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The arbuscular mycorrhizal fungus, *Glomus intraradices*, induces the accumulation of cyclohexenone derivatives in tobacco roots

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Abstract. Tobacco (*Nicotiana tabacum* L.) plants were grown with and without the arbuscular mycorrhizal fungus, *Glomus intraradices* Schenk & Smith. High-performance liquid chromatographic analyses of methanolic extracts from mycorrhizal and non-mycorrhizal tobacco roots revealed marked fungus-induced changes in the patterns of UV-detectable products. The UV spectra of these products, obtained from an HPLC photodiode array detector, indicated the presence of several blumenol derivatives. The most predominant compound among these derivatives was spectroscopically identified as 13-hydroxyblumenol C 9-*O*-gentiobioside (''nicoblumin''), i.e. the 9-*O*-(6′-*O*-β-glucopyranosyl)-β-glucopyranoside of 13-hydroxy-6-(3-hydroxybutyl)-1,1,5-trimethyl-4-cyclohexen-3-one, a new natural product. This is the first report on the identification of blumenol derivatives in mycorrhizal roots of a non-gramineous plant.

Key words: Arbuscular mycorrhiza – Blumenol derivative – Cyclohexenone derivative – *Glomus* – *Nicotiana* – Isoprenoid induction

There is increasing evidence that secondary compounds play an important role in various interactions between plants and their environment, including the symbiotic relationship between plant roots and arbuscular mycorrhizal (AM) fungi (Morandi 1996). In this respect, isoprenoid metabolism seems to play an as yet unknown role in AM roots of cereals and other members of the Poaceae (Maier et al. 1995, 1997). It was found that the AM fungus, *Glomus intraradices* Schenk & Smith, induces the accumulation of sesquiterpenoid cyclohexenone derivatives, with blumenin, i.e. the 9-*O*-(2′-*O*-β-glucuronosyl-β-glucopyranoside of 6-(3-hydroxybutyl)-1,1,5-trimethyl-4-cyclohexen-3-one, as the predominant constituent (Maier et al. 1995). The possible role of these compounds in mycorrhizal symbiosis is unknown. However, exogenously applied blumenin strongly inhibits colonisation and development of arbuscules in the early stages of barley mycorrhization (Fester et al. 1998), indicating that cyclohexenone derivatives might be involved in mycorrhizal control.

The biosynthetic origin of the cyclohexenone derivatives is unknown, but the structural similarity to abscisic acid of the aglycone of blumenin, blumenol C, indicates that they are products of a dioxygenase-catalysed carotenoid-cleavage reaction. This suggestion has been supported by feeding experiments with ^{13}C-labelled glucose, indicating that biosynthesis of blumenol C proceeds via the novel glyceraldehyde 3-phosphate/pyruvate pathway (Maier et al. 1998) known to lead also to carotenoids (Lichtenthaler et al. 1997). This is in line with our recent finding of strongly induced expression in mycorrhizal wheat roots of the gene encoding the key enzyme of this pathway, 1-deoxyxylulose-5-phosphate synthase (data not shown).

In our current studies on the accumulation of secondary compounds in AMs, we have found, and report here for the first time, AM-fungus-induced accumulation of cyclohexenone derivatives in a non-gramineous plant. Colonisation of roots of tobacco (Solanaceae) with *G. intraradices* induced the accumulation of various cyclohexenone derivatives. The predominant constituent was isolated and identified by spectroscopic methods as 13-hydroxyblumenol C 9-*O*-gentiobioside (''nicoblumin''), i.e. the 9-*O*-(6′-*O*-β-glucopyranosyl)-β-glucopyranoside of 13-hydroxy-6-(3-hydroxybutyl)-1,1,5-trimethyl-4-cyclohexen-3-one.

