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-[(6¢-O-b-glucopyranosyl)-b-glucopyranoside ("nicoblumin"), i.e. the 9-O-(6¢-O-b-glucopyranosyl)-b-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-b-glucuronosyl-b-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 arbuscles 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 13C-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-b-glucopyranosyl)-b-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, *Gnomus intraradices* Schenck & Smith, by application of propagules in expanded clay (isolate 49, provided by H. von Ailen 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 grown 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. 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 (1 g fresh weight) were treated twice for about 1 min with an Ultra Turrax homogenizer for about 1 min and the homogenates were 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), equipped with a Nucleosil 100-10 C18 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 C18 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 C18 column (4 μm; 100 mm long, 1 mm i.d.; ULTRASEP). For HPLC, a gradient system starting from H2O/CH3CN 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 and two dimensional (1D, 2D) correlation spectroscopy (COSY) and 13C (1D, 2D heteronuclear multiple bond correlation (HMBC); Bax et al. 1986) NMR spectra were recorded on a Bruker DMAX 600 NMR spectrometer at 300 K locked to the major deuterium resonance of the solvent, CD3OD. Chemical shifts are given in ppm relative to the relevant signals of the solvent (1H: residual proton signals at 3.35 ppm, 13C: 49.0 ppm) and coupling constants in Hz. The multiplicities of the 1H signals were deduced from the correlations in the HMBC spectrum.

Nicoblumin. For MS, the conditions were Re = 4.07 min, positive ion ES-MS m/z (rel. int.): 551 ([M + H]+, 100), 389 ([M + H-C2H3O2]-, 90), 295 (25), 227 ([M + H-C2H3O2]-, 62), 209 ([M + H-C2H3O2]-, 45); negative ion ES-MS m/z (rel. int.): 549 ([M-H]-, 100).

For 1H-NMR (CD2OD) δ = 6.10 [bs, H-4], 4.44 [dd, H-1′, J(1-2′) 7.8], 4.36 [dd, H-13A, J(13A-13B) 4.3], 4.36 [d, H-1′, J(1′-2′) 7.8], 4.20 [dd, H-13B, J(13B-14) 1.5], 4.15 [dd, H-6′, J(6′A-5) 1.8, J(6′A-6′B) 1.7], 3.91 [dd, H-6′A, J(6′A-6′B) 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 [dd, H-2A, J(2A-2B) 17.2], 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 13C-NMR (CD2OD), δ = 202.4 (s, C-3), 172.4 (s, C-5), 121.6 (d, C-4), 104.9 (d, C-15), 104.0 (d, C-17), 78.1, 78.0, 78.0 (d x 3, C-3, C-3′, C-3′), 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, C-11), 28.5 (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: H2A: 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 “C14 carotenoid” (’maccorradicin’; Klinger 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-graminaceous 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 medicinal 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 λmax values), indicated the presence of six