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Accumulation of sesquiterpenoid cyclohexenone derivatives induced by an arbuscular mycorrhizal fungus in members of the Poaceae

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Abstract. Sixty one members of the Poaceae, including various cereals, were grown in defined nutrient media with and without the arbuscular mycorrhizal (AM) fungus, *Glomus intraradices* Schenk & Smith. The roots of all species investigated were colonized by the AM fungus, however, to different degrees and independent of their systematic position. High-performance liquid chromatographic analyses of methanolic extracts from the roots of mycorrhizal and nonmycorrhizal species revealed dramatic changes in the patterns of UV-detectable products along with a widespread occurrence of AM-fungus-induced accumulation of sesquiterpenoid cyclohexenone derivatives. The latter occur most often in the tribes Poeae, Triticeae and Aveneae. Some additional control experiments on plant infection with pathogens (*Gaeumannomyces graminis*) and *Drechslera* sp.) or an endophyte (*Fusarium* sp.), as well as application of abiotic stress, proved that the metabolism of these terpenoids is part of a response pattern of many gramineous roots in their specific reaction to AM fungal colonization.

Key words: Arbuscular mycorrhiza – Cereal (root fungus) – *Glomus* – Poaceae – Terpenoid accumulation/induction

Introduction

Almost all terrestrial plants form mycorrhizas resulting in enhanced water uptake and assimilation of nutrients from the soil (Varma and Hock 1995). The molecular mechanisms controlling establishment and functional maintenance of mycorrhizas are virtually unknown. In line with increasing evidence that secondary compounds play a significant role in various interactions occurring between plants and their natural environment (Harborne 1993), including pathogenic plant-fungus interactions, one might expect an equivalent role in mycorrhizas.

In our recent studies, the arbuscular mycorrhizal (AM)-fungus-induced accumulation of sesquiterpenoid cyclohexenone glycosides in roots of barley was described (Maier et al. 1995; Peipp et al. 1996). One of these glycosides (blumenin) was also found to accumulate in mycorrhizas of oat, rye and wheat (Maier et al. 1995). These results, pointing to a specific role for terpenoid metabolism in gramineous mycorrhizas, are in line with related studies that show AM-fungus-induced accumulation of abscisic acid (ABA) (Danneberg et al. 1993) and a yellow-coloured "C_{14} carotenoid", termed "mycorradicin", in maize (Klingner et al. 1995a) and some other gramineous plants (Klingner et al. 1995b). Mycorradicin has repeatedly been observed in earlier studies (see references cited by Klingner et al. 1995b). Thus various aspects of the terpenoid metabolism of roots seem to be involved in mycorrhiza formation in members of the grass family (Poaceae).

The occurrence of blumenin in the above-mentioned cereal mycorrhizas prompted us to study its systematic distribution, together with that of two related structures, in members of the Poaceae. Preliminary results (Maier et al. 1995) indicated a restricted occurrence of the AM-fungus-induced accumulation of these terpenoids within the tribes Aveneae and Triticeae. Results of the present taxonomic study, however, revealed the widespread occurrence of this phenomenon in members of the Poaceae.

Materials and methods

Plant material and AM-fungus inoculation. Seeds of the plants studied came from the seed collection at the IPK (Gatersleben; Hammer 1993). Representative samples of the gramineous collection, comprising more than 37 000 accessions, were selected on the species level. The rich infraspecific variation, e.g. in the major

Dedicated to Professor Benno Parthier on the occasion of his 65th birthday

Abbreviations: AM = arbuscular mycorrhiza/arbuscular mycorrhizal; *Ggt* = *Gaeumannomyces graminis*

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cereals, has not been yet considered. The plants were cultivated in plastic pots filled with expanded clay and inoculated with the AM fungus *Glomus intraradices* Schenk & Smith by application of propagules (isolate 49) in expanded clay. Details of cultivation (generally six weeks) have been published previously (Maier et al. 1995). The mycorrhizal roots were stained with trypan blue in lactophenol according to a procedure described by Phillips and Hayman (1970). The approximate percentage values of mycorrhization were estimated microscopically by counting the frequency of colonization from 30 root pieces (2–3 cm).

