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Is stimulation of carotenoid biosynthesis in arbuscular mycorrhizal roots a general phenomenon?

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Abstract

The identification and quantification of cyclohexenone glycoside derivatives from the model legume *Lotus japonicus* revealed far higher levels than expected according to the stoichiometric relation to another, already determined carotenoid cleavage product, i.e., mycorradicin. Mycorradicin is responsible for the yellow coloration of many arbuscular mycorrhizal (AM) roots and is usually esterified in a complex way to other compounds. After liberation from such complexes it has been detected in AM roots of many, but not of all plants examined. The non-stoichiometric occurrence of this compound compared with other carotenoid cleavage products suggested that carotenoid biosynthesis might be activated upon mycorrhization even in plant species without detectable levels of mycorradicin. This assumption has been supported by inhibition of a key enzyme of carotenoid biosynthesis (phytoene desaturase) and quantification of the accumulating enzymic substrate (phytoene). Our observations suggest that the activation of carotenoid biosynthesis in AM roots is a general phenomenon and that quantification of mycorradicin is not always a good indicator for this activation.

Keywords: *Lotus japonicus*; *Medicago truncatula*; Arbuscular mycorrhiza; Glycosylated cyclohexenone derivatives; Phytoene

1. Introduction

The arbuscular mycorrhizal (AM) symbiosis is a mutualistic association of fungi from the order Glomales with roots of most plant species (Strack et al., 2003a). The establishment of this symbiosis is often connected to a more or less intense yellow to orange-brownish coloration of the roots that was described as early as by Jones (1924). The compound responsible for the yellow coloration of AM roots from *Zea mays* has been identified as the acyclic C_{14} polyene 'mycorradicin' (Klingner et al., 1995). Mycorradicin has been assumed to be derived from the oxidative cleavage of the C_{40} precursor carotenoids (Walter et al., 2000). The respective activation of carotenoid biosynthesis has been shown in AM roots of *Zea mays*, *Nicotiana tabacum* and *Medicago truncatula* (Fester et al., 2002b). The other fragments from the oxidative cleavage of carotenoids leading to mycorradicin are C_{13} cyclohexenone derivatives, which were identified in AM roots from barley (Maier et al., 1995), various other Poaceae (Maier et al., 1997), tobacco (Maier et al., 1999) and tomato (Maier et al., 2000). Given the structural similarity of the accumulating C_{13} cyclohexenone derivatives to the zygomycete mating factor trisporic acid, cyclohexenone derivatives might be involved in symbiotic signaling. Alternatively, the carotenoids may have structural functions (Walter et al., 2000) or are possibly involved in scavenging reactive oxygen species (Fester and Hause, 2005).

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Mycorradicin has a characteristic UV/VIS-spectrum and can be identified easily after alkaline hydrolysis of methanolic extracts using HPLC. The accumulation of this compound has been detected in AM roots of all monocots and of a number of dicots studied (Fester et al., 2002a). According to this analysis, the levels of accumulating mycorradicin are highly variable among various plant species. In particular, the model legumes *M. truncatula* and *Lotus japonicus* accumulate only small amounts of this compound in their AM roots, i.e., 7 and 2 nmol $(g\,fw)^{-1}$, respectively. To examine whether these low levels adequately reflect the extent of activation of carotenoid biosynthesis, we have identified and quantified the other products (C_{13} cyclohexenone derivatives) derived from oxidative carotenoid cleavage. Considerably higher levels were observed than expected for a simple 2:1 stoichiometric relation for the cleavage reaction. These results suggested that carotenoid biosynthesis might be activated even in plants not accumulating mycorradicin. We verified this possibility for a number of such plants by applying norflurazon, an inhibitor of phytoene desaturase, and by measuring the accumulating substrate of this key enzyme of carotenoid biosynthesis (Chamovitz et al., 1991).

