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Accumulation of scopoletin is associated with the high disease resistance of the hybrid *Nicotiana glutinosa* × *Nicotiana debneyi*

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Abstract. The high disease resistance of the amphidiploid hybrid of *Nicotiana glutinosa* × *Nicotiana debneyi* is associated with high constitutive levels of two phenolic compounds as analysed by high-performance liquid chromatography. The structures of these two compounds were elucidated by means of gas chromatography-tandem mass spectrometry, fluorescence- and light-spectrophotometry to be those of scopolin and scopoletin. They reached levels of 4 nmol·(g FW)$^{-1}$ and 35 nmol·(g FW)$^{-1}$, respectively, in leaf tissues of the hybrid, about 10–50 times the amount found in the parental species. Scopoletin showed a direct antimicrobial activity against *Cercospora nicotianae*, *Phytophthora parasitica* var. *nicotianae*, *Pseudomonas syringae* pvs. *tabaci* and *syringae* and tobacco mosaic virus when added to synthetic growth media, mixed with the inoculum or sprayed onto tobacco plants prior to inoculation. We postulate that the high amount of toxic phenolics in the leaves of the hybrid *N. glutinosa* × *N. debneyi* contributes to its high disease resistance.

Key words: Disease resistance – *Nicotiana* – Scopoletin – Scopolin

Introduction

The amphidiploid hybrid *Nicotiana glutinosa* × *Nicotiana debneyi* is far more resistant to viral, bacterial and fungal diseases than either parental species (Ahl and Gianinazzi 1982; Ahl Goy et al. 1992). It constitutively accumulates pathogenesis-related (PR) proteins (Ahl and Gianinazzi 1982) and an inhibitor of virus replication (IVR, Loebenstein et al. 1990). It also contains constitutively high levels of chitinase, β-1,3-glucanase, peroxidase and polyphenoloxidase (Ahl Goy et al. 1992). These proteins are usually not present in tobacco but appear after inoculation of the lower leaves with necrotizing viruses, during development of systemic acquired resistance to secondary infections (Van Loon 1975; Ward et al. 1991). Therefore, the metabolism of the hybrid resembles that of tobacco plants with systemic acquired resistance and it was postulated that IVR, hydrolases and oxidases could directly contribute to the high resistance of the hybrid *N. glutinosa* × *N. debneyi* against various pathogens (Loebenstein et al. 1990; Ahl Goy et al. 1992).

We were interested to study further the mechanism of the high disease resistance in the hybrid *N. glutinosa* × *N. debneyi*. In this report, we describe the possible role of phenolic compounds and present strong evidence that scopoletin could be involved in the high resistance of the hybrid.

Abbreviations: GC/MS-MS = gas chromatography – tandem mass spectrometry; HPLC = high-performance liquid chromatography; PR-proteins = pathogenesis-related proteins; TMV = tobacco mosaic virus; TNV = tobacco necrosis virus

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Materials and methods

Plant material. Seeds of *Nicotiana tabacum* cv. Xanthi-nc, *Nicotiana glutinosa* L., *Nicotiana debneyi* L. and of the amphidiploid *Nicotiana glutinosa* × *Nicotiana debneyi* (Ahl and Gianinazzi 1982) were all provided by the Institut National de la Recherche Agronomique, Dijon, France. Plants were grown in the greenhouse in 1-l pots in a peat-sand mixture (19:1, w/w). Plants were used eight to 10 weeks after sowing. Tobacco mosaic virus (TMV) was used to provoke local lesions on *N. glutinosa* and on *N. glutinosa* × *N. debneyi*. As *N. debneyi* is sensitive to TMV, tobacco necrosis virus (TNV) was used to provoke local lesions on this species.

Extraction and analysis of total phenols. One gram of leaf material from virus- or water-inoculated *N. glutinosa*, *N. debneyi* or *N. glutinosa* × *N. debneyi* was harvested 7 d after the inoculation and immediately frozen in liquid nitrogen. The leaves were then macerated with 2 ml of 80% methanol, allowed to stand overnight at −20° C, filtered and reextracted twice for 2 h with 1 ml 80% methanol. The three methanolic phases were combined for each extract and directly subjected to separation by high-performance liquid chromatography (HPLC) on a 5-μm C-18-octadodecylsilane column (250 mm long, 10 mm i.d.; Beckman, Geneva, Switzerland) equilibrated with 5% acetonitrile and 0.1% trifluoroacetic acid.

