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GC/MS ANALYSIS OF FUNGITOXIC TERPENOIDS FROM TOBACCO

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Key Word Index—*Nicotiana tabacum*, Solanaceae; TMV, sesquiterpenoids; capsidiol; solavetivone, 3-hydroxysolavetivone; 3-hydroxylubimin, rishitin; epirishitin, glutinosone; oxyglutinosone; phytoalexins, stress metabolites.

Abstract—Capsidiol, solavetivone, 3-hydroxysolavetivone, 3-hydroxylubimin, rishitin, epirishitin, glutinosone and oxyglutinosone were isolated from TMV-inoculated leaves of tobacco (cv Samsun NN) and identified by GC/MS analysis.

INTRODUCTION

In various studies, a number of different sesquiterpenoid and related phytoalexins or stress metabolites have been isolated from diseased or stressed tobacco plants, belonging to several species and cultivars, within the genus *Nicotiana* (Table 1) For the structural formulae of tobacco phytoalexins and their biosynthesis the reader is referred to recent reviews by Kuć [13] and Stoessl [14]

In an investigation on the possible role of phytoalexins in disease resistance in tobacco [15], we examined leaves of *Nicotiana tabacum* cv Samsun NN inoculated with several *Pseudomonas* species or with TMV, or treated with aqueous solutions of mercuric chloride. Preliminary experiments showed especially TMV-inoculated leaves to produce large numbers of fungitoxic substances which seemed worth closer examination.

RESULTS AND DISCUSSION

TMV-inoculated tobacco leaves were extracted as outlined below (see Experimental). The TLC bioassay used to detect fungitoxic compounds [16] revealed up to six bands with fungitoxic activity. Capsidiol (band 5, see below) was the only fungitoxic compound detected in tobacco leaves after 2 days and up to 13 days the quantity remained the same. Bands 1, 3 and 4 appeared after 3 days and had declined after 10 days especially in the cases of bands 3 and 4. Band 2 appeared some 8 days after inoculation and then declined. In view of the small scale of the experiments which resulted in only microgram quantities of the compounds of interest in each extract examined, GC/MS analysis was considered to be the most appropriate method for the further study and identification of these compounds. The mass spectra were

Table 1 Sesquiterpenoid phytoalexins and stress metabolites known to occur in various species and cultivars of *Nicotiana**

Species and cultivar	Pathogen or abiotic inducer	Phytoalexin/ stress metabolite	Reference†
N clevelandii	TNV	Capsidiol	[1]
N glutinosa	TMV	Glutinosone	[2]
N rustica	TMV	Phytuberol	[3]
N sylvestris	TMV, TRV	Phytuberin, phytuberol, solanascone, solavetivone	[4]
N tabacum cv KY 16	*Ps lachrymans*	Phytuberin	[5]
N. tabacum cv Samsun NN	Ethrel, TMV	Phytuberin, phytuberol	[6, 4]
	TMV	3-Hydroxysolavetivone, solanascone, solavetivone	[4, 7]
N tabacum cv White Burley	TNV	Capsidiol	[1]
N tabacum cv Xanthi-nc	TMV	Solavetivone	[8]
N. tabacum × *N glutinosa*	TMV	Glutinosone, 3-hydroxysolavetivone, phytuberin, phytuberol, solanascone, solavetivone	[4]

*In addition to the phytoalexins listed, rishitin and an isomer of rishitin have recently been reported from *Ph. parasitica* var *nicotianae*-inoculated tobacco callus tissues [9]. Several of the sesquiterpenoids mentioned have earlier been obtained in very small amounts from apparently healthy tobacco [10–12]

†Only earliest reference given

checked with spectral data reported in the literature or compared with those of authentic samples No attempts were made to quantify the components present.

Band 1 (R_f 0 82) contained, besides traces of glutinosone and solavetivone (both less than 5 % of extractable content of band 1), 3-hydroxysolavetivone as the major constituent These compounds had been reported earlier as stress metabolites from tobacco (cf Table 1)

Band 2 (R_f 0 51) was only visible in extracts of leaves more than 6 days after inoculation with TMV This band contained one compound with an elemental composition of $C_{17}H_{26}N_2O$ ([M]$^+$ 274 2050, calc 274 2045) The base peak in the mass spectrum (190 1093) was due to loss of hexane, while the next intense peak was at *m/z* 189 1018 (loss of C_6H_{13}). These fragments possibly originated from a compound containing a C_7H_{15} group attached to a highly unsaturated (six double bonds plus rings) $C_{10}H_{11}N_2O$ system

Analysis of band 3 (R_f 0 37) showed the presence of three compounds The main component was identified as oxyglutinosone Its mass spectrum was found to be essentially similar to that of authentic oxyglutinosone and the elemental composition found for the fragment [M $-H_2O$]$^+$ was $C_{14}H_{18}O_2$ (measured mass 218 1304, calc 218.1307), which agreed well for this norsesquiterpenoid This compound had been reported earlier as a phytoalexin in potato tubers [17] As for the other two compounds in this fraction, the relative concentrations varied widely for the various extracts The compounds are, as yet, unidentified isomers with elemental composition of $C_{15}H_{26}O_2$ (measured mass of the [M $-H_2O$]$^+$ fragment 220 1830; calc 220 1827) From the mass spectrum it seems probable that both oxygens are present in a 1,2-dihydroxyisopropyl group

The fungitoxic band 4 (R_f 0.28) contained two isomeric compounds, A and B, compound A in most extracts examined being more abundant than compound B. The mass spectra of both compounds showed the characteristic features (see Experimental) of that of rishitin Comparison with the R_f and mass spectrum of authentic rishitin showed the minor constituent (B) to be rishitin The mass spectrum of the main component (A) was almost identical to that of rishitin except for the intensity of the peak corresponding with the [M $-H_2O$]$^+$ fragment, which was much lower compared with the intensity in the spectrum of authentic rishitin (9 vs 52 %). Reaction of a 1 1 mixture of both compounds with acetone, in the presence of *p*-toluenesulfonic acid, gave a nearly complete conversion of A into the acetonide while only 25 % of B (rishitin) was converted This indicated that A was most probably 2- or 3-epirishitin Rishitin and an isomer of rishitin have recently been reported to accumulate in tobacco callus tissues after challenge with *Phytophthora parasitica* var *nicotianae* [9]

A third compound present in this band appeared to be 4,8,13-duvatriene-1,3-diol This diterpene is known as a constituent of tobacco leaf surface lipids [18].