2. Results and discussion

2.1. Analysis of cyclohexenone derivatives in AM roots of *M. truncatula* and *L. japonicus*

HPLC analysis of methanolic extracts revealed a number of compounds characterized by UV/VIS-spectra indicative of cyclohexenone derivatives accumulating specifically upon fungal colonization (Fig. 1). As UV/VIS-spectra of cyclohexenone derivatives are not as characteristic as those of mycorradicin and their HPLC retention times are dependent on the molecule's substituents, we characterized some of these derivatives. One component of *M. truncatula* coeluted with 4-(-3-*O*-β-glucopyranosylbutyl)-3-(hydroxymethyl)-5,5-dimethyl-2-cyclohexen-1-one (13-hydroxyblumenol C glucoside; Peipp et al., 1997) from methanolic extracts of AM roots from *Hordeum vulgare* (data not shown). For *L. japonicus*, three cyclohexenone derivatives were isolated and identified as 4-[3-*O*-(2′-*O*-β-glucuronoyl)-β-glucopyranosylbutyl]-3-(hydroxymethyl)-5,5-dimethyl-2-cyclohexen-1-one (**1**, 13-hydroxyblumenin = 13-hydroxyblumenol C glucuronoylglucoside), 4-[3-*O*-(2′-*O*-β-apiofuranosyl)-β-glucopyranosylbutyl]-3-(hydroxymethyl)-5,5-dimethyl-2-cyclohexen-1-one (**2**, 13-hydroxyblumenol C apiosylglucoside), and 4-[3-*O*-(2′-*O*-β-apiofuranosyl)-(6′-malonyl)-β-glucopyranosylbutyl]-3-(hydroxymethyl)-5,5-dimethyl-2-cyclohexen-1-one (**3**, 13-hydroxyblumenol C apiosyl-2-malonylglucoside) (Fig. 2) using a combination of NMR spectroscopic (Table 1) and mass spectrometric data (Experimental), together with methylation analysis to unambiguously identify the sugar moieties. The molecular weight of each compound was determined from the positive molecular ions in the electrospray mass spectra (ESI-MS). High mass accuracy ESI-MS data afforded the molecular formulae.

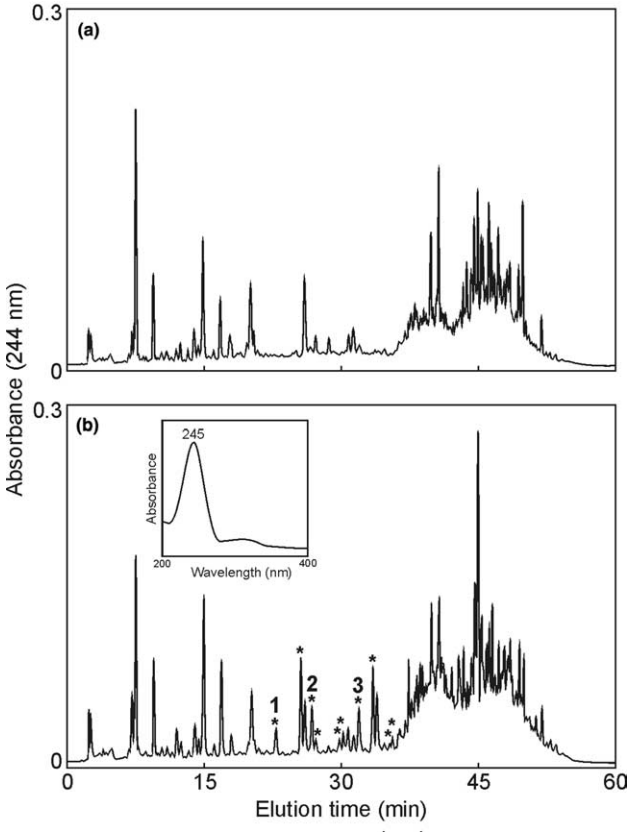

Fig. 1. HPLC profiles of MeOH extracts of non-mycorrhizal (a) and mycorrhizal (b) roots from *L. japonicus*. Compounds accumulating exclusively in mycorrhizal roots and characterized by a UV/VIS-spectrum (as given in the inset) indicative for cyclohexenone derivatives are marked with asterisks. 13-hydroxyblumenin (**1**), 13-hydroxyblumenol C apiosylglucoside (**2**), and 13-hydroxyblumenol C apiosyl-2-malonylglucoside (**3**) were identified as described in the text. Deviations from the base line are due to the steep gradient starting after 30 min.