Isolation of fungi from healthy barley plants. Roots of healthy looking barley plants grown in the greenhouse for 42 d were surface-sterilized for 1 min in ethanol (70%) and 5 min in NaOCl (1%) and washed twice for 10 min in sterile tap water. Segments of approx. 5 mm were placed on a biomalt antibiotic agar medium (Schulz et al. 1993). Successful sterilization was checked by making an imprint of the sterilized root on a separate plate of the same medium. Mycelia which grew out of segments within six weeks were isolated onto a biomalt (20 $g \cdot l^{-1}$) agar medium. Two of the most frequent isolates, *Fusarium* sp. and *Drechslera* sp., were selected for further experiments.

Infection of barley roots with the endophyte Fusarium sp. and the pathogen Drechslera sp. For surface sterilization, barley seeds were (i) suspended for 12 h in a solution of 0.11% formaldehyde in sterile water containing 0.1% Tween 20, (ii) washed three times for 5 min in sterile tap water, and (iii) dried for 15 min on sterile filter paper. The seeds were then placed on semi-solid (6 g agar $\cdot l^{-1}$) biomalt medium (50 $g \cdot l^{-1}$; Vitaborn, Hameln, Germany) and incubated for 6–7 d in the dark at room temperature. Four seedlings were then transferred into each sterile plastic pot (Phytacon, Sigma, Deisenhofen, Germany) containing Lecaton (2–5 mm particle size) and a liquid medium (Murashige and Skoog 1962) without sucrose. An inverted second pot served as a lid. In the first experiment, the pots were tightly sealed. In the second experiment, a hole (2 cm diameter) in the bottom of the inverted pot and fitted with a cotton plug provided limited ventilation. Simultaneously with the transfer of the seedlings to the plastic pots, the plants were inoculated with *Fusarium* sp. or *Drechslera* sp. by placing a segment (5 mm × 5 mm) of a 28 d fungal culture grown on a solid biomalt agar medium into the expanded clay along with the seedlings. For the controls, a segment of agar medium was substituted for fungal culture. The barley plants were grown in a photoperiod of 15 h, at 12 °C in the first experiment and in the second one at 15 °C during the day and at 10 °C at night.

Infection with the pathogen Gaeumannomyces graminis (*Ggt*). Barley plants were cultivated for six weeks as described above, except that the expanded clay was mixed with *Ggt* inoculum [*Triticum aestivum* caryopsis infected with *Gaeumannomyces graminis* (*Sacc.*) of Arex & Olivier var. *tritici* Walker]. The *Ggt* inoculum was a generous gift of Dr. H. Mielke (Biologische Bundesanstalt für Land- und Forstwirtschaft, Braunschweig, Germany).

Stress conditions. The barley plants were grown for four weeks in a greenhouse as described previously (Maier et al. 1995), followed by the application of various forms of abiotic stress such as heat (39 °C), cold (5 °C) and light stress (1320 instead of 210 µmol photons $\cdot m^{-2} \cdot s^{-1}$) in a phytotron for 24 h, drought (no watering) or heavy-metal stress (10^{-3} M $CuSO_4$) in the greenhouse for two weeks.

Root harvest and extraction. Mycorrhized roots of all the members of the Poaceae studied and Ggt-infected barley roots. For one experiment, freshly harvested whole mycorrhizal and non-mycorrhizal roots from ten plants were washed with water and cut into small pieces. Root aliquots (1 g fresh weight) were treated twice for about 1 min with an Ultra Turrax homogenizer in 5 ml 80% aqueous methanol, and the homogenates allowed to stand for 30 min with continuous stirring and centrifuged. The supernatants were used for HPLC analysis (20-µl aliquots).

Fusarium- and Drechslera-infected barley roots. The barley plants that had been infected with *Fusarium* sp. or *Drechslera* sp. were harvested at 7, 14, 21 and 28 d after inoculation (five infected plants and five non-infected control plants were used for each variant in each experiment). The roots were first washed with sterile water and dried on filter paper. Then two segments were cut from each separated root: a 10-mm segment was used for re-isolation of the inoculated fungus onto a biomalt agar medium, a 5-mm segment was used for microscopic examination. (In all cases the inoculated fungi could be re-isolated from the barley roots, proving that infection had occurred.) The remaining lengths of roots were frozen in liquid nitrogen, lyophilized and cut into small pieces, stored in a sealed vial (10-mg aliquots in 1.5-ml polyethylene test tubes) at room temperature prior to extraction. For extraction, 500 µl of 80% aqueous methanol and zirconium beads (1.0 mm diameter) were added, the vials cooled in liquid nitrogen, vigorously shaken (3 × 10 s at 5000 rpm) with a Bead Beater (Biospec Products, Bartlesville Okla., USA) and centrifuged. The supernatant was used for HPLC analysis (20-µl aliquots).