Elution at 2 ml · min^{-1} was programmed as a linear gradient of 5–50% acetonitrile over 40 min. Peak detection was by fluorescence (excitation, 290 nm; emission, 402 nm). To obtain leaf exudates, fully developed leaves of virus- or water-inoculated *N. glutinosa*, *N. debneyi* or *N. glutinosa* × *N. debneyi* were harvested 7 d after the inoculation, weighed and their petioles placed in a glass beaker filled with 6 ml of 0.1 M ammonium-acetate buffer, pH 6.0. The leaves were allowed to stand in the dark at room temperature and 100% relative humidity for 7 h. The buffers with the released exudates were filtered and lyophilised. The residues were resuspended in 200 µl of 50% methanol and analysed by HPLC as described above.

Isolation and identification of scopolin and scopoletin. The dominant peaks with retention times of 16.5 min and 23.4 min (peaks A and B in Fig. 1) were collected after HPLC separation, the solvents evaporated and the residues (300–900 µg) further analysed by gas chromatography-tandem mass spectrometry (GC/MS-MS) and fluorescence techniques. For GC/MS-MS, the dried HPLC fractions were dissolved in 100 µl pyridine and 100 µl acetic anhydride was added. The acetylation reaction proceeded at 50° C for 30 min. The excess of the reagent was removed by concentrating the solution to dryness. The residues were dissolved in dichloromethane to a final volume of 50 µl. An aliquot of 1 µl was used for the GC-MS/MS analysis. The analyses were performed on a Carlo Erba 4160 gas chromatograph (Carlo Erba, Milano, Italy) additionally equipped with a constant flow/constant pressure regulator (CP/CF 516; Carlo Erba), linked by a high-temperature interface to a Finnigan TSQ-45 triple-stage quadrupole mass spectrometer (Finnigan-MAT, San Jose, Cal., USA), as reported recently (Blum et al. 1990). The ionisation technique employed was chemical ionisation using isobutane as a reagent gas; argon was used as the target gas for tandem mass spectrometry (MS/MS) experiments (translational energy was about 23 eV). The mixtures were separated on a glass capillary column (20 m long, 0.3 mm i.d.) coated with SDPE-08 (OH-terminated sildiphenylether/siloxane copolymer, made according to Blum and Aichholz 1991). All samples were introduced by cold on-column injection. The carrier gas was hydrogen. The reference compound acetylscopoletin obtained from pure scopoletin (S-2500; Sigma, St. Louis, Mo., USA) following the acetylation procedure described above was employed for preliminary MS/MS experiments and coinjection. Trace analysis was carried out using a characteristic fragmentation pathway of acetylscopoletin (reaction monitoring of MH–CH_2=C=O, m/z 235→193).

Fluorescence measurements were performed on solutions of the residues with concentrations of 10–500 µg in 10 ml ethanol (Uvasol 02850, Fluka, Buchs, Switzerland), with or without 1% 1 N HCl or 1% 1 N NaOH, in square 1-cm cuvettes. The absorbance of the solutions did not exceed 0.1 above 250 nm. Fluorescence spectra were recorded for excitation in the range 250–450 nm with an interval of 20 nm. Excitation spectra were measured for the principal fluorescent compound(s), detecting emission at the corresponding fluorescence maximum. The slit widths at the entrances of the excitation and emission monochromators were 5 and 2.5–3 nm, respectively. Emission spectra were corrected for the wavelength dependency of the detector system.

The UV-visible spectra of peaks A and B were measured directly by separating the crude leaf extracts by HPLC as described above using a diodearray detector (Model 168, Beckman). Scans were performed from 220 to 390 nm. Once the identity of peaks A and B with scopolin and scopoletin, respectively, was established, the amounts of these compounds in plant extracts were determined by HPLC using a standard curve relating the peak area to known amounts of pure scopoletin (84792, Fluka) or scopolin. Scopolin was synthesized from scopoletin as described by Merz (1932).