The identity of the aglycone and one β-glucopyranosyl moiety in all compounds was readily elucidated from the 1D and 2D ^{1}H NMR data, and by comparison with our previous data for similar derivatives from other mycorrhizal root systems (Peipp et al., 1997; Maier et al., 1999, 2000). The shifts of the remaining ^{1}H signals in **1**, together with the molecular mass from the MS data, unambiguously identified the third moiety as a β-glucuronic acid moiety. This is a terminal unit as an initial loss of this moiety was observed in the MS–MS analysis. The linkages between the three moieties followed directly from correlations in the 2D ROESY spectrum.

For both **2** and **3** the ^{1}H signals of the third moiety had the characteristic shifts of a β-apiofuranosyl moiety

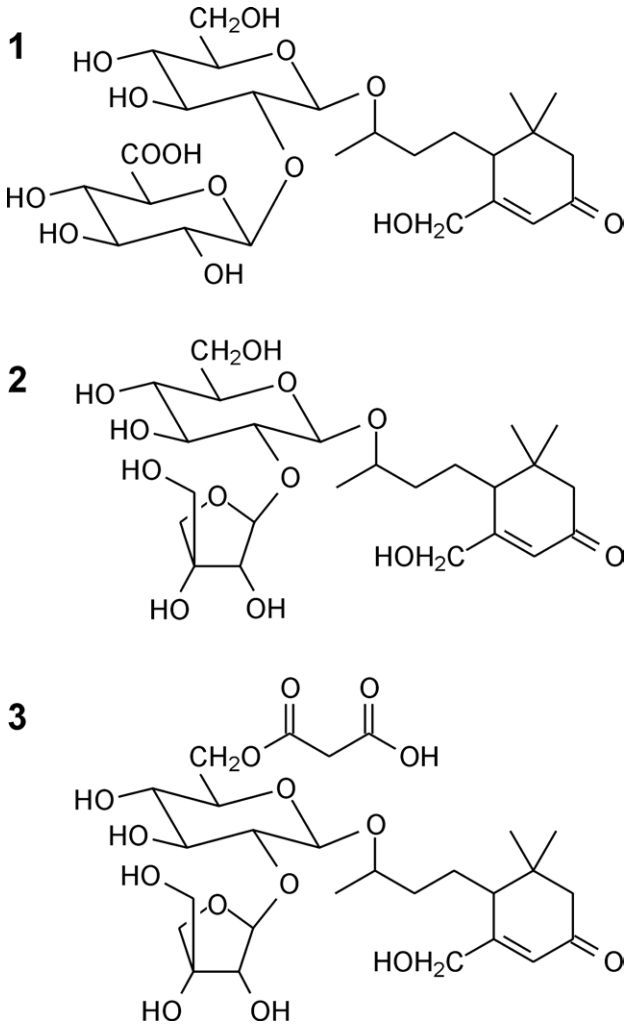

Fig. 2. Structure scheme of cyclohexenone derivatives **1**, **2** and **3**.

(Nahrstedt et al., 1995; Schwarz et al., 1996). Again in **2** the sequence was evident from the MS–MS analysis and the linkage of the β-glucopyranosyl moiety to the cyclohexenone moiety was evident from the correlation between the anomeric proton and H-9 in the 2D ROESY spectrum. A similar strong correlation of the apiose anomeric proton with H-2 of the glucose unit and weaker correlations to H-1 and H-3 of the same unit indicated the linkage of the apiose to C-2 of glucose. Internal correlations within the apiose unit of H-1 with H-4B and H-4A with H-5AB established the relative β-furanose configuration of this moiety. In contrast to **1**, a doubling of various signals in **2** (Table 1) was observed and was particularly evident for the anomeric proton of the β-glucose moiety. In a previous investigation, we have attributed this phenomenon to the presence of R- and S-isomers at C-9 (Maier et al., 2000).

A similar analysis of **3** indicated the presence of an identical arrangement of sugar and cyclohexenone units to that in **2** with the only difference being the low field shift of the H-6A/B of the glucose indicative of acylation at C-6. Although the methylene group of a malonyl group could not be unambiguously identified in the ^{1}H spectrum, probably as a result of exchange with the solvent, its presence was established from the molecular weight of the compound and its MS–MS fragmentation. The latter confirmed that this unit was attached to the hexose. Again a doubling of several signals in the ^{1}H spectrum was observed.