High-performance liquid chromatography. The HPLC system (Waters, Milford, Mass., USA), the Nucleosil C_{18} column (Macherey-Nagel, Düren, Germany), and quantification of the cyclohexenone derivatives by using ABA as the external standard compound were as described previously (Maier et al. 1995). The values listed in Table 1 are the means ± SD of three independent experiments.

Results and discussion

High-performance liquid chromatographic analyses of methanolic extracts from mycorrhizal and nonmycorrhizal roots of members of the Poaceae revealed dramatic AM-fungus-induced changes in the patterns of UV-detectable metabolites. Some of these compounds were identified in recent studies (Maier et al. 1995; Peipp et al. 1996) as agmatine and putrescine amides of hydroxycinnamic acids (4-coumaric and ferulic acids), as well as the cyclohexenone derivatives **1** (blumenin), **2**, and **3**, shown in Fig. 1.

Whereas the hydroxycinnamic acid amides transiently accumulate in the early stages of barley mycorrhization, perhaps reflecting initiation of a defense response (Peipp et al. 1996), the cyclohexenone derivatives accumulated continuously due to induction by the AM fungus (Maier et al. 1995; Peipp et al. 1996), indicating a correlation with the establishment of the barley mycorrhiza. One of these terpenoids, blumenin **(1)**, was also found to accumulate in mycorrhizal roots of oat, rye and wheat. This result prompted us to study the systematic distribution of **1**–**3** in members of the Poaceae. Preliminary results (Maier et al. 1995) indicated a restricted occurrence of this phenomenon within the tribes Aveneae and Triticeae. Results of the present taxonomic study, however, revealed the widespread, but sporadic occurrence of the induced accumulation of **1**–**3** in the different tribes of the Poaceae (Table 1).

The roots of all members of the Poaceae investigated in the present study were colonized by the AM fungus; however, the degree of colonization varied and it was independent of the grass's systematic position. For example, only 5% mycorrhization was found in one member of the tribe Poeae (*Poa badensis*) and one of the Phalarideae (*Phalaris phleoides*), whereas 80% was

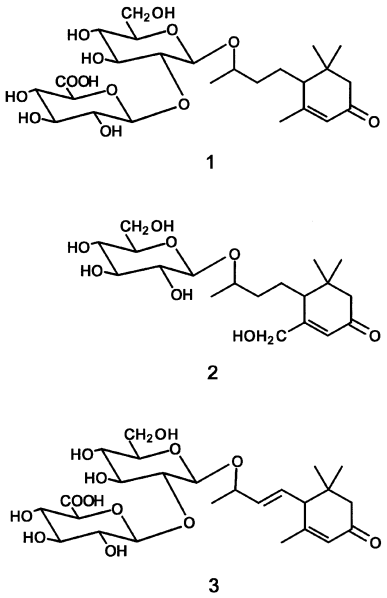

Fig. 1. Structure of the sesquiterpenoid cyclohexenone derivatives that were surveyed in mycorrhizal roots of members of the Poaceae: derivate **1** = 4-{-3-*O*-[2′-*O*-β-glucuronosyl)-β-glucopyranosyl]-butyl}-3,5,5-trimethyl-2-cyclohexen-1-one (blumenin); derivate **2** = 4-(-3-*O*-β-glucopyranosylbutyl)-3-(hydroxymethyl)-5,5-dimethyl-2-cyclohexen-1-one; derivate **3** = 4-{-3-*O*-[(2′-*O*-β-glucuronosyl)-β-glucopyranosyl]-1-butenyl}-3,5,5-trimethyl-2-cyclohexen-1-one

reached in *Avena sativa* (Aveneae), *Zea mays* (Maydeae), *Panicum miliaceum* and *Setaria italica* (Paniceae), as well as in *Triticum aestivum* (Triticeae). The highest mycorrhization rate (more than 90%) was reached with *Triticum spelta*.

The fungus-induced accumulation of the cyclohexenone derivatives was found in more than half of the species investigated and seems to occur most frequently in the tribes Aveneae, Poeae and Triticeae. Figure 2 shows two examples of the HPLC traces obtained from methanolic extracts from six-week-old noncolonized plant roots and the respective roots colonized with the AM fungus. *Poa nemoralis* roots accumulated AM-fungus-induced blumenin (compound **1**) along with compound **2**, *Lolium temulentum* roots only blumenin. The highest blumenin concentrations were found in mycorrhizal roots of *Danthonia decumbens* and *Triticum spelta* with approx. 470 and 540 nmol · g^{-1} root fresh weight, respectively.