The main compound in band 5 (R_f 0 20) was identified as capsidiol, a well-known phytoalexin of tobacco [1] For some extracts this band also contained minor quantities of 3-hydroxylubimin This sesquiterpenoid had earlier been reported to occur in diseased potato tubers [19]

Band 6 (R_f 0 09) was due to nicotine, present in relatively large amounts as compared with the other substances

Bioassays of extracts of water-inoculated control leaves revealed only two fungitoxic bands One band (R_f 0 30) was due to duvatrienediol, the other band (R_f 0.09) consisted of nicotine Except for these two compounds none of the other substances described above was found in the extracts of healthy leaves

Table 2 summarizes the main TLC, GC and mass spectral data on the fungitoxic terpenoids as found by us in TMV-inoculated tobacco leaves Of these, oxyglutinosone and 3-hydroxylubimin have not been isolated before from tobacco, epirishitin and rishitin have only been reported for tobacco callus tissues [9] Remarkably and as distinct from other reports (cf Table 1) we have never observed the presence of phytuberin nor of phytuberol

EXPERIMENTAL

Tobacco plants (*N tabacum* cv Samsun NN) were grown in the greenhouse at 22 *Ca* 6 8 weeks after sowing, when 3 4 leaves were fully expanded, three of these were inoculated with TMV strain WU-1 (courtesy Dr L C van Loon, Department of Plant Physiology, Agricultural University, Wageningen) At intervals from 1 to 13 days after inoculation, leaves were harvested and extracted in 40 % aq EtOH (× 2) for 2 hr These extracts were taken to dryness and the residues dissolved in 60 % aq MeOH The 60 % MeOH solns were shaken (× 3) with equal vols of $CHCl_3$, the combined $CHCl_3$ extracts were taken to dryness and

Table 2 Fungitoxic terpenoids in TMV-inoculated tobacco leaves

Compound	R_f-value*	Retention index†	Reference for mass spectral data
Glutinosone	0 82	2425	[2]
3-Hydroxysolavetivone	0 82	2524	[4]
Solavetivone	0 82	2425	[10]
Oxyglutinosone	0 37	2788	See text
4,8,13-Duvatriene-1,3-diol‡	0 30	2862	[20]
Epirishitin	0 28	2459	See text
Rishitin	0 28	2495	See text
Capsidiol	0 20	2618	See text
3-Hydroxylubimin	0 20	Decomp	See text

*TLC Si gel (Merck), $CHCl_3$–MeOH (19 1)

†OV-225 at 200°

‡Also present in water-inoculated control leaves

the residues dissolved in Me_2CO. Aliquots corresponding to 4·3 g (fr wt) of leaves were examined for the presence of fungitoxic compounds using a TLC bioassay [16] on TLC Al sheets precoated with Si gel 60 F_{254} (Merck), with $CHCl_3$–MeOH (19:1) as the solvent system and with *Cladosporium cucumerinum* as the test organism. After incubation of the TLC plates for 2 days, the bands showing fungitoxic activity were scraped off and the Si gel was then extracted with Me_2CO. If necessary, purification was achieved by a second TLC separation.

H_2O-inoculated control leaves were extracted similarly, however, the samples assayed for fungitoxic activity corresponded to 8 g (fr wt) of leaves.

The extracts of the TLC bands were analysed by GC and GC/MS. An OV-225 column (3% phase on Chromosorb-WHP 100–120, column dimensions 1.5 m × 2 mm diam.) was used either isothermally at 200° (GC) or in a programmed mode from 160° to 200° (GC/MS). The MS were taken with a VG MM 70–70 F mass spectrometer using electron impact ionization (70 eV, ion source temp. 200°). Accurate masses were measured with an AEI MS-902 mass spectrometer by means of peak matching.

MS oxyglutinosone *m/z* (rel. int.): 236 $[M]^+$ (5), 218 (11), 200 (5), 178 (100), 135 (36), 121 (41), 110 (26), 107 (17), 69 (20), 68 (32). The spectrum was in excellent agreement with the spectrum obtained by Murai *et al.* [personal communication].

MS rishitin *m/z* (rel. int.): 222 $[M]^+$ (10), 204 (52), 189 (15), 188 (7), 161 (69), 143 (100), 119 (77), 105 (67), 91 (70), 41 (61). The spectrum of epirishitin was almost the same, except for the much reduced intensities of *m/z* 222 $[M]^+$ (2) and 204 $[M - H_2O]^+$ (9).

MS capsidiol *m/z* (rel. int.): 236 $[M]^+$ (3), 221 (39), 218 (61), 203 (43), 200 (29), 157 (77), 143 (80), 107 (83), 105 (91), 93 (100), 91 (87), 41 (97) and for 3-hydroxylubimin: 252 $[M]^+$ (6), 234 (13), 209 (14), 165 (40), 163 (34), 136 (72), 135 (67), 107 (78), 93 (80), 41 (100). The full spectra were in good agreement with spectra obtained by Stoessl *et al.* [personal communication].

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