Malonylation of cyclohexenone glycosides and glycosylation of cyclohexenone derivatives by apiose has not been described before. Malonylation is a common modification of isoflavonoids in various legumes (Dixon, 1999), and has been observed for legume saponins as well (Huhmann and Sumner, 2002). Glycosylation by apiose is not restricted to the legume family and has been described for flavonoids (e.g., Hahlbrock et al., 1976; Cuyckens et al., 2002), saponins (e.g., Guo and Kenne, 2000) and betacyanins (Strack et al., 2003a,b).

In summary, individual glycosylated cyclohexenone derivatives reached concentrations of up to 60 nmol $(g\ fw)^{-1}$ in roots of *M. truncatula* and up to 100 nmol $(g\ fw)^{-1}$ in roots of *L. japonicus*. These concentrations are considerably higher than concentrations of accumulating mycorradicin, i.e., 7 and 2 nmol $(g\ fw)^{-1}$, respectively. This is comparable to those reported for various Poaceae (Maier et al., 1997), tobacco and tomato (Maier et al., 2000), although in some cases, these plants accumulate even higher levels. C_{13} cyclohexenone derivatives and C_{14} mycorradicin are thought to be derived from the cleavage of a common precursor carotenoid (Walter et al., 2000), producing 2 moles of C_{13} ketones for each mole of C_{14} dialdehyde. As the levels of mycorradicin are much lower than expected from this stoichometric relation, our findings suggested that either mycorradicin or the C_{14} dialdehyde is converted into compounds no longer detectable by our analytical method. Accordingly, the absence of mycorradicin is not indicative for a missing activation of carotenoid biosynthesis.

2.2. Inhibition of carotenoid biosynthesis in AM roots by application of norflurazon

We tested the activity of carotenoid biosynthesis in roots not accumulating mycorradicin by inhibiting phytoene desaturase, a key enzyme of carotenoid biosynthesis catalyzing the two-step oxidation of phytoene to ζ-carotene (Chamovitz et al., 1991). After application of the specific inhibitor norflurazon to *Z. mays*, *N. tabacum* and *M. truncatula*, phytoene has been reported to accumulate to a much greater extent in AM roots than in the respective non-mycorrhizal roots (Fester et al., 2002b). This demonstrated the mycorrhiza-specific activation of carotenoid biosynthesis. We observed a similar differential accumulation of phytoene in AM roots of plants accumulating no mycorradicin such as *Centaurea cyanus*, *Petroselinum crispum*, *Papaver somniferum*, *Tagetes*

Table 1
^{1}H NMR data of cyclohexenone derivatives from *L. japonicus* in CD_3OD