Abbreviations: AM = arbuscular mycorrhizal/mycorrhiza; COSY = correlation spectroscopy; 1D, 2D = one, two dimensional; HMBC = heteronuclear multiple bond correlation; NMR = nuclear magnetic resonance

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Plant material and AM-fungus inoculation. Tobacco (*Nicotiana tabacum* L. cv. Samsun NN) plants were grown in 1-L plastic pots (three plants per pot) filled with expanded clay (Lecaton; 2–5 mm

particle size; Fibo Exclay, Pinneberg, Germany) and inoculated with the AM fungus, *Glomus intraradices* Schenck & Smith, by application of propagules in expanded clay (isolate 49, provided by H. von Alten from the collection of the Institut für Pflanzenkrankheiten und Pflanzenschutz der Universität Hannover, Germany). The arbuscular mycorrhizal fungus, *G. intraradices*, was propagated in our laboratory by inoculation of *Tagetes erecta* roots and growth in expanded clay for 4 months. Mycorrhizas were induced in tobacco by growing the plants in expanded clay mixed with 10% (v/v) of the fungal inoculum. Details of plant growing conditions have been published previously (Maier et al. 1995). As a control, tobacco plants were mock-inoculated with expanded clay from non-mycorrhizal *Tagetes*. The approximate percentage values for mycorrhiza formation were estimated microscopically by counting the frequency of colonisation from 30 root pieces (2 cm) after staining with trypan blue in lactophenol according to a procedure described by Phillips and Hayman (1970).

Sample preparation. Analytical root extraction. Freshly harvested whole tobacco roots (15 weeks old) were washed with water, cut into small pieces, and aliquots (l g fresh weight) were treated twice for about 1 min with an Ultra Turrax homogenizer (Janke & Kunkel, Staufen, Germany) in 5 ml 80% aqueous methanol. The mixture was centrifuged and the supernatant was used for HPLC analysis (20-μl aliquots).

Preparative root extraction and fractionation. Freshly harvested mycorrhizal tobacco roots (24.5 g) were washed with water, cut into small pieces and transferred into 100 ml of 80% aqueous methanol. The plant material was treated twice with an Ultra Turrax homogenizer for about 1 min and the homogenates were allowed to stand for 30 min with continuous stirring. The suspension was filtered and the residue re-extracted twice. The combined extracts were evaporated to dryness at 40 °C in a vacuum, the residue re-dissolved in 8 ml of water, centrifuged, and the supernatant separated using a preparative HPLC apparatus (Beckman Instruments, System Gold with a photodiode array detector 168), equipped with a Nucleosil 100–10 C_{18} column (VarioPrep; 10 μm, 250 mm long, 40 mm i.d.; Macherey-Nagel, Düren, Germany). The compounds were separated at a flow rate of 15 ml min^{-1} with a linear gradient within 10 min from 30% solvent B (methanol) to 50% solvent B in solvent A (0.4% formic acid in water) followed by a 70-min isocratic elution.

High-performance liquid chromatography. The Waters 600 HPLC system (Waters, Milford, Mass., USA), the Nucleosil C_{18} column (Macherey-Nagel), and quantification of nicoblumin by using *cis*-abscisic acid as the external standard compound were as described by Maier et al. (1995). However, to optimize the separation of the extract components we used a different linear gradient elution system [at a flow rate of 1 ml min^{-1} within 30 min from 5 to 20% solvent B (acetonitrile) in solvent A (1.5% ortho-phosphoric acid in water)] adding 5 min at 20% B.

Mass spectrometry (MS) and nuclear magnetic resonance (NMR). The electrospray (ES) mass spectra were obtained from a Finnigan MAT TSQ 7000 instrument [electrospray voltage 4.5 kV (positive ions), 3.5 kV (negative ions); heated capillary 220 °C; sheath gas nitrogen] coupled with a Micro-Tech Ultra-Plus MicroLC system equipped with a C_{18} column (4 μm; 100 mm long, 1 mm i.d.; ULTRASEP). For HPLC, a gradient system starting from H_2O:CH_3CN 17:3 (containing 0.2% acetic acid) to 1:9 within 15 min at a flow rate of 70 μl min^{-1} was chosen . The ^1H one and two dimensional (1D, 2D) correlation spectroscopy (COSY) and ^{13}C [1D, 2D heteronuclear multiple bond correlation (HMBC); Bax et al. 1986] NMR spectra were recorded on a Bruker DMX 600 NMR spectrometer at 300 K locked to the major deuterium resonance of the solvent, CD_3OD. Chemical shifts are given in ppm relative to the relevant signals of the solvent (^1H: residual proton signals at 3.35 ppm, ^{13}C: 49.0 ppm) and coupling constants in Hz. The multiplicities of the ^{13}C signals were deduced from the correlations in the HMBC spectrum.

