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The effect of benzoic acid derivatives on *Nicotiana tabacum* growth in relation to PR-b1 production

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Summary

Using HPLC quantification, we have shown that benzoic acid derivatives stimulate PR-b1 protein synthesis in the leaf discs of *Nicotiana tabacum* Xanthi nc. The stimulation of PR-b protein synthesis during treatment with several benzoic acid derivatives is described for the first time in the root system of in vitro grown *Nicotiana tabacum* plantlets. In healthy in vitro grown plantlets the PR-b1 concentration is similar in roots and leaves (200 ng per gram of fresh material). During chemical treatment, however, the PR-b1 concentration increases to a lesser extent in roots than in leaves (10-fold higher in treated roots and 100-fold higher in treated leaves). Benzoic acid derivatives also have a detrimental effect on the growth of in vitro plantlets, which may be related to the accumulation of PR-b proteins.

Nicotiana tabacum; PR-b protein; Benzoic acid derivative; Chemical induction

Introduction

In their early work Gianinazzi et al. (1970) and Van Loon and Van Kammen (1970) reported the production of new soluble proteins called b-proteins or pathogenesis-related (PR) proteins in *Nicotiana tabacum* variants hypersensitive to infection with tobacco mosaic virus (TMV). Similar proteins were found in several *Nicotiana* species after infection with different viruses, viroids, fungi or bacteria

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(Van Loon, 1985). The occurrence of PR-b proteins was not pathogen-specific, but was determined by the reaction of the host plant to different pathological conditions, indicating that these proteins were of host origin (Gianinazzi and Ahl, 1983).

Several chemical substances such as polyacrylic acid and salicylic acid (Van Loon, 1983; White, 1979) stimulate PR-b protein synthesis when injected or applied onto leaves, watered on the soil, or taken up by detached leaves through the petiole or by leaf discs floating on a solution.

The PR-b proteins also accumulate in large amounts during flowering (Fraser, 1981) and in amorphous callus tissue grown in vitro (Antoniw et al., 1981). In the latter case the PR-b production was probably stimulated by the presence of plant hormones in the growth medium.

Although PR-b proteins are not the product of the N gene, which is responsible for the hypersensitive reaction to TMV (Ahl et al., 1982), they appear to be consistently related to the expression of resistance. Therefore, the use of a chemical which selectively stimulates synthesis of PR-b proteins could represent a powerful tool for research on the molecular mechanism involved in resistance.

The aim of this work was to investigate the effect of some benzoic acid derivatives on the relative growth rate of *N. tabacum* Xanthi nc and Samsun nn in relation to the production of PR-b1, the major PR-b protein.

Materials and Methods

Chemical products

In addition to benzoic acid, we also tested the following benzoic acid derivatives, all purchased from Sigma (St Louis, Missouri): 2-hydroxybenzoic acid; 2,6-hydroxybenzoic acid; 2-acetoxybenzoic acid; 2-hydroxy-5-nitrobenzoic acid; 2-hydroxy-5-chlorobenzoic acid; 2-hydroxy-5-aminobenzoic acid; benzoic acid; 2,3-hydroxybenzoic acid; 2,4-hydroxybenzoic acid; 2,5-hydroxybenzoic acid; 3-hydroxybenzoic acid; 4-hydroxybenzoic acid; 4-methoxybenzoic acid; 2-methoxybenzoic acid; 4-hydroxy-3-methoxybenzoic acid; 2-hydroxybenzamide.

Plant material

Leaf discs. The influence of benzoic acid derivatives on PR-b1 production was studied over a 6-day period with leaf discs floating on a solution with the chemicals. Leaf discs (2.5 cm diameter) were made from the 2 well developed leaves taken in the middle of the stem from 50-days-old *Nicotiana tabacum* Xanthi nc plants grown at 23°C (16 h photoperiod, 16000 lux). Twelve leaf discs were incubated in 100 ml of freshly prepared aqueous solutions (5×10^{-4} M) of benzoic acid derivatives adjusted to pH 6.5 with NaOH, in a Petri dish (140 mm diameter). The leaf discs floated on the solution with the lower surface in contact with the liquid. Controls consisted of twelve discs floating on distilled deionized water. The abilities of the benzoic acid derivatives to stimulate PR-b1 synthesis were tested by electrophoresis and by HPLC quantification measuring the PR-b1 concentration in the samples constituted from the twelve leaf discs.

