA did not stimulate ethylene production and hardly increased peroxidase activity. Induction of acquired resistance and PR's by SA developed equally well at 20 °C and at 32 °C. However, pricking leaves with needles moistened with the ethylene-releasing compound ethephon mimicked TMV infection in inducing acquired resistance and PR's in both treated and untreated leaves at 20 °C, but not at 32 °C. Ethephon increased peroxidase activity at both temperatures, but only at 20 °C dit it induce changes in both the anodic and the cathodic isoenzymes that were similar to those induced as a result of TMV infection. SA induced PR's and reduced TMV multiplication in 'Samsun' tobacco, and inhibited virus spread in 'Samsun NN' at 32 °C.

These observation indicate that neither the induction of PR's, nor the development of acquired resistance is temperature-sensitive. On the other hand, the effects of ethephon are temperature-sensitive in the same way as the hypersensitive response to TMV. It can thus be hypothesized that ethylene, produced naturally during the hypersensitive reaction of tobacco to TMV, leads to the temperature-sensitive synthesis or release of a presumably benzoic acid-type compound that functions as the natural inducer of PR's and acquired resistance. Although vanillic acid has been shown to accumulate in hypersensitively reacting tobacco leaves, it produced none of the effects of SA, and thus cannot be the natural inducer.

Additional keywords: Nicotiana tabacum, tobacco mosaic virus, local lesions, peroxidases, pathogenesis-related proteins, ethylene, temperature effects.

Introduction

The formation of necrotic local lesions on tobacco varieties that react hypersensitively to tobacco mosaic virus (TMV) is accompanied by a large burst of ethylene (Gáborjá-

nyi et al., 1971; Pritchard and Ross, 1975; De Laat et al., 1981). Subsequently, peroxidase activity increases and a new anodic isoenzyme appears (Simon and Ross, 1971; Van Loon and Geelen, 1971), new protein components, recently designated as pathogenesis-related proteins (PR's) become evident (Van Loon and Van Kammen, 1970; Gianinazzi et al., 1970; Antoniw et al., 1980), and resistance to further virus infection is acquired by both the inoculated and the noninoculated leaves (Ross, 1961a, 1961b, 1966). Treatment of tobacco leaves with the ethylene-releasing compound ethephon (2-chloroethylphosphonic acid) induces all these changes in the absence of virus, thus mimicking the effect of TMV infection. It has been proposed, therefore, that abundant ethylene synthesis during pathogenesis is a causative factor in redirecting the metabolism of hypersensitively reacting tobacco plants (Van Loon, 1977).

Increased peroxidase activity is not directly related to acquired resistance (Van Loon, 1976a), but a distinct correlation does exist between acquired resistance and the presence of PR's (Kassanis et al., 1974; Van Loon, 1975b). This correlation is strengthened by observations that polyacrylic acid and acetylsalicylic acid (aspirin) induce both the appearance of PR's and resistance to infection in 'Xanthi-nc' tobacco (Gianinazzi and Kassanis, 1974; White, 1979; Antoniw and White, 1980). Aspirin, as well as salicylic acid (SA) and benzoic acid, also induces both PR's and resistance in 'White Burley', 'Samsun' and 'Samsun NN' tobacco, whereas polyacrylic acid is ineffective in these cultivars (White, 1979; Antoniw and White, 1980).

Because SA is not considered to be a natural constituent of tobacco leaves, its mode of action was further investigated. Both treated and untreated leaves were analyzed for changes in protein constitution and peroxidase activity and for ethylene production, in order to compare the effects of SA with the alterations occurring after ethephon treatment or TMV infection. In earlier work on the effects of aspirin the reduction in numbers of lesions formed following inoculation with TMV has mainly been used as a measure of induced resistance, although it was noted that the few lesions that did occur, were very small (White, 1979; Antoniw and White, 1980). As acquired resistance induced by TMV is primarily characterized by smaller lesion size (Ross, 1966), and, in addition, the presence of virus-induced PR's has so far always been associated with a reduction in TMV lesion spread, the effect of SA treatment on lesion size was given particular attention.

In 'Xanthi-nc' tobacco reacting hypersensitively to TMV, methoxylated phenylpropanoids start to accumulate after local lesions become apparent. These phenols include vanillic acid, a benzoic acid derivative that appears to be absent from uninfected tobacco leaves (Tanguy, 1970; Tanguy and Martin, 1972). Since vanillic acid (VA) is structurally related to SA, it was also tested whether this compound could function as the natural inducer of PR's and acquired resistance in tobacco.

Materials and methods

Plant material. Tobacco (*Nicotiana tabacum* L.) cultivars Samsun NN and Samsun were grown from seed in a growth chamber at 18-20 °C (Van Loon, 1976a), and in a greenhouse at ambient temperature with a minimum of 19 °C (De Laat et al., 1981), respectively. For experiments at 32 °C, at least one day before treatment plants were transferred to a growth cabinet at 31-33 °C with standard conditions of light and rela-

tive humidity. Temperature and relative humidity were continuously monitored to be certain that the conditions had been adequately maintained, and especially that temperature had never dropped below 30 °C.

Treatments. Solutions of 1 mM SA or VA, adjusted to pH 6.5 with KOH, were injected into the intercellular space (Van Loon and Van Kammen, 1970) of three fully expanded leaves of intact 9-to-11-week-old 'Samsun NN' plants. Untreated plants, plants injected with water, plants pricked with needles moistened with 0.3 M ethephon (Van Loon, 1977) or water, and plants inoculated with a purified suspension of TMV W U1 (10 mg l^{-1}) (Van Loon and Van Kammen, 1970) or water served as references. Alternatively, selected leaves were detached and allowed to take up 2 ml of solution from individual vials through their petioles (Van Loon, 1979). Afterwards, the leaves were incubated in large Petri dishes on wet filter paper under standard conditions of light and temperature. Leaf discs, 25 mm in diameter, were floated on solutions or water under similar conditions. In some experiments with 'Samsun NN', and in all experiments with 'Samsun', solutions of SA were sprayed on both upper and lower surfaces of selected leaves to the point of run-off, or watered on the soil. Occasionally, SA solutions were inoculated on the leaves, using carborundum as an abrasive.

Analysis of PR's. Seven or 14 days after treatment, both treated and untreated leaves were harvested and divided along the midrib. One set of leaf halves was homogenized at 2 °C in 1.25 vol. (v/w) of standard phosphate-citrate buffer (pH 3.0) containing 0.5 M NaCl and 0.1% (v/v) mercaptoethanol (Van Loon, 1976b). The homogenate was centrifuged according to Van Loon and Van Kammen (1970); the resulting supernatant was dialysed successively against 100 vol. (v/v) of 5 mM Tris-glycine buffer (pH 8.6) and 50 vol. (v/v) of the same buffer containing 0.5 M sucrose, and centrifuged again for 30 min at 30 000 *g*. Aliquots of 0.5 ml of the final supernatant, designated as the pH 3-soluble protein fraction, were subjected to electrophoresis in 10% polyacrylamide gels as described previously (Van Loon and Van Kammen, 1970; Van Loon, 1976b). Gels were stained with a saturated solution of amido black in 0.3 M trichloroacetic acid and destained by repeated washings in 7% acetic acid (Van Loon, 1973).