Nicoblumin. For MS, the conditions were R_t = 4.07 min, positive ion ES-MS m/z (rel. int.): 551 ($[M+H]^+$, 100), 389 ($[M+H-C_6H_{10}O_5]^+$, 90), 295 (25), 227 ($[M+H-2C_6H_{10}O_5]^+$, 62), 209 ($[M+H-2C_6H_{10}O_5-H_2O]^+$, 45); negative ion ES-MS m/z (rel. int.): 549 ($[M-H]^-$, 100).

For ^1H-NMR (CD_3OD) δ = 6.10 [bs, H-4], 4.44 [d, H-1″, J(1″-2″) 7.8], 4.36 [dd, H-13A, J(13A-4) 1.3, J(13A-13B) 17.7], 4.36 [d, H-1′, J(1′-2′) 7.8], 4.20 [dd, H-13B, J(13B-4) 1.5], 4.15 [dd, H-6′A, J(6′A-5) 1.8, J(6′A-6′B)11.7], 3.91 [dd, H-6″A, J(6″A-5″) 1.9, J(6″A-6″B) 12.1], 3.86 [m, H-9], 3.83 [dd, H-6′B, J(6′B-5) 5.8], 3.70 [dd, H-6″B, J(6″B-5) 5.5], 3.48 [m, H-5′], 3.39 [m, H-3″], 3.38 [m, H-3′, H-4′], 3.33 [m, H-4″], 3.30 [m, H-5″], 3.25 [dd, H-2″, J(2″-3″) 9.1], 3.19 [dd, H-2′, J(2′-3′) 9.0], 2.63 [d, H-2A, J(2A-2B) 17.7], 2.06 [d, H-2B], 1.96 [t, H-6, J(6–7) 5.1], 1.86 [m, H-7A], 1.71 [m, H-7B], 1.75–1.63 [m, H-8AB], 1.28 [d, H-10, J(10–9) 6.3], 1.15 [s, H-11], 1.06 [s, H-12]. Long-range couplings were observed in the 2D COSY spectrum between H-4 and H-13A/B, H-4 and H-2B, and H-2B and H-6. The chemical shifts of H-3′, H-4′, H-3″–H-5″ in the region 3.4 to 3.3 overlap with solvent signals and were taken from the cross peaks in the COSY spectrum.

For ^{13}C-NMR (CD_3OD), δ = 202.4 (s, C-3), 172.4 (s, C-5), 121.6 (d, C-4), 104.9 (d, C-1″), 104.0 (d, C-1′), 78.1, 78.0, 78.0 (d × 3, C-3′, C-3″, C-5″), 77.6 (d, C-9), 77.1 (d, C-5′), 75.3 (d, C-2′), 75.0 (d, C-2″), 71.6 (d, C-4″), 71.5 (d, C-4′), 69.8 (t, C-6′), 65.1 (t, C-13), 62.8 (t, C-6″), 48.5 (t, C-2), 48.0 (d, C-6), 37.4 (s, t, C-1, C-8), 28.8 (q, C-12), 27.7 (q, C-11), 27.1 (t, C-7), 22.0 (q, C-10).

The HMBC correlations (sequential correlations are shown in bold type for the relevant carbon and only correlations relevant for the structure elucidation are documented) were as follows: H-2A: C-1/C-3/C-11/C-12; H-2B: C-1/C-3/C-4/C-11/C-12; H-4: C-5/C-6/C-13; H-6: C-1/C-2/C-4/C-5/C-7/C-8/C-12; H-9: C-7/C-8/C-10/**C-1′**; H-11(and H-12): C-1/C-2/C-6; H-13A/B: C-3(Small)/C-4/C-5/C-6; H-1′: **C-9**; H-6′A (and H-6′B): **C-1″**; H-1″: **C-6′**.

Our previous studies on changes of the accumulation of secondary products in AMs indicated the restricted occurrence of the AM-fungus-induced accumulation of sesquiterpenoid cyclohexenone derivatives in cereals and other members of the Poaceae (Maier et al. 1995, 1997). The possible involvement of isoprenoids in the formation of gramineous AMs has also been indicated by AM-fungus-induced accumulation of abscisic acid (Danneberg et al. 1993) and a "C_{14} carotenoid" ('mycorradicin'; Klingner et al. 1995a,b), a component of the 'yellow pigment', frequently observed in AMs (Becker and Gerdemann 1977).