In vitro grown plantlets. *N. tabacum* cv Xanthi nc and cv Samsun nn cultivars plantlets were grown and monthly multiplied by node cuttings on agar culture media as described by Murashige and Skoog (1962) and modified by Cardin et al. (1983). The plantlets were grown in glass containing 10 ml medium placed in a growth chamber (23°C, 16 h of light; 2500 lux).

We used cuttings from the terminal and the first axillary bud. We examined the relative growth rate of the in vitro grown plantlets by two methods. In the first method, we chose five benzoic acid derivatives representing the three classes described in Results: 2-hydroxybenzoic acid (high ability to produce PR-b proteins), 2-hydroxy-5-chlorobenzoic acid and 2-hydroxy-5-aminobenzoic acid (moderate ability to stimulate PR-b proteins synthesis) and 2,3- and 2,4-hydroxybenzoic acids (low ability to stimulate PR-b proteins synthesis).

Each of these benzoic acid derivatives was added to the agar medium (final concentration: 5×10^{-4} M) on which cuttings of *N. tabacum* Samsun nn were placed. Each treatment was applied to twelve plantlets. After thirty days of incubation, we measured both the fresh leaf weights and the PR-b1 production of these plantlets. In the second method, cuttings of *N. tabacum* Xanthi nc and Samsun nn rooted for one week in agar medium without benzoic acid derivatives were transferred into liquid medium (medium used by Cardin et al. (1983) without agar) containing 5×10^{-4} M of 2-hydroxybenzoic acid. The influence of this compound was studied by measuring both the fresh weight of roots and leaves and the amount of PR-b1 after 10, 20 and 30 days after start of the 2-hydroxybenzoic acid treatment.

HPLC determination of PR-b1

Leaf discs were ground in a mortar and pestle with a 84 mM citric acid - 0.3 M sodium phosphate pH 2.8 buffer (Antoniw and White, 1980). The homogenate was filtered through muslin and centrifuged at 20000 × *g* for 20 min. The proteins were precipitated with 50% (W/V) ammonium sulphate (30 min, 4°C) and centrifuged at 10000× *g* for 10 min. The pellet was suspended in Tris-HCl buffer (pH 8) (5 g fresh material (gFM)/ml).

After centrifugation (5000 × *g*, 10 min), the final supernatant was stored at 4°C for electrophoresis, or directly injected for high performance liquid chromatography (HPLC) assay as described previously (Abad et al., 1985).

Electrophoresis

The samples obtained as described earlier were also analyzed by polyacrylamide gel electrophoresis (PAGE) in native conditions (Davis, 1964). Electrophoresis was performed for 3 h at 150 V and 4°C.

ELISA determination

Leaves or roots of in vitro grown tobacco were homogenized in 84 mM citric acid, 0.3 M sodium phosphate buffer, pH 2.8. The homogenate was centrifuged for 10 min at 5000 × *g* and the supernatant consisting of crude samples was tested in an enzyme linked immunosorbent assay (ELISA). The ELISA procedure described by Clark and Adams (1977) was slightly modified; the wells of polystyrene

Table 1

PR-bl synthesis (μg/gFM) in leaf discs of *N.tabacum* Xhanti nc floating on a solution with benzoic acid derivatives (5.10^{-4} M); PR-bl synthesis measured by HPLC.