Determination of peroxidase activity and isoenzyme patterns. The other set of leaf halves was extracted with 1.25 vol. (v/w) of a buffer (pH 8.0) containing 0.1 M Tris-HCl, 0.5 M sucrose, 5.7 mM ascorbic acid and 6.3 mM cysteine. The extract was centrifuged as described above. Peroxidase activity in the resulting total soluble protein fraction was determined spectrophotometrically with guaiacol as the hydrogen donor, and polyacrylamide gel electrophoresis of anodic and cathodic peroxidase isoenzymes was performed as described earlier (Van Loon, 1976a, 1979).

Tests for acquired resistance. Localized effects of SA or VA tested for by inoculating as well as appropriate control leaves with TMV. In another type of experiment, plants were trimmed to three consecutive young, expanding leaves, inoculated with TMV (1 mg l^{-1}), and sprayed with SA, VA or water 12 h after inoculation.

To test for systemic resistance, either the second, third and fourth, or the fourth,

fifth and sixth leaves above the ones treated 7 or 14 days previously with SA, VA, ethephon, or TMV, as well as the corresponding leaves from control plants, were inoculated with TMV (1 mg l^{-1}). 'Samsun NN' plants treated at 32 °C, were returned to 20 °C within an hour after challenge inoculation.

Seven days after inoculation lesion sizes were measured with a stereoscopic microscope equipped with an ocular micrometer at an enlargement of 10 times, as described previously (Van Loon, 1976a). Differences were statistically analysed using Student's *t*-test.

The extent of TMV multiplication in 'Samsun' plants was measured by purifying the virus and determining its concentration spectrophotometrically (Van Loon and Dijkstra, 1976). The infectivity of the virus in both a sample from the original crude extract and in the purified preparation was then assayed on leaf halves of *N. glutinosa* at a concentration corresponding to 5 mg TMV l^{-1}.

Measurement of ethylene production. Detached leaves were incubated in water-locked 750-ml Petri dishes on wet filter paper under standard conditions of light and temperature. Ethylene accumulated in the dishes over the past 24 h was determined daily by withdrawing 1-ml gas samples through a sealed hole in the lid and injecting these into a gas chromatograph equipped with an alumina column and a flame ionization detector (De Laat et al., 1981). After measurement, the dishes were flushed with fresh air. Corrections were made for the amount of ethylene present in ambient air.

Chemicals. Salicylic acid (Analar) was obtained from BDH, Poole, England, vanillic acid from Fluka, Buchs, Switzerland, and ethephon (Amchem 68-250) from Amchem Products, Ambler, PA., USA. All solutions were made up in deionized water, sterilized by filtration through a Millipore-Q water purification system.

Results

Effects of SA, ethephon and TMV on PR's and peroxidase. Of the four PR's known to occur in virus-infected tobacco plants, aspirin has been reported to induce PR 1a, b and c, but not PR 2 in 'Samsun NN' tobacco (White, 1979; Antoniw and White, 1980). However, as shown in Fig. 1, in leaves injected 7 days previously with SA, all four new protein components were present, the electrophoretic protein pattern being both qualitatively and to a large extent quantitatively similar to the patterns resulting from pricking with ethephon or inoculation with TMV. Noteworthy less PR 1c was induced after treatment with either SA or ethephon than as a result of TMV infection. All four PR's were similarly induced in detached leaves that had taken up SA through the petiole (Fig. 1), in leaf discs floating on SA solution, and in whole plants that were either sprayed on the leaves, or watered with SA solution on the soil (results not shown). In contrast, no PR's were induced when VA was injected, sprayed on the leaves, or used as incubating solution for leaf discs (Fig. 1).

In leaves injected with SA, the activity and isoenzyme patterns of peroxidase were not substantially different from those of corresponding leaves of untreated control plants (Table 1, Figs. 2 and 3). A minor increase in peroxidase activity was of the same order of magnitude as the one occurring as a response to the injury resulting from injecting water. In some experiments, injection with SA caused more injury around the

Fig. 1. Electrophoretic patterns in 10% polyacrylamide gels of pH 3-soluble proteins from 'Samsun NN' tobacco leaves: (from left to right) untreated control, leaf discs floated on vanillic acid solution, leaves injected with salicylic acid solution, pricked with ethephon, inoculated with TMV, and detached leaf after uptake of salicylic acid solution through the petiole.

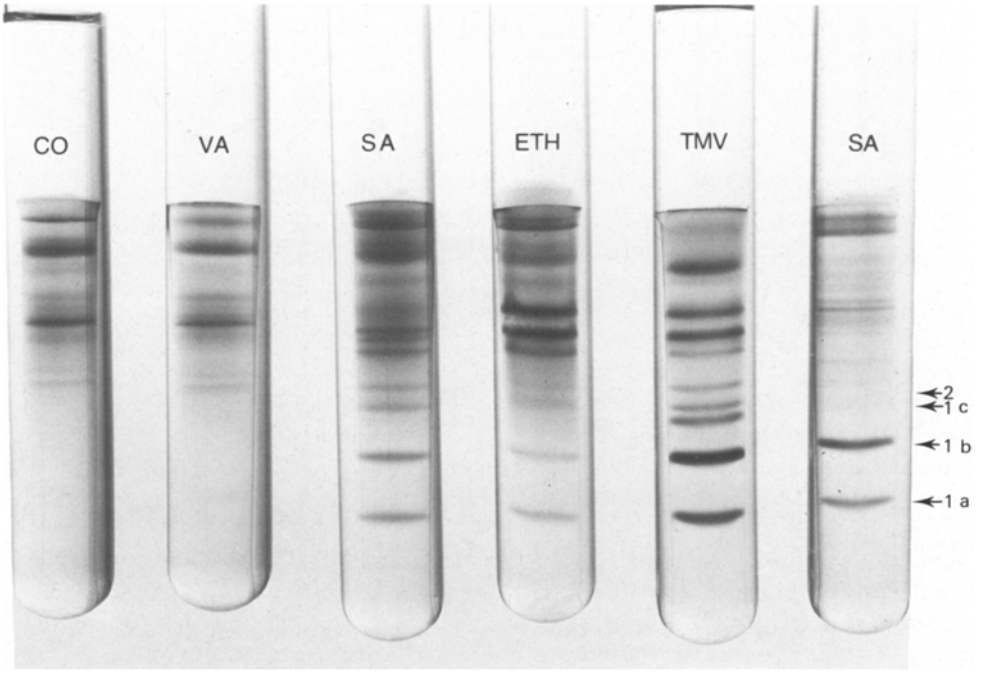

Fig. 1. Elektroforesepatronen in 10% polyacrylamidegels van de bij pH 3 oplosbare eiwitten uit 'Samsun NN'-tabaksbladeren: (van links naar rechts) onbehandelde controle, bladponsjes drijvend op een oplossing van vanillinezuur, bladeren ingespoten met een oplossing van salicylzuur, geprikt met naalden gedoopt in een oplossing van ethefon, geïnoculeerd met TMV, en afgesneden blad na opname van een oplossing van salicylzuur door de bladsteel.