Biological activity of scopolin and scopoletin. The antimicrobial activities of scopolin and scopoletin against *Cercospora nicotianae* Ellis and Everhart (ATCC strain No. 18366), *Phytophthora parasitica* var. *nicotianae* van Breda de Haan (ATCC strain No. 15409), *Pseudomonas syringae* pv *syringae* van Hall (Ciba strain No. 548) and *Pseudomonas syringae* pv *tabaci* Wolf and Forster (Ciba strain No. 511) were tested in vitro in microtiter plates. Scopoletin was dissolved in water with 10% acetone and scopolin in water at the appropriate concentrations; 10 µl of the solutions was added to 90 µl of nutrient broth (L11; Oxoid Basingstoke, Hampshire, UK) preinoculated with $3 \cdot 10^5 - 10^6$ cfu · ml^{-1} *(P. syringae)*, to 90 µl of 10% Vegetable-8 (V-8)-juice broth (Campbell, Felegara, Italy; Staub and Young 1980) preinoculated with 10^4 zoospores per ml of *P. parasitica* var. *nicotianae* or to 90 µl of Czapek Dox V-8-juice broth (Campbell; Cooke and Jones 1970) preinoculated with 10^4 spores per ml of *C. nicotianae*. The final concentrations of scopolin and scopoletin in the media ranged from 0.02 to 5.6 mM. The plates were incubated in darkness at 22° C, 20 h for the bacteria and 2 d for the fungi. The growth of the microorganisms was recorded by measuring the absorbance at 595 nm with an enzyme-linked immunosorbent assay (ELISA) plate reader (model 2550; BioRad Glattbrugg, Switzerland). Streptomycin and the fungicides TILT and Ridomil (Ciba, Basle, Switzerland) were included in the tests as positive controls. Six replicates were used for each substance and concentration. To measure the activity of scopoletin on TMV, the substance was directly added to the inoculum (1 µg TMV · ml^{-1}) at the final concentration of 10 mM. The inoculum was allowed to stand for 15 min before inoculation on three leaves of *N. tabacum* cv. Xanthi-nc tobacco plants as described previously (Ahl and Gianinazzi 1982); nine replicates were made. Control plants were inoculated with TMV alone. The numbers and the sizes of the viral lesions were recorded 7 d later.

To test the activity of scopolin and scopoletin in vivo, three to four Xanthi-nc tobacco plants were sprayed or drenched with scopolin or scopoletin at various concentrations. Four days later, the plants were inoculated with *C. nicotianae* or TMV, incubated and rated as described by Neuhaus et al. (1991) and Ahl and Gianinazzi (1982), respectively. Control plants were left untreated. For comparison with induced resistance, three tobacco plants were also treated with 2,6-dichloro-isonicotinic acid, recently described as a powerful resistance inducer (Métraux et al. 1991; Staub et al. 1993), or inoculated first on three lower leaves with TMV and then reinoculated on the three next-uppermost leaves with *C. nicotianae* or TMV as just indicated. To see if treatment with scopolin or scopoletin induces the appearance of the PR-proteins in tobacco, 0.5 g of leaf was harvested from the treated plants just before the challenge inoculation and assayed for the presence of the PR-1a protein by ELISA. The indirect procedure described by Torrance (1980) using an alkaline-phosphatase goat antirabbit conjugate was followed, except that monoclonal antibodies raised against the PR-1a protein (Ciba, Research Triangle Park, N.C., USA) were used to trap the antigen.

Results

Comparison of the phenolic contents of leaves and leaf exudates of N. glutinosa, N. debneyi and their hybrid. The phenol contents of leaves of *N. glutinosa*, *N. debneyi* and *N. glutinosa* × *N. debneyi* with or without viral lesions were analyzed by HPLC with fluorescence detection. Leaf exudates were analysed, too.

As shown in Fig. 1A, the chromatogram from the hybrid *N. glutinosa* × *N. debneyi* showed two major peaks with retention times of 16.5 and 23.4 min, which were present only as minor peaks on the chromatograms from the water-inoculated parents. These peaks, called A and B, largely increased on the chromatograms from virus-inoculated leaves of *N. glutinosa* and *N. debneyi* (Fig. 1B). Leaf exudates from the hybrid similarly showed a much larger amount of peak B and, to a lesser extent, of peak A than the parental species (Fig. 1C, D).