Benzoic acid derivatives			1 day	2 days	3 days	4 days	6 days
	2-Hydroxybenzoic	P	31%	49%	62%	77.4%	100%
H		Q	28.2	43.8	55.2	68.9	89
I	2,6-Hydroxybenzoic	P	29.6%	57.7%	52.3%	235%	171%
G		Q	26.4	51.4	46.6	210	152.6
H	2-Acetoxybenzoic	P	39%	67.4%	126%	177%	57%
		Q	34.8	60	107.4	157.8	50.7
M	2-Hydroxy	P	ND	ND	ND	ND	19.3%
	5-Nitrobenzoic	Q	ND	ND	ND	ND	17.2
O	2-Hydroxy	P	ND	ND	ND	ND	13.9%
	5-Chlorobenzoic	Q	ND	ND	ND	ND	12.4
D	2-Hydroxy	P	ND	ND	ND	ND	12.3%
	5-Aminobenzoic	Q	ND	ND	ND	ND	10.96
E	Benzoic	P	6.5%	5.9%	4.1%	6%	14.2%
		Q	5.76	5.18	3.62	5.38	12.7
R	2,3-Hydroxybenzoic	P	ND	ND	ND	ND	2.7%
		Q	ND	ND	ND	ND	2.4
A	2.4-Hydroxybenzoic	P	ND	%ND	ND	ND	2.1%
		Q	ND	ND	ND	ND	1.88
T	2,5-Hydroxybenzoic	P	ND	ND	ND	ND	5.2%
		Q	ND	ND	ND	ND	4.6

E	3-Hydroxybenzoic	P	3.2%	5%	2.3%	2.8%	3.4%
		Q	2.82	4.48	2.06	2.54	3
L	4-Hydroxybenzoic	P	ND	ND	ND	ND	3.1%
O		Q	ND	ND	ND	ND	2.72
W	4-Methoxybenzoic	P	ND	ND	ND	ND	5.2%
		Q	ND	ND	ND	ND	4.64
	2-Methoxybenzoic	P	ND	ND	ND	ND	2%
		Q	ND	ND	ND	ND	1.8
	4-Hydroxy	P	ND	ND	ND	ND	1.6%
	3-Methoxybenzoic	Q	ND	ND	ND	ND	1.4
	2-Hydroxybenzamide	P	ND	ND	ND	ND	4%
		Q	ND	ND	ND	ND	3.48
	Control	0.02**	ND	ND	ND	ND	0.18

Q = PR-bl protein expressed in μg/gFM. P = Percentage of PR-bl protein induced by benzoic acid derivatives compared with PR-bl protein obtained after 6 days of incubation with 2-hydroxybenzoic acid.

* = The 100% value represents the PR-bl concentration in discs floating on 2-hydroxybenzoic acid for six days.

** = PR-bl concentration at the start of treatment in the control discs (expressed in μg/gFM.) ND = not determined.

microtitre plates (Falcon) were coated with 200 µl of rabbit anti PR-b1 IgG (5 µg/ml) at 37°C for 3 h. After washing (four washes in PBS containing 0.05% Tween 20), 200 µl of crude plantlet extracts were loaded into wells during 2 h and the wells were then rinsed as before. Alkaline phosphatase conjugated to anti PR-b1 IgG using glutaraldehyde as described by Avrameas (1969) was added at a dilution of 1/500 in PBS, 0.05% Tween 20 containing 1% BSA (PTB), pH 7. Plates were incubated at 37°C for 2 h. Unreacted enzyme conjugated antibody was removed in four washings and 200 µl of a substrate solution (*p*-nitrophenylphosphate, 1 mg/ml) were added per well. After 2 h incubation, the plates were read at 405 nm using a Titertek twin-reader. Each sample was assayed in a range of ten 2-fold dilutions in PTB. Each dilution was assayed in triplicate.

Sample concentrations were estimated from the standard on the same plate by plotting absorbance against log standard concentration and fitting a logistic curve.

Results

Chemical PR-b1 production in leaf discs after treatment with benzoic acid derivatives

The results obtained by incubating leaf discs in solutions with benzoic derivatives are summarized in Table 1. The PR-b1 concentration was measured by HPLC. A significant production of PR-b1 protein was observed in the twelve leaf discs 24 h after treatment with 2-hydroxybenzoic acid (28.2 µg/gFM), 2-acetoxybenzoic acid (34.8 µg/gFM) and 2,6-dihydroxybenzoic acid (26.4 µg/gFM).

Maximum accumulation of PR-b1 protein was reached 4 days after the start of treatment with 2,6-hydroxybenzoic acid (210 µg/gFM), 2-acetoxybenzoic acid (157.8 µg/gFM), and 6 days after start of treatment with 2-hydroxybenzoic acid (89 µg/gFM).