Table 1. Relative peroxidase activities[a] of treated lower and untreated upper leaves from differently treated 'Samsun NN' tobacco plants.

Treatment	Peroxidase activity	
	treated leaves	untreated leaves
Untreated control	100	100
Water	115 ± 3	107 ± 11
Salicylic acid	124 ± 27	116 ± 21
Ethephon	585 ± 370	498 ± 199
TMV	349 ± 121	269 ± 94

[a] Average peroxidase activity (mean ± s.d.) as percentage of enzyme activity in corresponding untreated control leaves.

Tabel 1. Relatieve peroxidaseactiviteiten van behandelde onder- en onbehandelde bovenbladeren van verschillend behandelde 'Samsun NN'-tabaksplanten.

Fig. 2. Electrophoretic patterns in 7.5% polyacrylamide gels of anodic peroxidases from tobacco leaves: (from left to right) untreated control, injected with water, injected with salicylic acid solution, pricked with ethephon, and inoculated with TMV. The position of the new isoenzyme is indicated by the arrow.

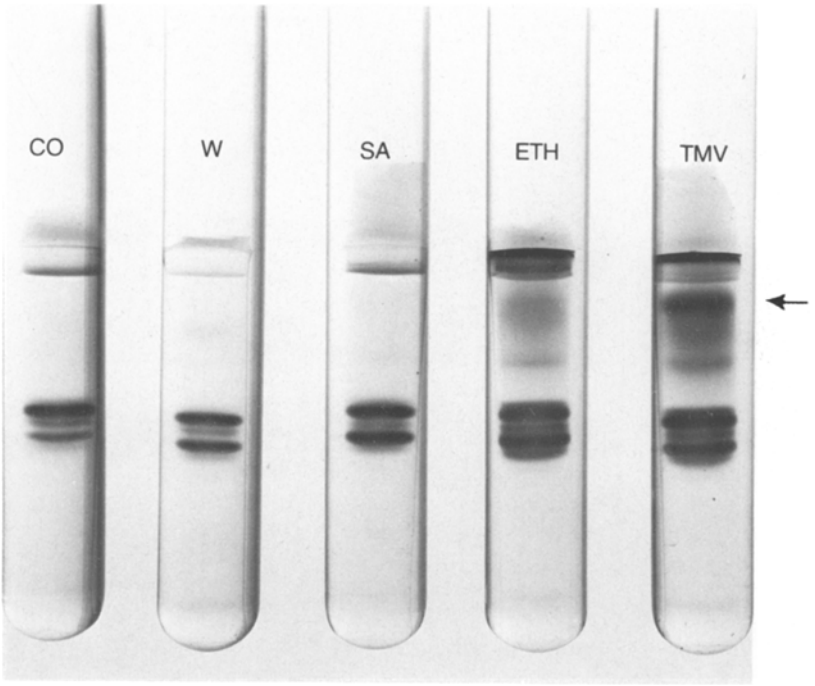

Fig. 2. Elektroforesepatronen in 7,5% polyacrylamidegels van de anodische peroxidasen uit tabaksbladeren: (van links naar rechts) onbehandelde controle, ingespoten met water, ingespoten met een oplossing van salicylzuur, geprikt met naalden gedoopt in een oplossing van ethefon, en geïnoculeerd met TMV. De positie van het nieuwe isoenzym is aangeduid met de pijl.

injection sites than injection with water, as reflected by increased activity of both anodic and cathodic isoenzymes and the manifestation of an additional, more rapidly migrating cathodic zone of activity (Fig. 3). Similar effects were evident when SA was applied as a single spray on the leaves or by continuous watering on the soil, the relative peroxidase activities being 111 and 131, respectively. These results indicate that SA itself causes a slight increase in peroxidase activity.

In contrast, both ethephon and TMV caused a very large, comparable increase in peroxidase activity, due to large increases in most of the isoenzyme bands, the appearance of a new anodic isoenzyme (cf. Van Loon and Geelen, 1971), and similar quantitative alterations in the cathodic isoenzymes. Quantitatively, however, differences in the relative proportions of individual isoenzymes were evident. The activity of the new anodic isoenzyme induced by ethephon pricking was generally less than after TMV infection. Peroxidase activity was similarly increased in untreated leaves from ethephon-pricked or TMV-inoculated plants (Table 1). However, the new anodic isoenzyme did not occur in untreated leaves.

Fig. 3. Electrophoretic patterns in 7.5% polyacrylamide gels of cathodic peroxidases from tobacco leaves: (from left to right) untreated control, injected with water, injected with salicylic acid solution, pricked with ethephon, and inoculated with TMV.

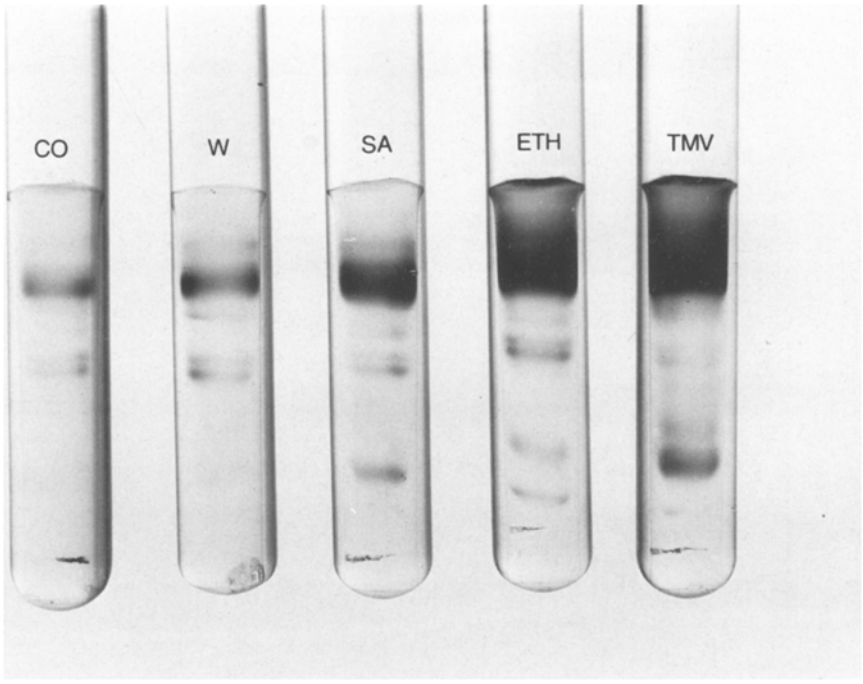

Fig. 3. Elektroforesepatronen in 7,5% polyacrylamidegels van de kathodische peroxidasen uit tabaksbladeren: (van links naar rechts) onbehandelde controle, ingespoten met water, ingespoten met een oplossing van salicylzuur, geprikt met naalden gedoopt in een oplossing van ethefon, en geïnoculeerd met TMV.

Hence, SA resembled ethephon and TMV in inducing the same four PR's but, in contrast, did not cause a substantial increase in peroxidase activity. Neither induction of PR's, nor appreciable changes in peroxidase occurred in plants inoculated, sprayed, pricked or injected with water (cf. Table 1, Figs. 2 and 3).