Compound	**1**[e]		**2**		**3**[d]	
	Shifts (ppm)	*J* (Hz)	Major/minor[a] shifts (ppm)	Major/minor[a] *J* (Hz)	Major/minor[b] shifts (ppm)	Major/minor[b] *J* (Hz)
H-2A	2.59	(2A–2B) 17.5	2.65/2.63	(2A–2B) 17.5/17.5	2.62/2.65	(2A–2B) 17.5/17.5
H-2B	2.05	(4–13A) 1.3	2.06	(4–13A) 1.3/1.3	2.06	(4–13A) 1.6/1.5
H-4	6.08	(4–13B) 1.3	6.11/6.10		6.10/6.11	(4–13B) 1.4
H-6	2.08	(6–7) 4.4, 4.4	1.96	(6–7) 5.0, 5.0	1.96	
H-7A	1.84	(9–10) 6.3	1.86	(9–10) 6.3/6.2	1.85	
H-7B	1.63	(13A–13B) 17.6	1.75–1.58	(13A–13B) 17.7/17.7	1.75–1.58	(13A–13B) 17.7/17.7
H-8A	1.75		1.75–1.58		1.75–1.58	
H-8B	1.63		1.75–1.58		1.75–1.58	
H-9	3.92		3.88		3.79/3.81	
H-10	1.27		1.28/1.28		1.26/1.25	
H-11	1.15		1.16/1.15		1.15/1.16	
H-12	1.06		1.06		1.06	
H-13A	4.36		4.36/4.35		4.36/4.37	
H-13B	4.31		4.20		4.20	
H-1′	4.53	(1′–2′)7.7	4.44/4.35	(1′–2′) 7.7/7.8	4.35/4.44	(1′–2′) 7.7/7.7
H-2′	3.49	(2′–3′) 9.0	3.31/3.18	(2′–3′) 9.1	3.19/3.32	(2′–3′) 9.0
H-3′	3.58	(3′–4′)9.0	3.49/3.39–3.24		3.37/3.37–3.29	
H-4′	3.38	(4′–5′) 9.8	3.39–3.24		3.37–3.29	
H-5′	3.29	(5′–6′A) 2.2	3.39–3.24		3.50	(5′–6′A) 1.9
H-6′A	3.88	(5′–6′B) 5.7	3.88	(5′–6′B) 5.9/5.9	4.49	(5′–6′B) 6.4/6.4
H-6′B	3.68	(6′A–6′B) 11.9	3.68/3.69	(6′A–6′B) 11.8/11.8	4.30/4.28	(6′A–6′B) 11.8/11.8
H-1″	4.64	(1″–2″) 7.8	H-1″ 5.35	(1′–2′) 2.1	5.35	(1′–2′) 2.1
H-2″	3.37	(2″–3″) 8.9	H-2″ 4.00	(4″A–4″B) 9.5	4.00/3.99	(4″A–4″B) 9.5
H-3″	3.44	(3″–4″) 8.9	H-4″A 4.05		4.04	
H-4″	3.50	(4″–5″) 9.7	H-4″B 3.74		3.74	
H-5″	3.62		H-5″3.64[b]		3.64[c]	

[a] Major 62%/minor 38% from the signal intensities. Data for the minor component are given when these can be distinguished.
[b] Major 53%/minor 47% from the signal intensities.
[c] This is broad and integrates for two protons.
[d] The signals from the malonyl moiety could not be unambiguously identified and presumably these had exchanged with the solvent deuterium atoms.
[e] Correlations in the ROESY spectra indicated the β-glucopyranose moiety was attached to C-9 of the cyclohexenone system and the glucuronic acid moiety was attached to C-2 of the β-glucopyranose moiety.

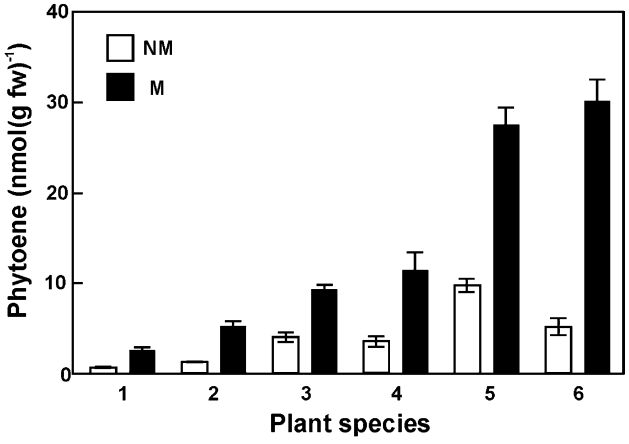

Fig. 3. Accumulation of phytoene in mycorrhizal (M) and non-mycorrhizal (NM) roots from *C. cyanus* (**1**), *P. crispum* (**2**), *P. somniferum* (**3**), *L. japonicus* (**4**), *T. erecta* (**5**), and *R. graveolens* (**6**) after application of norflurazon. Standard deviations of 4 different individual plants are given.

erecta or *Ruta graveolens* (Fig. 3). The levels of phytoene in AM roots of these plants range between 2.6 and 30 nmol (g fw)$^{-1}$ and were comparable to those reported for *Z. mays*, *N. tabacum* or *M. truncatula*, that are 79, 31, and 17 nmol (g fw)$^{-1}$, respectively.

The extent of the AM-specific activation of carotenoid biosynthesis in colonized roots is apparently difficult to assess. All accessible parameters (the levels of mycorradicin, cyclohexenone derivatives, phytoene after application of norflurazon) may be affected by various factors that are difficult to control. Nevertheless, our combined data unambiguously demonstrate that the AM-induced activation of carotenoid biosynthesis in roots is more widespread than previously thought and suggest that this activation is a general feature of the AM symbiosis.