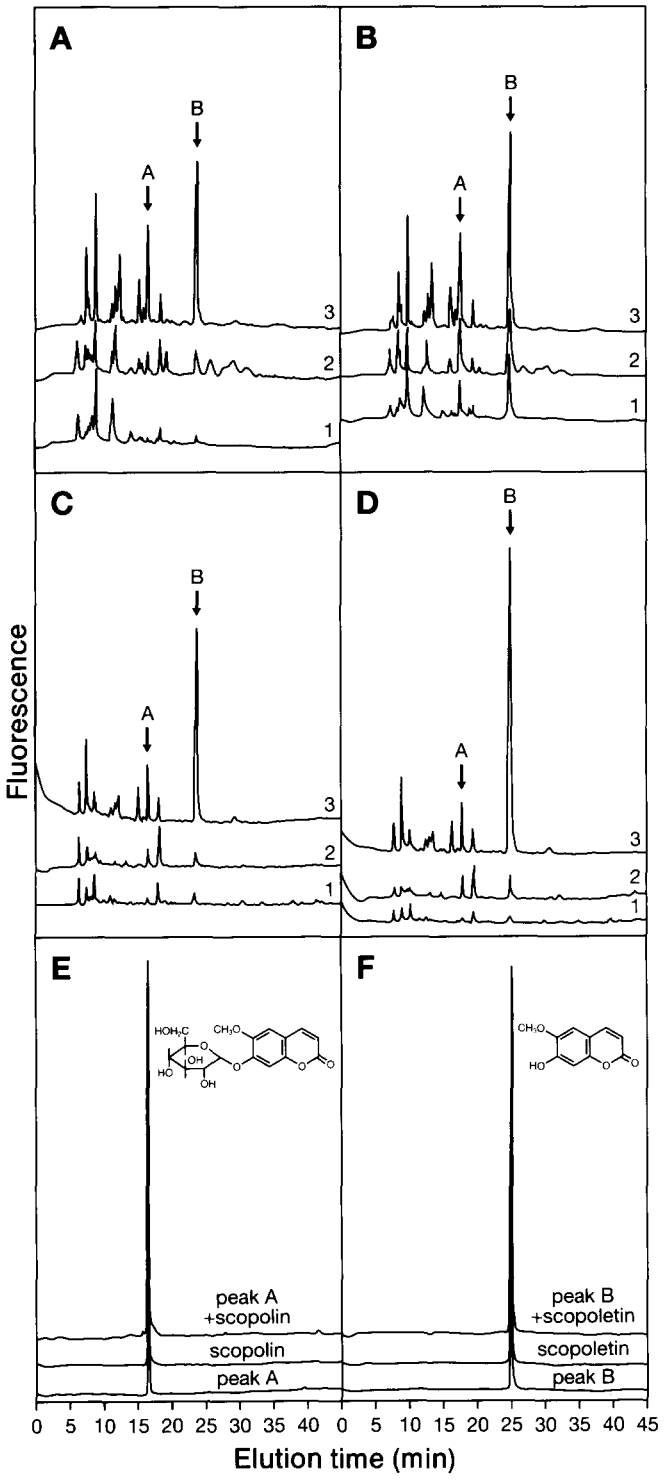

Fig. 1A–D. Chromatograms of leaf extracts (**A, B**) and leaf exudates (**C, D**) from *Nicotiana glutinosa (1), Nicotiana debneyi (2)* and the hybrid *Nicotiana glutinosa* × *Nicotiana debneyi (3)* after inoculation with water (**A, C**) or necrotizing viruses (**B, D**). The *arrows* indicate the major differences between the chromatograms. **E, F** Chromatograms of scopolin, scopoletin and of the isolated peaks A and B

Isolation and identification of peaks A and B. The fluorescent fractions A and B were collected separately. Elucidation of the structure of compounds by means of GC/MS-MS, using chemical ionisation, was feasible after acetylation of each fraction with acetic anhydride. In both fractions, acetylated 6-methoxy-7-hydroxycoumarin (scopoletin) could be found as traces in variable amounts. However, for the unequivocal identification of scopoletin in the complex matrix, a characteristic fragmentation pathway of acetylscopoletin was monitored by MS-MS in order to exclude errors due to impurities, and the GC/MS-MS results were corroborated by co-injection of acetylscopoletin with peaks A and B.