Induction of resistance by SA, ethephon and TMV. When leaves were injected with SA and inoculated with TMV 7 or 14 days later, few lesions developed and lesion size reached only 57% of that on corresponding leaves previously left untreated or injected with water (Table 2). Watering plants with SA solution was also effective, but to a lesser extent, whereas inoculation of the leaves with SA had only a marginal effect. Inhibitions of lesion enlargement similar to those due to injection were obtained when detached leaves were inoculated immediately after uptake of SA solution through the petiole, when inoculated leaf discs were floated on SA solution, or when trimmed plants were inoculated and sprayed with SA solution 12 h later. Therefore, SA was still effective in reducing lesion spread when applied shortly after inoculation. However, upon spraying with a concentration range from 1 mM to 0.1 μM SA, only the

Fig. 4. Electrophoretic patterns in 10% polyacrylamide gels of pH 3-soluble proteins from upper leaves of 'Samsun NN' tobacco plants, of which three lower leaves had been (from left to right) left untreated, injected with salicylic acid solution, and pricked with ethephon.

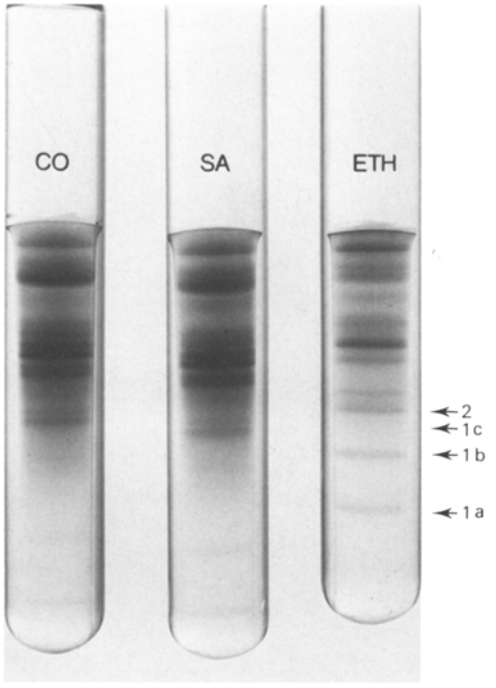

Fig.4. Elektroforesepatronen in 10% polyacrylamidegels van de bij pH 3 oplosbare eiwitten uit bovenbladeren van 'Samsun NN'-tabaksplanten waarvan drie onderbladeren (van links naar rechts) onbehandeld waren gelaten, ingespoten waren met een oplossing van salicylzuur, en geprikt waren met naalden gedoopt in een oplossing van ethefon.

highest concentration turned out to consistently reduce lesion enlargement to a significant extent. VA was ineffective when applied after inoculation, and significantly stimulated lesion enlargement when injected 7 days previously.

Since watering with SA solution induced both PR's and resistance throughout the entire plant, SA appeared to be easily transported. However, no PR's were ever detected in extracts from untreated upper leaves of plants from which three expanded lower leaves were either inoculated or injected with SA 7 or 14 days earlier, indicating that under these conditions SA was confined to the treated leaves. Ethephon and TMV were also restricted to the treated leaves, but their effects were not, since both induced PR's and increased peroxidase activity systemically (Fig. 4, Table 1). Thus, it became of interest to compare the abilities of SA, ethephon and TMV to induce resistance systemically.

When untreated upper leaves were tested for the development of systemic acquired resistance by inoculation with TMV 7 or 14 days after treatment of the lower leaves, the effects on lesion size were more variable (Table 3) than upon challenge-inoculation of lower leaves (Table 2). Both water inoculation and water injection of lower leaves

Table 2. Localized acquired resistance[a] against TMV induced by salicylic acid in treated leaves of 'Samsun NN' tobacco.

Treatment	Period between treatment and inoculation[b] (days)	Induction of PR's[c]	Relative lesion size[d]
Untreated control		−	100
Water injection	7 or 14	−	102 ± 18
Water inoculation	7	−	107 ± 3
Salicylic acid, injection	7 or 14	+ + +	57 ± 8***
Salicylic acid, inoculation	7	+	88 ± 5
Salicylic acid, watering on soil	7 - 0	+ +	79***
Salicylic acid, uptake by detached leaf	0	+ + +	40***
Salicylic acid, uptake by floating leaf discs	0 - −7	+ + +	66 ± 9***
Salicylic acid, spraying on trimmed plants	−0.5	+ +	59 ± 16***
Vanillic acid, injection	7	−	145 ± 4***
Vanillic acid, uptake by floating leaf discs	0 - −7	−	127 ± 25
Vanillic acid, spraying on trimmed plants	−0.5	−	94 ± 16

[a] Localized acquired resistance expressed as average TMV lesion size (mean ± s.d.) in treated leaves relative to TMV lesion size in corresponding untreated control leaves. Lesion sizes were measured 7 days after inoculation.

[b] Negative values indicate day(s) of treatment *after* inoculation.

[c] The number of + signs indicates increasing amounts of PR's (TMV: + + + +); −: no PR's detectable.

[d] ***: statistically significant at the 0.1% level.

Tabel 2. Verworven lokale resistentie tegen TMV geïnduceerd door salicylzuur in behandelde bladeren van 'Samsun NN'-tabak.

tended to increase lesion size in untreated upper leaves. In contrast, pricking of the lower leaves with water slightly reduced lesion size on upper leaves. Inoculation of untreated leaves from SA-injected plants produced lesions which, on an average, remained about 20% smaller than those on comparable leaves from water-injected plants. Out of a total of ten experiments, in five lesions developed to a size no different from that on untreated control or water-injected plants (111 ± 11), whereas in the other five experiments, lesions remained significantly smaller (74 ± 9***). These observations indicate that in 50% of these experiments systemic resistance was induced by SA, whereas in the other 50% it was not. This discrepancy was unrelated to the positions of the challenge-inoculated leaves on the stem or the time between the inducing and the challenge inoculation, to particular SA solutions, the growth chamber used or the time of the year, or to differences in the absolute size of the lesions produced on different batches of plants. Unlike injection, inoculation of lower leaves with SA never induced a significant resistance in upper leaves. No systemic resistance was induced when lower leaves were injected with VA (Table 3).

In contrast, both ethephon and TMV always induced a stong, quantitatively similar systemic resistance in upper leaves, whether corrected for the effects of water pricking and water inoculation, respectively, or not.

Table 3. Systemic acquired resistance[a] against TMV induced in differently treated 'Samsun NN' tobacco plants.