We have now found that the AM-induced accumulation of cyclohexenone derivatives also occurs in non-gramineous plants, i.e. solanaceous tobacco plants. Tobacco was considered as a prime candidate for this investigation since various cyclohexenone derivatives are found to occur constitutively in leaves of various tobacco species (Kodama et al. 1984), which thus have the potential for the metabolic reaction upon AM formation.

High-performance liquid chromatographic analyses of methanolic extracts from 15-week-old mycorrhizal (80% frequency of colonisation) and non-mycorrhizal tobacco roots revealed marked AM-fungus-induced changes in the patterns of UV-detectable products (Fig. 1). The UV spectra of these products, obtained from an HPLC photodiode array detector (see legend of Fig. 1 for λ_{max} values), indicated the presence of six

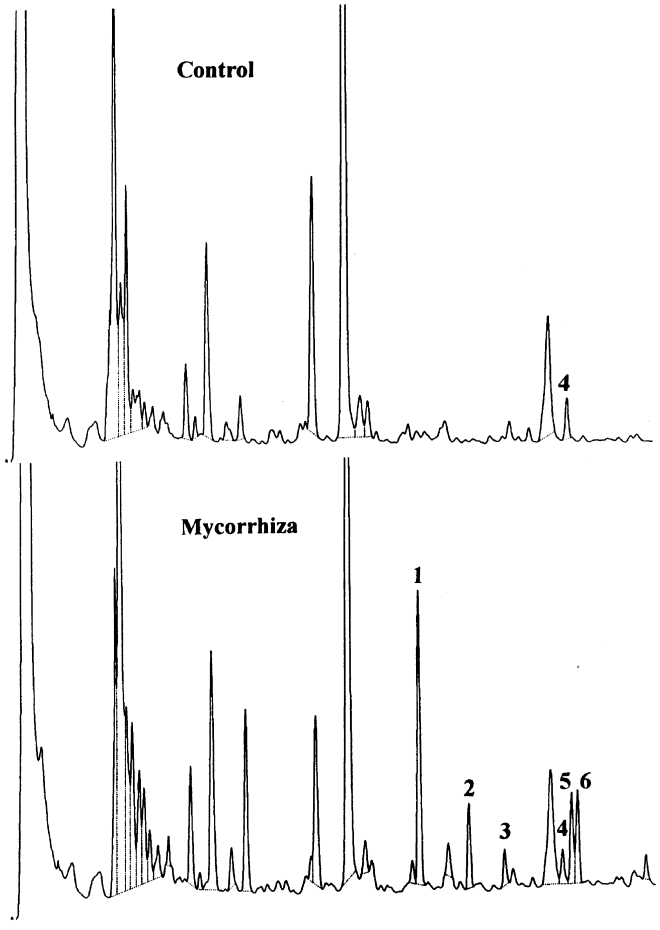

Fig. 1. High-performance liquid chromatographic traces (35 min) of methanolic extracts from 15-week-old non-colonised tobacco roots (*Control*) and the respective roots colonised with *Glomus intraradices* (*Mycorrhiza*). Of 5 ml of extraction volume from 1 g of root material, 20 μL was injected. Compounds were traced by maxplot detection between 210 and 400 nm (0.1 full-scale absorbance). The peaks **1** (nicoblumin; λ_{max} 243) and **2** to **6** (λ_{max} between 243 and 245) correspond to cyclohexenone derivatives

different blumenol derivatives. The most predominant compound among these derivatives, reaching 310 nmol g^{-1} fresh weight, was found to be 13-hydroxyblumenol C 9-*O*-gentiobioside, i.e. the 9-*O*-(6′-*O*-β-glucopyranosyl)-β-glucopyranoside of 13-hydroxy-6-(3-hydroxybutyl)-1,1,5-trimethyl-4-cyclohexen-3-one. To the best of our knowledge, this is a new natural compound which we call "nicoblumin" (Fig. 2).