3. Experimental

3.1. Plant cultivation and AM-fungus inoculation

Medicago truncatula Gaertn. var. Jemalong (from Perkiss seeds, Australia), *Centaurea cyanus* L. (from N.L. Chrestensen, Erfurt, Germany), *Petroselinum crispum* (Miller) A.W. Hill (from N.L. Chrestensen, Erfurt,

Germany), *Papaver somniferum* L. (from Prof. T. Kutchan, Halle, Germany), *Lotus japonicus* (Regel) K. Larsen (from Prof. M. Parniske, University Munich, Germany), *Tagetes erecta* L. (from the institute's garden), *Ruta graveolens* L. (from the botanical garden, Halle, Germany) were grown in 250-ml plastic pots filled with expanded clay (Lecaton, 2–5 mm particle size, Fibo Exclay, Pinneberg, Germany) and inoculated with the AM fungus *Glomus intraradices* Schenck & Smith (from H. von Alten, University Hannover, Germany). Details of plant growing conditions have been published elsewhere (Maier et al., 1995). Approximate percentage values for mycorrhiza formation were estimated microscopically after staining with trypan blue in lactophenol according to a procedure described by Phillips and Hayman (1970).

3.2. Extraction and quantification of cyclohexenone derivatives (analytical HPLC)

Cyclohexenone derivatives were extracted and analyzed as described by Maier et al. (1999). Freshly harvested roots were washed with water and 1 g of root tissue was ground in liquid nitrogen and extracted once for 30 min with 2 ml 80% aq. MeOH. The mixture was centrifuged and subjected to analytical HPLC. The liquid chromatograph (600-MS system controller, Waters, Milford, USA) was equipped with a 5-μm Nucleosil C_{18} column (250 × 4 mm i.d.; Macherey-Nagel, Düren, Germany). Injections of 20 μl were carried out with an automatic sampler (717 autosampler, Waters, Milford, USA). Cyclohexenone derivatives were eluted using a linear gradient elution system at a flow rate of 1 ml min^{-1} within 30 min from 5% to 20% solvent B (100% MeCN) in solvent A (1.5% *ortho*-phosphoric acid in H_2O). Further compounds were eluted from the column by applying a gradient for 18 min from 20% to 80% solvent B in solvent A, adding 2 min 80% of solvent B. Compounds were detected photometrically at 245 nm with a Waters (Milford, USA) 996 photodiode array detector. Quantitative values were calculated as described in Maier et al. (1995) from external standardization with *cis*-abscisic acid using the Millenium software 2010 (Millipore, Eschborn, Germany).

3.3. Isolation of cyclohexenone derivatives from L. japonicus (preparative HPLC)

Methanolic extracts (80% aq. MeOH) from mycorrhizal roots from *L. japonicus* (about 130 g fw) were filtered, evaporated at 40 °C (in vacuo) close to dryness, and redissolved in 80% aq. MeOH. For preparative HPLC, a liquid chromatograph (System Gold; Beckman Instruments, München, Germany) equipped with a Nucleosil 100-10 C_{18} column (VarioPrep; 10 μm, 250 × 40 mm i.d.; Macherey-Nagel, Düren, Germany) was used. Compounds were eluted using a linear gradient from solvent A (1% aq. HOAc, 30% MeOH) to solvent B (90% aq. MeOH) at a flow rate of 10 ml min^{-1} within 70 min and further 40 min with solvent B. The eluate was monitored at 244 nm, fractions were collected and evaporated at 40 °C (in vacuo) to dryness. They were redissolved in 0.4 (**1**) or 0.6 ml (**2**, **3**) 80% aq. MeOH and further analyzed and purified by analytical HPLC using isocratic elution at 8% (**1**) or 22% (**2**, **3**) MeCN in 1% aq. HOAc. Purified compounds were collected and evaporated at 40 °C (in vacuo) to dryness.