Treatment[b]	Presence of PR's[c]	Relative lesion size[d]
Untreated control	−	100
Water injection	−	113 ± 26
Water inoculation	−	109 ± 27
Water pricking	−	87 ± 5*
Salicylic acid, injection	−	93 ± 22[e]
Salicylic acid, inoculation	−	115 ± 35
Ethephon pricking	+ +	44 ± 11***
TMV inoculation	+ +	47 ± 9***
Vanillic acid, injection	−	119 ± 26

[a] Systemic acquired resistance expressed as average TMV lesion size (mean ± s.d.) in untreated upper leaves relative tot TMV size in corresponding upper leaves from untreated control plants.
[b] Three lower leaves per plant were treated 7 or 14 days prior to inoculation of upper leaves.
[c] The number of + signs indicates increasing amounts of PR's in the leaves before challenging (TMV, inoculated leaves: + + + +); −: no PR's detectable.
[d] *, ***: statistically significant at the 5 and 0.1% level, respectively.
[e] But see text.

Tabel 3. Verworven systemische resistentie tegen TMV geïnduceerd in verschillend behandelde 'Samsun NN'-tabaksplanten.

Effect of SA on multiplication of TMV in 'Samsun' tobacco. In SA-sprayed 'Samsun' plants, all four PR's were induced in the relative proportions characteristic of this cultivar, i.e. PR 1a being by far the predominant component (cf. Van Loon, 1975b; Antoniw and White, 1980). To test whether resistance would also be expressed in 'Samsun' plants, three expanded leaves were inoculated with TMV 7 days after spray-

Table 4. Influence of salicylic acid treatment[a] on TMV synthesis in 'Samsun' tobacco.

Experiment number	Relative infectivity of crude extracts[b]	Amount of TMV ($\mu g\ g^{-1}$ leaf)[c] after treatment with		Percentage of control
		water	salicylic acid	
I	55	236	126	53
II	28	566	167	29
III	47	529	161	30
IV	42	627	247	39

[a] Plants were sprayed with salicylic acid or water 7 days before inoculation.
[b] Four days after inoculation with TMV, centrifuged extracts from the inoculated leaves were assayed on 12 leaf-halves of *N. glutinosa*. Infectivity from water-sprayed plants = 100.
[c] The amount of virus was determined spectrophotometrically after purification.

Tabel 4. Invloed van behandeling met salicylzuur op de vermeerdering van TMV in 'Samsun'-tabak.

ing of the plants with SA solution. Four days after inoculation, the amount of virus synthesized in the inoculated leaves was determined. A substantially lower infectivity was found in crude extracts from SA-sprayed as opposed to water-sprayed leaves (Table 4). This difference was maintained through virus purification, indicating that SA did not act by inducing inhibitors of infection. Thus, leaves from plants sprayed with SA contained only about 40% of the amount of virus synthesized in water-sprayed leaves.

Effects of SA and TMV on ethylene production. To determine how far the effects or SA might be mediated by SA-induced ethylene synthesis, ethylene production of detached leaves after uptake of SA was measured and compared with ethylene synthesis after uptake of water, or due to TMV infection. Water-inoculated, water-fed leaves produced low levels of ethylene at a constant rate (Fig. 5). Leaves infected with TMV showed a large burst of ethylene between 1 and 3 days after inoculation, around the time local lesions became visible. Thereafter, ethylene production remained elevated during subsequent lesion expansion. In contrast, SA-fed leaves did not show any increase in ethylene synthesis over the water control. When analyzed for proteins at day 7, these leaves contained amounts of PR's similar to those in SA-injected leaves (Fig. 1). Untreated upper leaves from plants treated 7 days earlier on lower leaves by injection with water or SA, pricking with water, or inoculation with water or TMV, did not show increased ethylene production. Only after treatment of the lower leaves with ethephon did upper leaves evolve increased amounts of ethylene.

Effects of treatments with SA, ethephon, and TMV at 32 °C. At temperatures above 28 °C the *N* gene, conferring hypersensitivity to TMV, is no longer expressed. Consequently, 'Samsun NN' plants do not develop local lesions in response to TMV, but react with systemic mosaic symptoms, similar to 'Samsun' tobacco. Under these conditions, no PR's appear (Van Loon, 1975a), the peroxidase activity and isoenzyme patterns were similar to those of 'Samsun' (results not shown), and ethylene produc-

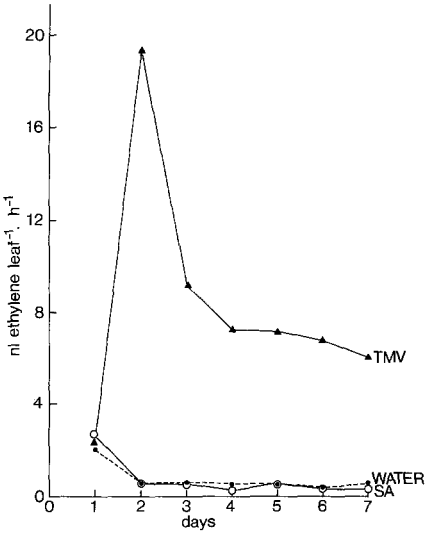

Fig. 5. Ethylene production of detached 'Samsun NN' tobacco leaves that had taken up salicylic acid solution (○) or water (●, ▲) through the petiole, and were inoculated with TMV (▲) or water (○, ●).

Fig. 5. Ethyleenproduktie van afgesneden 'Samsun NN'-tabaksbladeren die een oplossing van salicylzuur (○) of water (●, ▲) hadden opgenomen door de bladsteel, en waren geïnoculeerd met TMV (▲) of water (○, ●).

tion remains at the level of water-inoculated plants (De Laat and Van Loon, 1983).

When 'Samsun NN' plants were injected with SA and kept above 30 °C for 7 days, PR's accumulated in the injected leaves in the same proportions as at 20 °C. In contrast, no PR's became apparent in plants pricked with ethephon at 32 °C (Fig. 6), even when protein extracts were concentrated severalfold by ultrafiltration before electrophoretic analysis. Ethephon still increased peroxidase activity to 370 ± 30% at this temperature. However, the increase in activity was confined to the rapidly migrating anodic isoenzymes and no new isoenzymes were induced (Fig. 7). As at 20 °C, peroxidase activity was slightly increased (128%), whereas PR's were not induced in untreated leaves of SA-injected plants (Fig. 6).

Plants treated and kept above 30 °C for 7 days were transferred to 20 °C and challenged with TMV to test for the development of acquired resistance. As shown in Table 5, both treated un untreated leaves from SA-injected plants had developed a resistance quantitatively similar to that at 20 °C. Leaves from plants pricked with ethephon had not developed resistance during 7 days at 32 °C.

To investigate whether SA reduced TMV multiplication in systemically reacting

Fig. 6. Electrophoretic patterns in 10% polyacrylamide gels of pH 3-soluble proteins from 'Samsun NN' tobacco plants kept at 32 °C: (from left to right) lower leaves untreated, injected with salicylic acid solution, and pricked with ethephon; and upper leaves from the same plants.