For further characterisation of fractions A and B, fluorescence excitation and emission spectra were measured. A neutral, an acidic and a basic solvent were used in order to detect protonation. The fluorescence in fraction A was dominated by a single emission with a maximum at 420–425 nm and an excitation maximum at 340–345 nm, not affected by acid or base. A very similar fluorescence was observed for fraction B in acidic ethanol; in basic ethanol, the emission and excitation maxima were at 465 and 395 nm, respectively. Thus the principal fluorescent compounds in fractions A and B were expected to be different derivatives of the same chromophoric system. Comparison of the observed excitation and fluorescence maxima with data in the literature (Wolfbeis 1985) pointed to 6,7-di- and 6-mono-substituted 6,7-dihydroxy-coumarins for A and B, respectively. Subsequent measurement of the emission and excitation spectra with comparable solutions of scopolin and scopoletin were in perfect agreement with the spectra of A and B, respectively. This was further confirmed by comigration of peak A with scopolin and peak B with scopoletin on HLPC (Fig. 1E, F), and by the identical UV-visible spectrum of peak A and scopolin (maxima at 226, 252, 288 and 338 nm) and of peak B and scopoletin (maxima at 229, 255, 297 and 343 nm). Scopolin was not detected by GC/MS-MS analysis, presumably because of its lower volatility compared with scopoletin.

Quantification and localisation of scopolin and scopoletin in N. glutinosa, N. debneyi and N. glutinosa × *N. debneyi.* The hybrid *N. glutinosa* × *N. debneyi* contains constitutively about 4 nmol · $(gFW)^{-1}$ scopolin and 35 nmol · $(gFW)^{-1}$ scopoletin (Table 1). This represents 3–18 times the amount of scopolin and 10–50 times the amount of scopoletin present in the lower leaves of *N. debneyi* or *N. glutinosa.* The concentration gradient between the lower and the upper leaves was less pronounced in the hybrid than in the parents. Inoculation with necrotizing viruses induced locally a 25-fold and 35-fold increase of both compounds in *N. glutinosa* and *N. debneyi,* respectively, but no significant changes in the hybrid. Leaf exudates from *N. glutinosa* × *N. debneyi* contained much larger amounts of scopolin and scopoletin than exudates from the parental species. Expressed on a fresh-weight basis, the total amount of scopolin and scopoletin in the leaf exudates represented up to 25% of the total amount found in the leaves for the hybrid but less than 1% for *N. glutinosa* and *N. debneyi.*

Biological activity of scopolin and scopoletin. As the hybrid *N. glutinosa* × *N. debneyi* contains a high amount of

Table 1. Levels of scopolin and scopoletin in leaves and leaf exudates from *N. glutinosa*, *N. debneyi* and from the hybrid *N. glutinosa* × *N. debneyi*

Plant	Treatment	Leaf[a]	Level of (pmol · g FW^{-1})[b]	
			Scopolin	Scopoletin
N. glutinosa	H_2O	lower	230 ± 180	700 ± 290
		upper	20 ± 10	0.0
		exudate	5 ± 5	5 ± 4
N. debneyi	H_2O	lower	1530 ± 730	3560 ± 500
		upper	340 ± 140	1200 ± 980
		exudate	6 ± 5	3 ± 3
N. glutinosa × *N. debneyi*	H_2O	lower	4190 ± 2910	35500 ± 9410
		upper	2440 ± 290	32700 ± 1700
		exudate	619 ± 105	7790 ± 4970
N. glutinosa	TMV	lower	5500 ± 2740	16470 ± 4090
		upper	340 ± 80	1870 ± 490
		exudate	6 ± 4	15 ± 6
N. debneyi	TNV	lower	48340 ± 19570	141230 ± 39540
		upper	460 ± 240	6650 ± 950
		exudate	11 ± 5	18 ± 16
N. glutinosa × *N. debneyi*	TMV	lower	4160 ± 1340	47800 ± 7600
		upper	1250 ± 170	32930 ± 3690
		exudate	999 ± 237	11720 ± 6520

[a] Three lower leaves per plant were inoculated with water or necrotizing viruses. The samples were taken from these leaves (lower) or from the next non-inoculated leaves (upper). The exudates were collected from the inoculated leaves

[b] Data are means ± SD of nine (lower leaves) or three (upper leaves, exudates) measurements. The whole experiment was carried out twice

Table 2. Comparison of the in-vitro inhibitory activities of scopolin and scopoletin towards various fungal and bacterial pathogens with those of antifungal and antibacterial substances

Substance	EC-50 value (mM) against[a]			
	Cercospora nicotianae	*Phytophthora parasitica*	*Pseudomonas syringae* pv	
			syringae	*tabaci*
Scopolin	> 5.6	> 5.6	> 5.6	> 5.6
Scopoletin	1.8	4.2	0.8	2.5
TILT	0.001	–	–	–
Ridomil	–[b]	0.020	–	–
Streptomycin	–	–	< 0.001	< 0.001

[a] Concentration provoking 50% growth reduction

[b] not tested

scopolin and scopoletin and is very resistant to various pathogens, it seemed logical to test whether scopolin and scopoletin are toxic to tobacco pathogens or if these compounds are constitutive signals for induced resistance and might therefore be responsible for the constitutive presence of the PR-proteins in the hybrid.