The molecular weight of **1** was indicated by peaks at m/z 551 ($[M+H]^+$) in the positive-ion electrospray (ES) mass spectrum and m/z 549 ($[M\text{-}H]^-$) in the negative-ion ES mass spectrum. The key ions at m/z 389 and 227 ($[aglycon+H]^+$) are formed by consecutive losses of the two glucosyl moieties. The cyclohexenone derivatives **2** to **6**, marked in Fig. 1, were tentatively identified by liquid chromatography (LC)/MS (not documented) as 13-hydroxyblumenin (**2**; MW 564), 13-hydroxyblumenol C glucoside (**3**; MW 388), 13-hydroxyblumenol C glucuronide (**4**; MW 402) and two isomers of blumenol C diglucoside (**5** and **6**; MW 534).

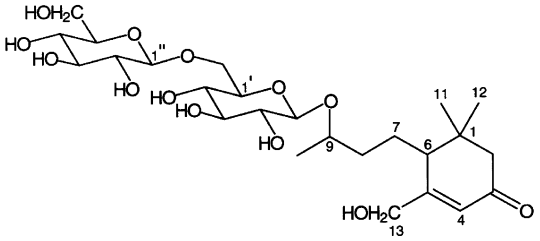

Fig. 2. Structure of the predominant cyclohexenone derivative, nicoblumenin, in mycorrhizal tobacco roots

The definite structure of **1** was elucidated from the 1D and 2D ^{1}H and ^{13}C-NMR data. The 1D ^{1}H, 2D ^{1}H-COSY and 1D ^{13}C-NMR spectra showed characteristic signals corresponding to fragments of the 13-hydroxyblumenol C part (Peipp et al. 1997) and two β-hexopyranose moieties. The through-bond correlations in the HMBC (^{1}H-detected long-range ^{13}C-^{1}H correlation) spectrum not only allowed the full assignment of the ^{13}C spectrum but disclosed the arrangement of atoms in the various moieties from the two- and three-bond intra-moiety correlations. Thus the hydroxyblumenol structure was confirmed together with the presence of the two sugar systems. The overlap of several ^{1}H signals of these two systems did not allow distinction between β-glucopyranose and β-galactopyranose from the coupling constants although the ^{1}H chemical shifts of H-4 and the ^{13}C chemical shifts were only compatible with the former system in both cases. Finally, the sequential arrangement of the three moieties followed from unambiguous three-bond inter-moiety correlations in the same HMBC spectrum.

The possible role of the sesquiterpenoid cyclohexenone derivatives in mycorrhizal symbiosis is unknown, although there is some indication that they might be involved in mycorrhizal control. Exogenously applied blumenin strongly inhibits colonisation and formation of arbuscules in the early stages of mycorrhiza formation in barley (Fester et al. 1998). The cyclohexenone derivatives might be related to the origin of mycorradicin (Klingner et al. 1995a). Placing the structure scheme of mycorradicin (C_{14}) between those of two aglycones of cyclohexenone derivatives ($2 \times C_{13}$) results in an almost perfect C_{40} carotenoid structure. We therefore speculate that both mycorradicin and the aglycones of the cyclohexenone derivatives originate from the same carotenoid precursor. This is in line with the assumption that the cyclohexenone derivatives ['C_{13}-nor-carotenoid glucosides', Kodama et al. 1981] are generated by oxidative degradation of carotenoids in flue-curing and ageing of tobacco (Wahlberg et al. 1977). They were also found in fresh leaves of tobacco (Kodama et al. 1984). Future experiments will have to find the putative carotenoid structures which might be the precursors of AM-fungus-induced dioxygenase-catalyzed reactions leading to mycorradicin and the cyclohexenone derivatives.

Finally, it is interesting to note that the present results with tobacco plants open up new avenues for research

on the role of the putative carotenoids and their degradation products in AMs. Many molecular probes for studying biosynthetic steps are available from solanaceous plants. Tobacco plants are easily amenable to transformation, and loss-of-function approaches in studies on the role of isoprenoids in AMs are now feasible, e.g. by antisense-RNA expression experiments. The potential for the use of transgenic tobacco plants in AM research has been shown previously, e.g. focusing on root chitinase expression (Vierheilig et al. 1993) or pathogenesis-related proteins (Vierheilig et al. 1995).

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