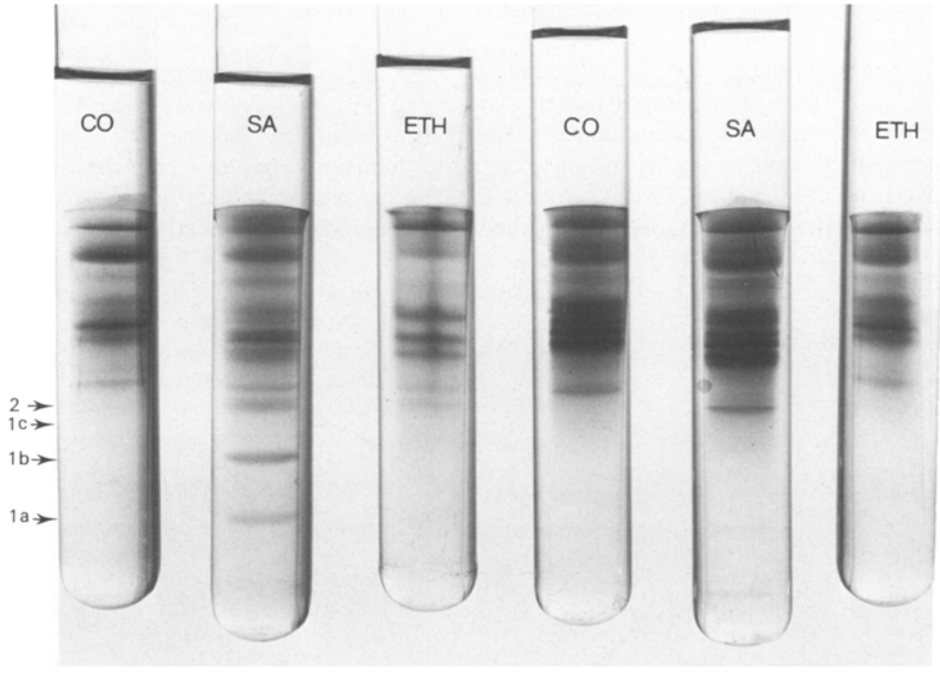

Fig. 6. Elektroforesepatronen in 10% polyacrylamidegels van de bij pH 3 oplosbare eiwitten uit bij 32 °C gehouden 'Samsun NN'-tabaksplanten: (van links naar rechts) onderbladeren onbehandeld, ingespoten met een oplossing van salicylzuur, en geprikt met naalden gedoopt in een oplossing van ethefon; en bovenbladeren van dezelfde planten.

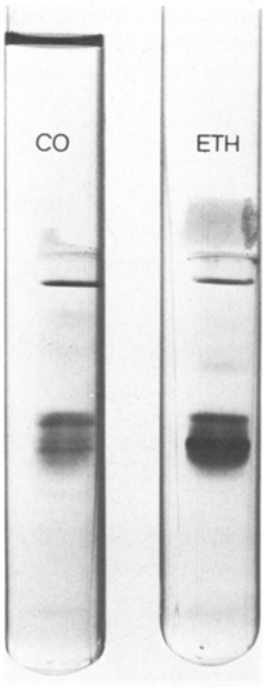

Fig. 7. Electrophoretic patterns in 7.5% polyacrylamide gels of anodic peroxidases from 'Samsun NN' tobacco plants kept at 32 °C: (left) untreated control, (right) pricked with ethephon.

Fig. 7. Elektroforesepatronen in 7,5% polyacrylamidegels van de anodische peroxidasen uit bij 32 °C gehouden 'Samsun NN'-tabaksplanten: (links) onbehandelde controle, (rechts) geprikt met naalden gedoopt in een oplossing van ethephon.

Table 5. Acquired resistance [a] against TMV induced by salicylic acid or ethephon in 'Samsun NN' tobacco plants at 20 °C and 32 °C.

Treatment [b]	Relative lesion size [c]			
	experiment I		experiment II	
	20 °C	32 °C	20 °C	32 °C
Treated leaves				
Untreated control	100	100	100	100
Salicylic acid injection	57***	62***	65***	62***
Untreated leaves				
Untreated control	100	100	100	100
Salicylic acid injection	72***	73***	70***	63***
Ethephon pricking	n.d.[d]	118	29***	107

[a] Acquired resistance expressed as average lesion size (mean ± s.d.) in treated (localized acquired resistance) lower or untreated (systemic acquired resistance) upper leaves relative to TMV lesion size in corresponding leaves from untreated controls.
[b] Three lower leaves were treated 7 days prior to inoculation of both lower and upper leaves.
[c] ***: statistically significant at the 0.1% level.
[d] n.d.: not determined.

Tabel 5. Verworven resistentie tegen TMV geïnduceerd door salicylzuur of ethefon in 'Samsun NN'-tabaksplanten bij 20 °C en bij 32 °C.

Fig. 8. Leaves of 'Samsun NN' tobacco plants sprayed with (left) water, and (right) salicylic acid solution and kept at 32 °C. Seven days after spraying the leaves were inoculated with TMV, and 60 h later the temperature was lowered to 20 °C. Leaves were detached and photographed one day later.

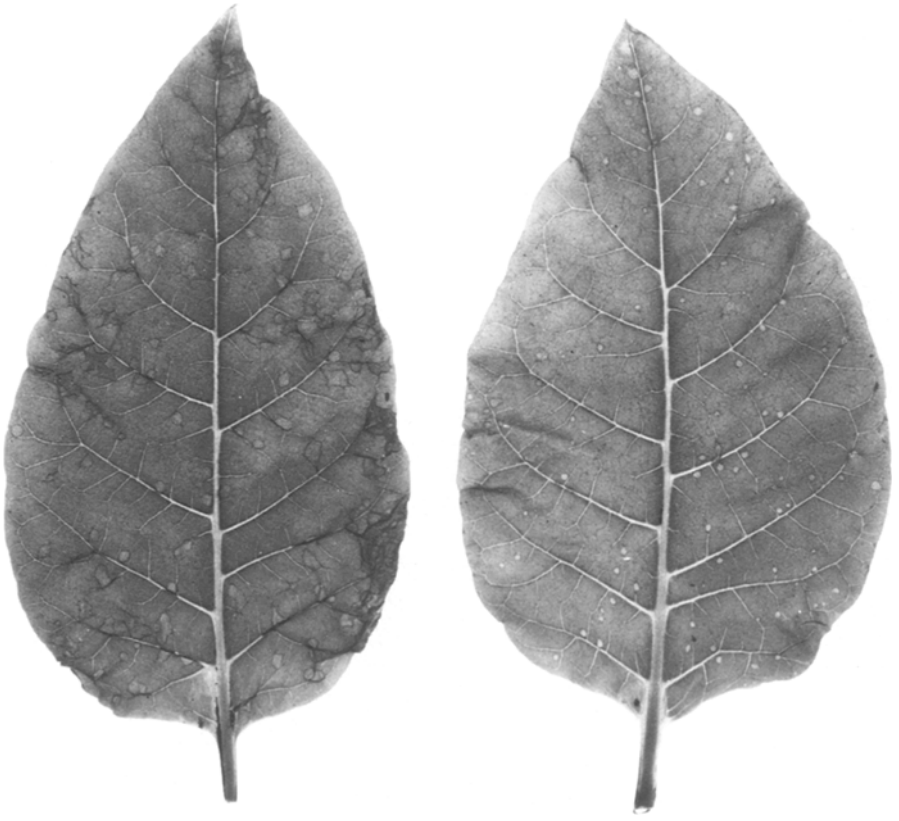

Fig. 8. Bladeren van bij 32 °C gehouden 'Samsun NN'-tabaksplanten bespoten met (links) water en (rechts) een oplossing van salicylzuur. Zeven dagen na bespuiting werden de bladeren geinoculeerd met TMV en 60 uur later werd de temperatuur verlaagd tot 20 °C. Een dag later werden de bladeren afgeplukt en gefotografeerd.

plants through a reduction of the number of infectible sites or through a decrease in virus spread, 'Samsun NN' plants were sprayed with SA and kept at 32 °C. After 7 days, three expanded leaves were inoculated with TMV. Sixty hours after inoculation, the temperature was lowered to 20 °C and necrosis ensued at the sites where TMV was multiplying. As shown in Fig. 8, no differences in lesion numbers were apparent, but in the water-sprayed plants the virus had spread considerably faster from the point of penetration than in SA-sprayed plants. Whereas necrotic spots readily coalesced in the water-sprayed controls, lesions remained distinct on SA-treated leaves. These results clearly indicate that SA reduced the rate of virus spread in the leaf.