Scopoletin decreased by 50% the in-vitro growth of *C. nicotianae* (frog-eye disease), *P. parasitica* var. *nicotianae* (black-shank disease), *P. syringae* pvs *tabaci* (wildfire disease) and *syringae* at 1.8, 4.2, 2.5 and 0.8 mM, respectively, and totally inhibited the growth of these microorganisms at 10 mM. Scopolin showed no activity at the concentrations tested (Table 2). When sprayed on *N. tabacum* cv. Xanthi-nc plants 4 d prior to inoculation with *C. nicotianae*, scopoletin retarded the development of frog-eye symptoms at both 3 and 10 mM, but scopolin was inactive (Fig. 2). For comparison with induced resistance, plants were also preinoculated on the lower leaves with TMV or treated with 2,6-dichloro-isonicotinic acid (foliar spray 0.3 mM, or soil drench 0.03 mM), a resistance inducer (Métraux et al. 1991; Staub et al. 1993), prior to challenge inoculation with *C. nicotianae*. These treatments did not induce the production of endogenous scopolin and scopoletin in the upper leaves (data not shown) but protected them against frog-eye in a much more efficient way than the scopoletin spray.

The activity of scopolin and scopoletin against TMV was also tested, either by applying the substances on the plants or by mixing it with the inoculum. Scopoletin decreased by 40% and 25% the number of local lesions when sprayed on the plant at the concentration of 10 mM or 3 mM, respectively, but did not affect the size of the lesions. Scopolin was inactive. 2,6-Dichloro-isonicotinic acid (foliar spray 0.3 mM, or soil drench 0.03 mM) or a