The structure of the benzoic acid had a major impact on the production of the PR-b1 protein. A slight structural alteration of 2-hydroxybenzoic acid markedly changed its ability to stimulate PR-b1 synthesis. The hydroxyl group as well as the carboxyl group of 2-hydroxybenzoic acid affected PR-b1 production. When the 2-hydroxyl group was transferred to position 3 or 4 of the aryl moiety (3-hydroxybenzoic or 4-hydroxybenzoic acid), a complete loss of activity was observed.

Moreover, the addition of a second hydroxyl group at position 3, 4 or 5 in the 2-hydroxybenzoic acid dramatically depressed the activity. On the other hand, when a second hydroxyl group was added at position 6 (2,6-hydroxybenzoic acid), there was an increase in PR-b1 synthesis by 235% (Table 1).

When comparing the activities of 2-hydroxybenzoic acid, 2-methoxybenzoic acid and 2-acetoxybenzoic acid, we noted that acetylation of the hydroxyl group at position 2 increased PR-b1 synthesis (177%) while its methylation led to an inactive compound.

Conversion of the carboxyl group into a carboxamide (2-hydroxybenzamide) resulted in a significant loss of activity (from 89 µg/gFM for 2-hydroxybenzoic acid to 3.48 µg/gFM for 2-hydroxybenzamide).

Substitution at position 5 of 2-hydroxybenzoic acid of a NO_2 group, as in 2-hydroxy-5-nitrobenzoic acid, a Cl group, as in 2-hydroxy-5-chlorobenzoic acid, or a NH_2 group, as in 2-hydroxy-5-aminobenzoic acid caused a substantial loss of activity.

Consequently, these benzoic acid derivatives were classified as having high, moderate or low activity (Table 1).

In controls (leaf discs floating on distilled water), the PR-b1 concentration did not vary significantly during the experiment. The initial concentration of 0.02 μg/gFM increased to 0.18 μg/gFM after six days of incubation on water.

Stimulation of PR-b1 synthesis by benzoic acid derivatives in relation to the relative growth rate

We measured the amount of PR-b1 protein in control plantlets grown in vitro. The PR-b1 concentration was measured by ELISA. The values in the roots were 0.15 μg/gFM after 10, 20, 30 days of incubation. The leaves of one and the same plant had the same PR-b1 protein content (Fig. 3).

In the apical shoots of *N. tabacum* Samsun nn placed in agar medium containing benzoic acid derivatives, we noted a good correlation between PR-b1 synthesis in leaves and relative growth rate. Benzoic acid derivatives which did not affect growth stimulated PR-b1 synthesis moderately. For example, 2,3- and 2,4-hydroxybenzoic acids that reduced fresh leaf weight by 12% and 24% had a weak effect on the PR-

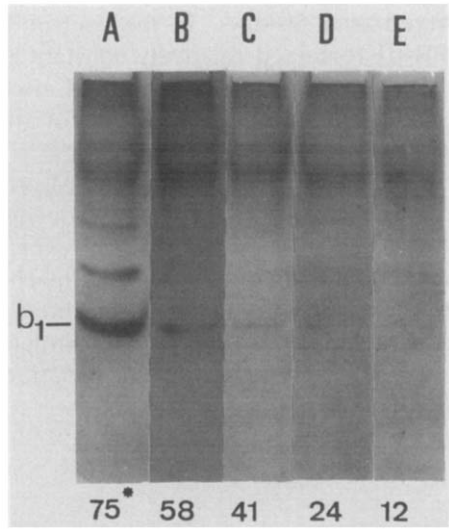

Fig. 1. Stimulation of PR-b synthesis by benzoic acid derivatives after 30 days of incubation in plantlets of *N. tabacum* Samsun nn grown in vitro.
Electrophoretic pattern of PR-b proteins on PAGE 15%. A, 2-hydroxybenzoic acid 5×10^{-4} M; B, 2-hydroxy-5-aminobenzoic acid 5×10^{-4} M; C, 2-hydroxy-5-chlorobenzoic acid 5×10^{-4} M; D, 2,4-hydroxybenzoic acid 5×10^{-4} M; E, 2,3-hydroxybenzoic acid 5×10^{-4} M.
* Values indicate % inhibition of fresh weight material.

b1 protein accumulation (Fig. 1, lanes D and E). Growth was significantly impaired by 2-hydroxy-5-chlorobenzoic acid and 2-hydroxy-5-aminobenzoic acid: the fresh leaf weight reduction was 41% and 58%, respectively, while appreciable amounts of PR-b proteins accumulated (Fig. 1, lanes B and C). The most significant growth inhibition was obtained following treatment with 2-hydroxybenzoic acid (75%). This was accompanied by a high PR-b1 production (Fig. 1, lane A).