The results of this study are summarized in Table 6.

Table 6. Effects of salicylic acid, ethephon, or TMV on induction of PR's, peroxidase activity, ethylene production, and development of acquired resistance in 'Samsun NN' tobacco plants[a]

Treatment	PR's	Increased peroxidase activity	Increased ethylene production	Acquired resistance
Treated leaves, 20 °C				
Salicylic acid	+ + +	+	−	+ + +
Ethephon	+ + +	+ + + +	+ + + +	+ + + +[b]
TMV	+ + + +	+ + +	+ + +	+ + + +[c]
Treated leaves, 32 °C				
Salicylic acid	+ + +	+	n.d.	+ + +
Ethephon	−	+ + +	+ + + +	−
TMV	−	−	−	−
Untreated leaves, 20 °C				
Salicylic acid	−	+	−	− — + + +
Ethephon	+ +	+ + + +	+ + + +	+ + + +
TMV	+ +	+ + +	−	+ + + +
Untreated leaves, 32 °C				
Salicylic acid	−	+	n.d.	+ +
Ethephon	−	+ + +	n.d.	−
TMV	−	−	−	−

[a] The number of + signs indicates increasing quantitative effects; −: not different from untreated control; n.d.: not determined.
[b] Cf. Ross and Pritchard, 1972; Pritchard and Ross, 1975; Van Loon, 1976a, 1977).
[c] Cf. Ross, 1961a.

Tabel 6. Effecten van salicylzuur, ethefon en TMV op de inductie van 'pathogenesis-related proteins', peroxidaseactiviteit, ethyleenproduktie en ontwikkeling van verworven resistentie in 'Samsun NN'-tabaksplanten.

Discussion

It was reported previously (White, 1979; Antoniw and White, 1980) that lesion numbers after TMV inoculation are reduced on aspirin-injected leaves. This study demonstrates that, in addition, SA also reduced lesion size and, therefore, induced a resistance similar to the acquired resistance induced by TMV itself or by ethephon (Van Loon, 1977). 'Samsun NN' plants sprayed or watered with SA solution developed resistance in all leaves, similar to that observed in 'Xanthi-nc' sprayed or watered with aspirin (White, 1979). Resistance was likewise induced in detached leaves after uptake of SA through the petiole or in leaf discs floating on SA solution. However, inoculation with SA was only marginally active, probably because of the low dose thus applied. In contrast, injection proved to be very effective, resulting in strong acquired resistance in the treated leaves. A similar resistance was induced in untreated upper leaves in half of the experiments. It appears unlikely that this systemic resistance was caused by a) spillage of SA solution on leaves or soil, b) the use of non-sterile solutions, or c) transport of SA out of the injected leaf. Any of these conditions would have resulted in

detectable amounts of PR's in the leaves to be challenged, but these were never observed. Apparently, SA is readily transported throughout the entire plant after entering the xylem of the roots, but is unable to leave inoculated or injected leaves by the phloem. The type of signal responsible for the induction of systemic resistance in the untreated upper leaves of plants on which lower leaves were injected with SA, and the reason why this signal is transmitted only occasionally, remains to be elucidated.

Pricking 'Samsun NN' leaves with needles moistened with ethephon mimicked TMV infection in inducing PR's and acquired resistance in both treated and untreated leaves at 20 °C, but not at 32 °C. In contrast, induction of PR's by SA was confined to the treated leaves and the induction of both PR's and resistance developed equally well at 20 °C and at 32 °C. Although there was no significant reduction in lesion numbers in aspirin-treated plants kept at 32 °C for 2 days (White, 1979), J.F. Antoniw, R.F. White and R.D. Woods (in preparation) found that aspirin also induced PR's and a reduction in lesion size in 'Samsun NN' and 'Xanthi- nc' at both 20 °C and 32 °C. Thus, our observations indicate that, unlike the expression of the *N* gene, the inductions of PR's and of acquired resistance are not temperature-sensitive. The absence of PR's in untreated leaves of SA-injected plants, even though those leaves sometimes show acquired resistance, may not be consistent with the proposed involvement of PR's in systemic acquired resistance (Van Loon and Van Kammen, 1970; Kassanis et al., 1974; Van Loon, 1975b). Fraser (1981a,b) recently reached a similar conclusion when demonstrating development of acquired resistance in respone to TMV well before detectable accumulation of PR's, as well as a lack of correlation between the concentration of PR's and lesion number or size. Although these observations seem difficult to reconcile with a direct action of PR's on virus multiplication and/or spread, it is still possible that only very minor amounts, not detectable with the stains used, are required to exert a full effect. In this respect, it may be significant that systemic resistance may be induced by as few as 16 lesions per leaf (Ross, 1961b) and is almost maximal after inoculation with 0.1 mg TMV l^{-1} (Van Loon, 1976a), whereas the amount of PR's increases strongly with increasing lesion density up to 10 mg TMV l^{-1} (Van Loon and Van Kammen, 1970). Alternatively, PR's could be enzymes generating products that may be transported within the plant; in SA-injected plants, PR's were present in the treated leaves for several days before upper leaves were challenged with TMV.

A further association between the presence of PR's and the reduction of TMV multiplication and spread was evident in SA-sprayed 'Samsun' plants at 20 °C. Furthermore, J.F. Antoniw, R.F. White and R.D. Woods (in preparation) showed that there was a reduction in TMV multiplication in aspirin-injected half-leaves compared with the water-injected opposite half-leaves as measured by both the infectivity of the leaf extracts and the number of virus particles. A similar reduction of TMV multiplication in 'Samsun' plants was found earlier when PR's had been induced systemically by local infection with tobacco necrosis virus (Van Loon and Dijkstra, 1976). As 'Samsun NN' and 'Samsun' plants react identically to TMV at 32 °C, the effect of the SA treatment on virus multiplication and spread under conditions that eventually lead to a systemic reaction, could be visualized directly by transferring 'Samsun NN' plants from 32 to 20 °C. Under these circumstances, as in a localized infection, virus spread was reduced considerably. Similar results were obtained in both 'Samsun NN' and 'Xanthi-nc' leaves injected with aspirin by J.F. Antoniw, R.F. White and R.D.