3.4. Structure elucidation of the cyclohexenone derivatives isolated from L. japonicus

1D and 2D (COSY and ROESY) ^1H NMR spectra were recorded at 300 K on a Bruker AVANCE DMX 600 NMR spectrometer locked to the major deuterium signal of the solvent, CD_3OD. Chemical shifts are reported in ppm relative to TMS but were determined relative to the signals of the solvent (^1H: 3.35 ppm) and coupling constants in Hz. Positive ion electrospray mass spectra (ESI-MS) were recorded on a Micromass QTOF^2 mass spectrometer. High resolution ESI-MS were recorded on the same instrument. Sugars were identified using a standard micro-methylation technique on a Finnigan GCQ GC–MS spectrometer as described previously (Nimtz et al., 1996). Apiin was used as reference material for the detection of apiose.

1: NMR see table. Sugar analysis: glucose and glucuronic acid. HR ESI-MS: *m/z* (rel. int.) 587.233 $[M + Na]^+$ (5) (calc. for $C_{25}H_{40}O_{14}Na$: 587.2316); ESI-MS/MS: *m/z* (rel. int.) 411.21 $[M + Na - HexA]^+$. **2**: NMR see table. Sugar analysis: glucose and apiose. HR ESI-MS: *m/z* (rel. int.) 543.244 $[M + Na]^+$ (5) (calc. for $C_{24}H_{40}O_{12}Na$: 543.2417); ESI-MS/MS: *m/z* (rel. int.) 411.21 $[M + Na - Pent]^+$, 317.09 $[Hex + Pent + Na]^+$, 185.05 $[Hex + Na]^+$.**3**: NMR see table. Sugar analysis: glucose and apiose. HR ESI-MS: *m/z* (rel. int.) 629.242 $[M + Na]^+$ (calc. for $C_{27}H_{42}O_{15}Na$: 629.2421); ESI-MS/MS: *m/z* (rel. int.) 585.26 $[M + Na - CO_2]^+$, 543.25 $[M + Na - Mal]^+$, 453 $[M + Na - Pent - CO_2]^+$, 404.11 $[Hex + Pent + Mal + Na]^+$, 317.09 $[Hex + Pent + Na]^+$, 227.05 $[Hex + Mal - CO_2 + Na]^+$.

3.5. Application of norflurazon and extraction and quantitation of phytoene

Norflurazon was applied after cultivation with and without mycorrhizal inoculum for 3 months (*L. japonicus*, *P. crispum*, *P. somniferum*) or 4 months (*R. graveolens*, *T. erecta*, *C. cyanus*). The plants were watered with 40 ml of 82 μM norflurazon every second day over 10 days, when leaves showed the first signs of bleaching. Extraction and subsequent HPLC of carotenoids was performed according to Fraser et al. (2000). Root sam-

ples (1 g) were rinsed with water and mortared in liquid nitrogen. Methanol (1.5 ml) was added and the suspension was repeatedly mixed for 5 min. Then, 1.5 ml of a buffer solution (50 mM Tris/HCl, pH 7.5, 1 M NaCl) was added, the sample mixed for 10 min, 4 ml chloroform added and the sample mixed again for 10 min. Phase separation was achieved by centrifugation at 3000*g* for 5 min. The lower phase (3.5 ml) was removed, evaporated at room temperature (in vacuo) to dryness and dissolved in ethyl acetate prior to HPLC analysis. The liquid chromatograph (Waters 600-MS system controller) was equipped with a S-5 μm C_{30} column (270 × 4 mm i.d.; YMC, Schwermbeck, Germany) coupled to a S-5 μm C_{30} precolumn. Injections of 20 μl were carried out by an automatic sampler (Waters 717 autosampler). Mobile phases consisted of 0.2% ammonium acetate in H_2O/MeOH (20/80 by volume) (B) and *tert*-butyl methyl ether (C) in MeOH (A). Compounds were separated by a two-step linear gradient from 95% A, 5% B, 0% C, first to 75% A, 5% B, 20% C in 4 min and then to 25% A, 5% B, 70% D in 40 min. Compounds were detected photometrically (maxplot between 200 and 600 nm) by a Waters 996 photodiode array detector. Data were collected and analyzed using the Millenium software 2010 (Millipore, Eschborn, Germany). Phytoene was quantified as described by Fester et al. (2002b) from external standardization with authentic phytoene.

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