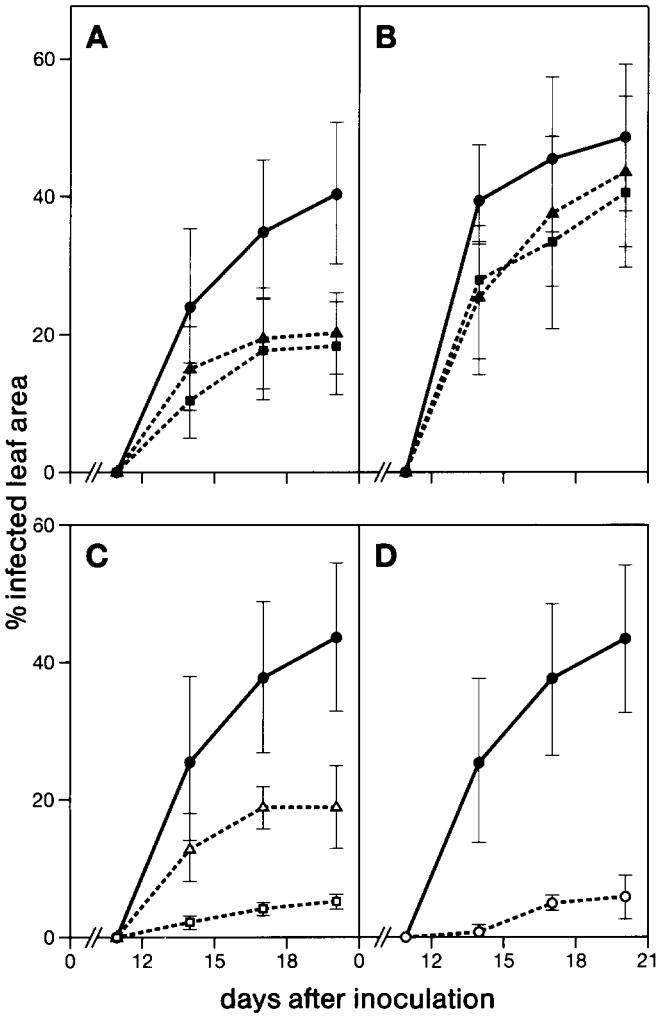

Fig. 2A–D. Development of symptoms on *Nicotiana tabacum* cv. Xanthi-nc plants inoculated with *Cercospora nicotianae* after treatment with scopoletin (**A**), scopolin (**B**), 2,6-dichloro-isonicotinic acid (**C**) or preinoculation on the lower leaves with TMV (**D**). **A–D** ●–●, control. **A** ■----■, spray, 10 mM; ▲----▲, spray, 3 mM. **B** ■----■, spray, 5.6 mM; ▲–▲, spray, 1.6 mM. **C** △----△, spray, 0.3 mM; □----□, soil drench, 0.03 mM. **D** ○----○, TMV preinoculation. Each datum point represents the mean of 12 determinations (4 plants treated, 3 leaves per plant inoculated)

preinoculation with TMV greatly reduced both the number and the size of the viral lesions (Table 3). Mixing scopoletin directly with the inoculum at a concentration of 10 mM also decreased the number of TMV local lesions by 50%.

Finally, samples removed from the treated plants just before challenge inoculation were analysed for the presence of the PR-1a protein. Application of scopolin or scopoletin at concentrations up to 5.6 and 10 mM, respectively on Xanthi-nc tobacco plants did not induce the appearance of the PR-1a protein in the leaves, whereas drenching with 0.03 mM of 2,6-dichloro-isonicotinic acid or preinoculation with TMV induced the production of $2.8 \pm 1.5\ \mu g \cdot (gFW)^{-1}$ and $23.4 \pm 2.0\ \mu g \cdot (gFW)^{-1}$, of PR-1a, respectively, in the upper leaves.

Discussion

The disease-resistant hybrid of *N. glutinosa* × *N. debneyi* contains constitutively high levels of scopolin ($4\ nmol \cdot (gFW)^{-1}$) and scopoletin ($35\ nmol \cdot (gFW)^{-1}$), whereas the parental species contain much smaller amounts of these compounds. Inoculation with necrotizing viruses provoked the appearance of well-developed local lesions on *N. glutinosa* and *N. debneyi* and increased the amounts of scopolin and scopoletin to levels comparable to or higher than those found in the hybrid. Inoculation of the hybrid resulted in the development of very few tiny lesions (Ahl and Gianinazzi 1982) and did not change substantially the amounts of scopolin and scopoletin.

Scopolin and scopoletin have already been cited with respect to diseases in *N. tabacum:* both compounds were reported to accumulate in roots attacked by *Phytophthora parasitica* var. *nicotianae* (Snook et al. 1991), in stems infected with *Pseudomonas solanacearum* (Sequeira and Kelman 1962) and in leaves infected with TMV (Fritig et al. 1972) or *Peronospora tabacina* (Reuveni and Cohen 1978). Only scopolin increased in tobacco callus inoculated with *Thielaviopsis basicola,* the concentration of scopoletin remaining unchanged (Gasser et al. 1988). The exact role of scopolin/scopoletin in these plant-pathogen interactions is unclear. On the one hand, cultivars resistant or susceptible to *P. parasitica* var. *nicotianae* or *P. solanacearum* both accumulated scopolin and scopoletin in a similar way after infection (Sequeira and Kelman 1962; Snook et al. 1991); on the other hand, only the TMV-resistant cultivars accumulated scopoletin after inoculation with TMV (Tanguy and Martin 1972) and only the *T. basicola*-resistant cultivars accumulated scopolin after inoculation with this fungus (Gasser et al. 1988). Cohen and Kuc (1981) also showed that tobacco plants systemically protected against blue mold by a stem injection with conidia of *P. tabacina* accumulated scopoletin in their stem and leaves prior to challenge inoculation.

We have tested the possible involvement of scopolin and scopoletin in the resistance of the hybrid *N. glutinosa* × *N. debneyi* to diseases. Spraying scopolin or scopoletin on Xanthi-nc tobacco plants did not induce the production of PR-proteins. Therefore, unlike salicylic acid in *N. tabacum* (Malamy et al. 1990), these compounds do not seem to act as signals for induced resistance in the hybrid *N. glutinosa* × *N. debneyi;* their presence in phloem exudates also does not seem to make them responsible for the graft transmissibility of the resistance from the hybrid *N. glutinosa* × *N. debneyi* into other tobacco plants, which is linked to the production of PR-proteins (Gianinazzi and Ahl 1983).