Woods (in preparation). It cannot be decided, however, whether this reduction was caused primarily by a decrease in the rate of virus multiplication, or whether virus multiplication was hampered due to neighbouring cells being more resistant to virus entry.

Whereas SA, like TMV, induced PR's and resistance, unlike TMV, it did not stimulate ethylene production, and only hardly increased peroxidase activity. However, the changes in peroxidase isoenzymes, although minor, resemble those characteristic of leaves with systemic acquired resistance due to TMV (cf. Van Loon, 1976a), pointing again to a similarity between the effects of SA and TMV. Moreover, induction of stronger resistance by SA tended to be associated with a greater increase in peroxidase activity. Since greater stimulation of peroxidase activity occurred when injury around the injection sites was more prominent, injury may be a contributing factor in the action of SA. Although no acute toxicity of SA was apparent, the older leaves on SA-sprayed plants usually lost chlorophyll more readily than comparable leaves on control plants. A senescence-promoting effect of SA would also enhance peroxidase activity as well as contribute to a reduction in the rate of lesion enlargement (Van Loon, 1976a). But localized acquired resistance was always clearly expressed by SA-injected leaves, whether peroxidase activity was stimulated or not. These observations, therefore, verify our earlier conclusion, obtained indirectly (Van Loon, 1976a), that increased peroxidase activity is a reflection of a physiological state rather than being directly responsible for acquired resistance.

It stands out clearly that none of the effects of SA can be ascribed to increased ethylene production. At first sight, it might therefore be concluded that the induction of PR's and acquired resistance by TMV does not depend on abundant local ethylene synthesis, as was suggested earlier (Van Loon, 1977). On the other hand, ethylene does appear to be directly responsible for the large increase in peroxidase activity and the appearance of the new peroxidase isoenzyme. However, ethephon treatment, mimicking the burst of ethylene produced during TMV infection, not only leads to changes in peroxidase, but also induces PR's and resistance. Induction of PR's and resistance by ethylene are no longer evident above 30 °C, indicating that this part of the action of ethylene is temperature-sensitive in the same way as the *N* gene is. Precisely these latter effects resulted from treatment with SA, indicating that SA simulates these temperature-sensitive effects of ethylene action. Furthermore, contrary to the action of ethylene, the mechanism with which SA induces PR's and acquired resistance is not temperature-sensitive. Ethylene is known to both enhance membrane permeability and stimulate aromatic biosynthesis (Abeles, 1973; Hanson and Kende, 1975; Reuveni and Cohen, 1978). It is conceivable, therefore, that the action of ethylene is either a liberation, through decompartmentalization (Weststeijn, 1978) or a promotion of the synthesis of a, presumably aromatic, compound functioning as the more immediate inducer of PR's and resistance, and that this action of ethylene is prevented by high temperature. Moreover, in 'Samsun' plants, lacking the *N* gene, but also in 'Samsun NN' plants kept at 32 °C, no stimulation of ethylene production occurs in response to TMV infection (De Laat and Van Loon, 1983) and neither PR's, nor resistance develop.

Benzoic acid, SA, and aspirin induce PR's whereas methyl salicylate does not (White, 1979), suggesting that there may be a naturally active compound similar to benzoic acid in structure. However, vanillic acid, the major benzoic-acid derivative in

hypersensitively reacting tobacco (Tanguy, 1970; Tanguy and Martin, 1972) induced neither PR's, nor resistance in treated leaves, and, therefore, cannot be the natural inducer. Snook et al. (1981) recently reported that flue-cured tobacco leaves contain several benzoic acid derivatives, including salicylic, *m,* and *p*-hydroxybenzoic and 2,5- and 3,4-dihydroxybenzoic acids. Of these, *p*-hydroxybenzoic and 2,5-dihydroxybenzoic acids have also been identified in hypersensitively reacting 'Xanthi-nc' tobacco (Tanguy, 1970; Tanguy and Martin, 1972). This resemblance suggests that phenylpropanoid metabolism during flue-curing and during a hypersensitive reaction may be rather similar. If so, it cannot be excluded that SA itself is produced upon infection of 'Samsun NN' tobacco with TMV.

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Samenvatting

Vergelijking van de effecten van salicylzuur en ethefon met door een virus geïnduceerde overgevoeligheid en verworven resistentie in tabak

Inductie van een overgevoeligheidsreactie in 'Samsun NN'-tabak door tabaksmozaïekvirus (TMV) bij 20 °C leidt tot de ontwikkeling van een verworven resistentie die zowel lokaal als systemisch werkzaam is, en gaat samen met het verschijnen van 'pathogenesis-related proteins' (PR's) en sterke toename in de activiteit van peroxidase en de produktie van ethyleen. Salicylzuur (SA) induceerde een vergelijkbare resistentie in behandelde plantedelen en af en toe ook in niet behandelde bovenbladeren van planten waarvan drie onderbladeren waren ingespoten. SA induceerde ook dezelfde vier PR's, maar deze waren beperkt tot de behandelde bladeren. Er bestaat dus geen directe samenhang tussen de aanwezigheid van PR's en de remming van de vermeerdering en uitbreiding van TMV in de plant.

In tegenstelling tot TMV stimuleerde SA de ethyleenproduktie niet en verhoogde het de peroxidaseactiviteit nauwelijks. Inductie van verworven resistentie en PR's door SA trad even goed op bij 32 °C als bij 20 °C. Net als infectie met TMV leidde aanprikken van bladeren met naalden die gedoopt waren in een oplossing van ethefon – waaruit in het blad ethyleen vrijkomt – echter tot inductie van verworven resistentie en PR's in zowel behandelde als onbehandelde bladeren bij 20 °C, maar niet bij 32 °C. Ethefon verhoogde de peroxidaseactiviteit bij beide temperaturen, maar alleen bij 20 °C induceerde het veranderingen in zowel de anodische als de kathodische isoënzymen die vergelijkbaar waren met die welke geïnduceerd werden als gevolg van infectie met TMV. SA induceerde PR's en verminderde de vermenigvuldiging van TMV in 'Samsun' tabak, en remde de uitbreiding van het virus in 'Samsun NN' bij 32 °C.

Deze waarnemingen tonen dat noch de inductie van PR's, noch de ontwikkeling van verworven resistentie een temperatuurgevoelig proces is. Daarentegen zijn de effecten van ethefon op dezelfde wijze temperatuurgevoelig als de overgevoeligheidsreactie op

TMV. Men kan daarom veronderstellen dat ethyleen, dat op natuurlijke wijze geproduceerd wordt tijdens de overgevoeligheidsreactie van tabak op TMV, aanleiding geeft tot een temperatuurgevoelig proces, namelijk de synthese of het vrijkomen van een verbinding, vermoedelijk een benzoëzuurderivaat, dat fungeert als de natuurlijke inductor van PR's en verworven resistentie. Hoewel is aangetoond dat vanillinezuur zich ophoopt in overgevoelig reagerende tabaksbladeren, veroorzaakte deze verbinding geen enkel van de effecten van SA. Vanillinezuur kan dus niet de natuurlijke inductor zijn.

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