Kinetics of PR-b1 production and growth inhibition in roots and leaves induced by 2-hydroxybenzoic acid

In roots, differences in growth inhibition between control and treated plants were observed after 10 days of culture (Fig. 2). They increased during the following 10 days in *N. tabacum* Samsun nn and during the next 20 days in *N. tabacum* Xanthi nc. Weight reductions were 61% and 60%, respectively (Fig. 2).

In leaves, differences in fresh weight between treated and untreated plantlets were smaller. We did not observe any difference during the first 10 days in *N. tabacum* Samsun nn and during the first 20 days in *N. tabacum* Xanthi nc. However, after 30 days of 2-hydroxybenzoic acid treatment, we observed a weight reduction of 19% in *N. tabacum* Xanthi nc and of 14% in *N. tabacum* Samsun nn (Fig. 2).

PR-b1 accumulation in leaves of *N. tabacum* Xanthi nc and *N. tabacum* Samsun nn was measured during the 2-hydroxybenzoic acid treatment (Fig. 3). In *N. tabacum* Xanthi nc, we found a PR-b1 concentration of 6 μg/gFM after 10 days and of 30 μg/gFM after 20 days. After 30 days the PR-b1 concentration decreased to 15 μg/gFM. However, in *N. tabacum* Samsun nn the maximal PR-b1 production (15 μg/gFM) was observed after 30 days. In the control *N. tabacum* Xanthi nc plantlets, the rate of PR-b1 remained relatively constant during the culture: we noted 0.21 μg/gFM at the beginning and 0.14 μg/gFM after 30 days. This PR-b1 level was higher in *N. tabacum* Samsun nn (0.74 μg/gFM after 30 days) than in *N. tabacum* Xanthi nc (Fig. 3).

In the root system of *N. tabacum* Xanthi nc, no PR-b1 production was observed during the first 20 days while a substantial PR-b1 production was observed in *N. tabacum* Samsun nn after 10 days (1.3 μg/gFM). However, in both systems maximal PR-b1 production was obtained after 20 days and reached identical levels (2.3 μ/gFM). Then, the PR-b1 concentration decreased in both *N. tabacum* Xanthi nc and Samsun nn. Values obtained in roots of control plantlets were similar to those found in leaves (0.15 μg/gFM), and concentration variations were not significant during in vitro incubation (Fig. 3).

Discussion

This study provides new information on PR-b proteins production in *N. tabacum* tissue. So far, the presence of PR-b proteins in roots of *Nicotiana* species has not been demonstrated. Using ELISA, we showed the presence of PR-b1 protein in the root system of healthy in vitro grown plantlets. The PR-b1 concentrations found in the root and leaf system of healthy plantlets grown in vitro were identical (about 0.15 μg/gFM).