Scopolin or scopoletin could protect the hybrid by being directly toxic to the pathogens. We showed that, in vitro, scopoletin is toxic to *Cercospora nicotianae, P. parasitica* var. *nicotianae, P. syringae* pvs *tabaci* and *syringae* and TMV, five pathogens towards which the hybrid is highly resistant (Ahl Goy et al. 1992). Moreover, spraying scopoletin at concentrations close to the in-vitro EC-50 values on Xanthi-nc tobacco plants prior

Table 3. Activity of scopolin and scopoletin against TMV on *N. tabacum* cv. Xanthi-nc

Substance	Treatment and concentration (mM)	Number of TMV lesions per leaf[a]	Diameter of TMV lesions (mm)[a]
Control	–	427±104	1.20±0.51
Scopoletin	leaf spray, 10	265± 59*	1.13±0.31
	leaf spray, 3	335±111	1.09±0.31
	soil drench, 0.3	240± 85*	1.10±0.33
	soil drench, 0.1	474±142	1.41±0.28
Scopolin	leaf spray, 5.6	520± 43	1.41±0.42
	leaf spray, 1.6	537± 98	1.22±0.34
2,6-dichloro-isonicotinic acid	leaf spray, 0.3	180± 69*	0.76±0.36*
	soil drench, 0.03	121± 39*	0.71±0.20*
TMV[b]	–	148± 41*	0.73±0.27*
Control	–	261±124	2.51±0.37
Scopoletin	mixed with inoculum, 10	139± 73*	2.47±0.32

[a] Data are means±SD; for each determination, 9 leaves were used and 30 to 40 lesions were measured. The values which are significantly different from the control values at $P<0.05$ are indicated by*

[b] TMV was inoculated on the lower leaves 4 d prior to the challenge inoculation

to inoculation with *C. nicotianae* or TMV reduced the number of lesions. Scopolin was inactive in all tests. These results are in good agreement with earlier reports on the toxicity of scopoletin (Mikulska-Macheta 1976; Gasser et al. 1988; Snook et al. 1991).

The exact mechanism of scopoletin toxicity in tobacco plants is not known. Scopoletin acts in vitro in the millimolar range, whereas it is present in the hybrid at the concentration of 35 nmol · $(gFW)^{-1}$, which corresponds to 50–100 µM. Therefore, two hypotheses may be advanced. The first hypothesis implies an uneven repartition of scopoletin within the leaves; local concentrations, e.g. near the leaf surfaces which are the first contact points for the pathogens, could be 10–50 times higher than the global concentration, reaching levels toxic for pathogens according to the in-vitro data. This would indicate the importance of the amount of scopoletin at the time of the infection and not just of its accumulation after infection; it would also explain why the constitutive level of scopoletin in the hybrid is lower than the levels reported to accumulate in tobacco plants after inoculation with *P. tabacina* (Reuveni and Cohen 1978), TMV (Fritig et al. 1972), or here with TNV in *N. debneyi*, but is nevertheless sufficient to provide good protection. The second hypothesis is that the toxicity of scopoletin could be enhanced in situ by peroxidation reactions. Scopoletin was found to be an endogenous substrate for a specific peroxidase isoenzyme in tobacco (Reigh et al. 1973); the hybrid *N. glutinosa* × *N. debneyi* has a much higher peroxidase activity than the parental species and contains additional isoperoxidases (Ahl Goy et al. 1992). Although we have not checked if a "scopoletin peroxidase" as defined by Reigh et al. (1973) is present in the hybrid, it may be hypothesized that such an enzyme exists and catalyses a reaction giving rise to the formation of products with a higher toxicity than scopoletin itself.

The reason that the hybrid *N. glutinosa* × *N. debneyi* accumulates high amounts of scopoletin and scopolin is unclear. The activity of the phenylalanine ammonia-lyase, the first enzyme involved in the synthesis of coumarin derivatives, is not enhanced in the hybrid compared with the parental species (Ahl Goy et al. 1992). A lack of correlation between the activity of the phenylalanine ammonia-lyase and the amount of scopolin and scopoletin was also reported by Massala et al. (1980). Therefore, other steps must be important for the regulation of the synthesis of coumarin derivatives.

In conclusion, the hybrid *N. glutinosa* × *N. debneyi* shows the metabolic characteristics of an induced tobacco plant (e.g. accumulation of PR-proteins, chitinase, peroxidase) with toxic phenolics in addition. It may be postulated that all these parameters contribute to the high and broad disease resistance of this plant.

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