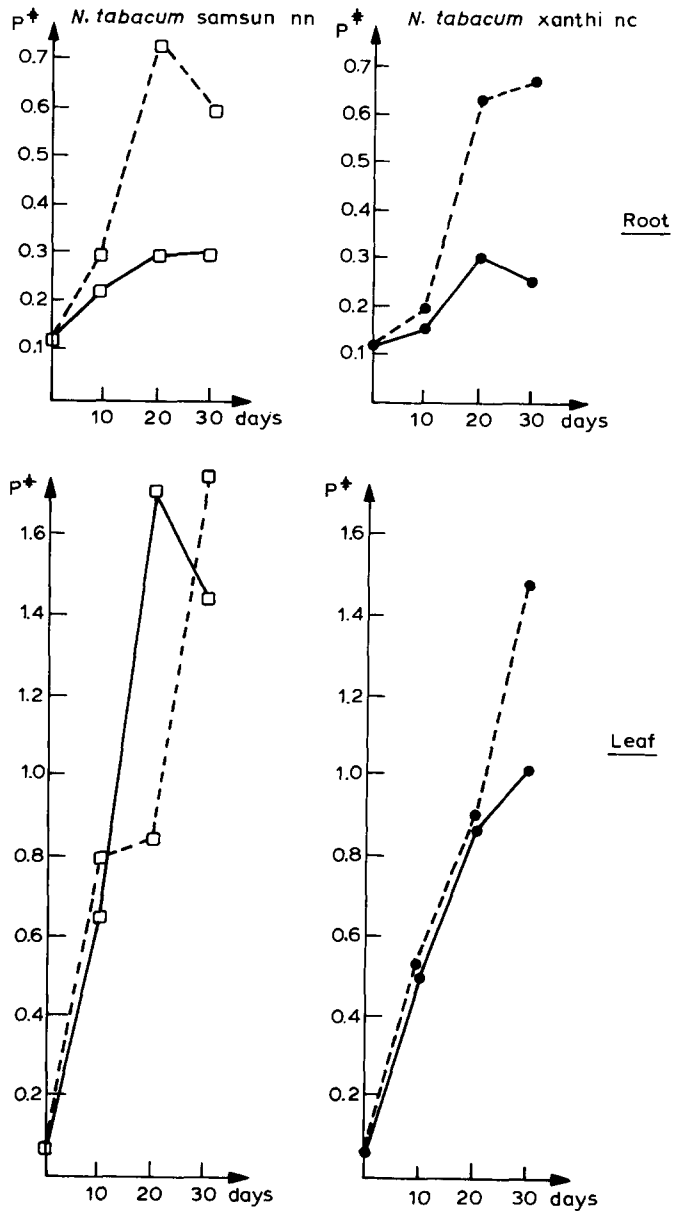

Fig. 2. Effect of 2-hydroxybenzoic acid (5×10^{-4} M) on growth in vitro of roots (top) and leaves (bottom) of tobacco plantlets. Tobacco grown without (---) and with 2-hydroxybenzoic acid (——); ● = *N. tabacum* Xanthi nc; □ = *N. tabacum* Samsun nn; P^* = Average leaf fresh weight of six plantlets (expressed in μg).

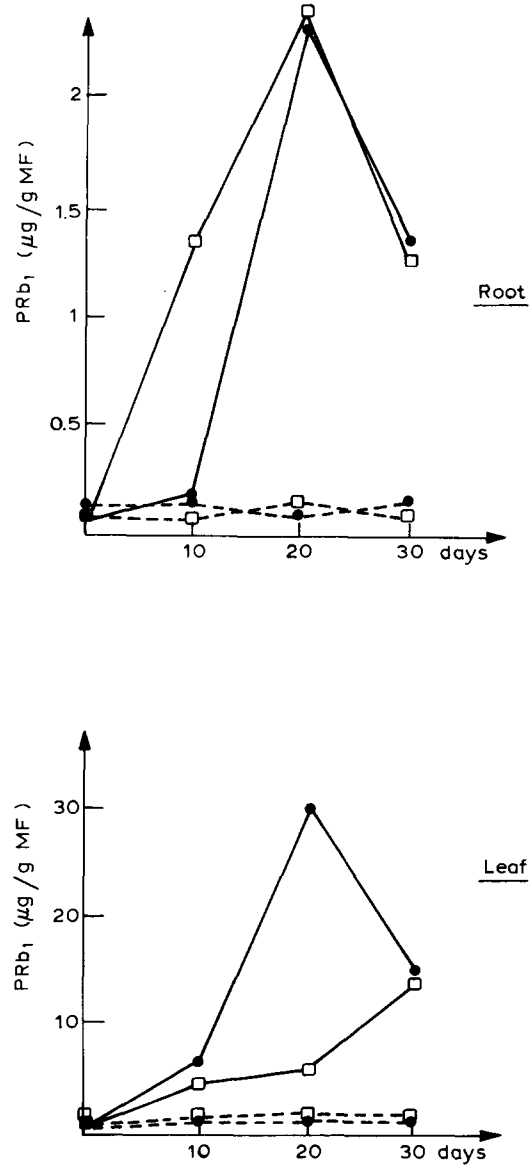

Fig. 3. PR-b1 synthesis in root and leaf tissue of tobacco plantlets grown in the presence of 2-hydroxybenzoic acid (5×10^{-4} M). PR-b1 synthesis was measured by ELISA. Tobacco growth without (---) and with 2-hydroxybenzoic acid (——); ● = *N. tabacum* Xanthi nc; □ = *N. tabacum* Samsun nn.

However, compared to those described in tobacco grown in pots (Abad et al., 1985), the PR-b1 values of in vitro grown plantlets were about 10-fold higher. The important amount of PR-b1 protein detected in healthy plantlets grown in vitro

may reflect inadequate biological or environmental conditions.

Bonnet et al. (1986) have previously demonstrated PR-b1 production during incompatible *Phytophthora* species–tobacco interactions. In the study of root resistance to infection by the fungus *Thielaviopsis basicola,* Ahl (1983) observed no occurrence of PR-b in roots despite the substantial PR-b production in leaves and concluded that PR-b metabolism is not involved in the root's resistance to this fungus. In addition, our results demonstrate PR-b1 production in both leaves and roots after a chemical stress, i.e. exposure to benzoic acid derivatives, suggesting an homology in the resistance mechanism to pathogens in both tissues. However, the roots of in vitro plantlets were exposed to light, whereas Ahl (1983) used roots growing in the dark. These different environmental conditions could explain the absence of PR-b1 as observed by Ahl (1983), if we assume that light regulates PR-b1 synthesis (Abod et al., 1986). However, recent observations with ELISA test have indicated the occurrence of PR-b1 in roots grown in the dark from plants grown in pots (unpublished results). The discrepancy between our results and those found by Ahl (1983) seems to be due to the sensitivity threshold of the techniques employed. ELISA is more sensitive than electrophoresis.

Stimulation of PR-b1 synthesis occurred more rapidly after benzoic derivative treatments of tobacco leaf discs than after infection of *N. tabacum* leaves with TMV (Abad et al., 1985). In the latter case PR-b1 production was not observed until 48 h after infection. This difference can be expected since the virus had to multiply before the plant could respond. Furthermore, the PR-b1 production kinetics are highly dependent on the structure of the benzoic acid.

A structure–function analysis permits classification of the benzoic acid derivatives into three groups, depending on their PR-b inducing ability. The position of the hydroxyl groups critically determines the activity of the hydroxybenzoic acids. Van Loon (1983) indicated that 2,3-, 2,4- and 2,5-hydroxybenzoic acids did not stimulate PR-b synthesis because they were oxidized by peroxidase present in the intercellular space, giving rise to reddish brown products. However, our results revealed no oxidation symptoms with 2,3-hydroxybenzoic acid and strong oxidation with moderately stimulatory compounds of PR-b synthesis such as 2-hydroxy-5-aminobenzoic and 2-hydroxy-5-nitrobenzoic acids. The results indicate that the PR-b1 inducing ability of benzoic acid derivatives is not correlated with peroxidase inactivation.

As far as plantlet growth was concerned, roots were more affected than leaves. This is probably due to a direct contact with the benzoic acid derivatives. PR-b1 synthesis seems to be correlated with the relative growth rate. In our conditions, however, the occurrence of PR-b1 in *N. tabacum* Xanthi nc leaves preceded the growth impairing symptoms. On the other hand, with *N. tabacum* Samsun nn, the maximal PR-b1 production concurred with maximal inhibition of plantlet growth (after 20–30 days). In the root system, PR-b1 production was smaller than in the leaf system. The amplification factor was 10 in roots as compared to 100 in leaves, suggesting a different response of these tissues to the chemicals used. The maximal PR-b1 production was noted at the time that root growth was most severely impaired.

Our results suggest that 2-hydroxybenzoic acid and related compounds can stimulate active defence mechanisms in plants, as already suggested by Pennazio et al. (1985), and that PR-b proteins are produced in response to chemical stress.

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