The sporadic occurrence of the cyclohexenone derivatives in the plants studied raises the question whether or not the accumulation of the cyclohexenone derivatives is causally related to mycorrhiza formation. If this is the case, should this phenomenon not necessarily occur in all members of the Poaceae investigated? The answer might be that their possible role in gramineous plants is an example of convergent functional evolution in secondary metabolism, a phenomenon that has also been discussed for the role of flavonoids in plant reproduction (Burbulis et al. 1996). In addition, a comparison with phenomena observed in pathogenic plant-fungus interactions shows that although certain chemical interactions between plants and microorganisms are generally characteristic for a given family, e.g. in phytoalexin production, there are also examples of fungus-induced accumulation of different types of chemical structures in the same family (Harborne 1993). This might also apply for compounds correlated with AM formation as a result of co-evolution of the host plants with their fungal symbionts, an assumption that is supported by the various dramatic changes in the patterns of as yet unknown root components (increase or decrease). An example is illustrated by the HPLC traces from *Danthonia decumbens* extracts in Fig. 3. It should be worthwhile identifying these unknown compounds, possibly raising new questions on the regulation of mycorrhiza formation in the Poaceae. Some of these compounds are most likely further sesquiterpenoid cyclohexenone derivatives, since their HPLC-online UV spectra (λ_{max} 241–244 nm) are almost identical to those traced in this paper. The occurrence of these unknown compounds was also observed with most of the other plants studied, suggesting that there might be various other unknown cyclohexenone derivatives that are involved in gramineous mycorrhization. One must keep in mind, however, that it is difficult to define which compounds are involved in the control of gramineous mycorrhizas, since the symbiotic root responses are most likely multicomponent, as is the case for induced phenylpropanoids in plant defense (Dixon and Paiva 1995).

In the present study using plants up to 6 weeks, we never observed the appearance of the yellow-coloured "C_{14} carotenoid" mycorradicin (Klingner et al. 1995a,b), considered to be widespread in AM mycorrhizas. In search of this phenomenon we did experiments using older mycorrhizal plants grown under different conditions of culture. Preliminary results obtained from 3-month-old fruiting maize plants suggest that accumulation of mycorradicin depends (i) on the age of the mycorrhizal plants and (ii) on certain conditions of culture (data not shown).

Occasionally, we observed accumulation of the cyclohexenone glycosides in the control cereals, but only once among the other members of the Poaceae (*Danthonia decumbens*). The cereals might possibly be more susceptible to cross-colonization than the noncereal species under the growth conditions applied. This has been assumed by Klingner et al. (1995b) in a study on the AM-fungus-induced accumulation of mycorradicin. On the other hand, other fungal infections or certain environmental factors (abiotic stressors?) might also induce accumulation of these compounds, as shown in stress-induced phenylpropanoid metabolism (Dixon and Paiva 1995). To check this possibility, we infected barley plants with two root pathogens and a root endophyte, as well as applying various forms of abiotic stress, such as heat, cold, drought, high intensities of light and heavy-metal treatment, and compared their effects with those of the fungal inoculation.

The pathogens were *Gaeumannomyces graminis* (*Ggt*), the causative agent of take-all disease (Jensen and Jorgensen 1973), and *Drechslera* sp.. The endophyte belongs to the genus *Fusarium*, one of the most common genera to be isolated from the roots of a number of plants (Gams 1967; Summerbell 1987; Zhang et al.

Table 1. Distribution of sesquiterpenoid cyclohexenone glycosides in AMs of members of the Poaceae

Taxon[b]	Fungal colonization (%)[c]	Terpenoids (nmol)[a]		
		1	**2**	**3**
Andropogoneae				
Bothriochloa ischaemum (L.) Keng	50	–[d]	–	–
Sorghum bicolor (L.) Moench	50	–	–	–
Aveneae				
Agrostis tenuis Sibth.	50	38 ± 7[e]	–	–
Ammophila arenaria (L.) Link	50	53 ± 11	117 ± 26	–
Apera spica-venti (L.) P. Beauv.	70	62 ± 1	–	–
Arrhenatherum elatius (L.) J. et C. Presl.	25	71 ± 8	–	–
A. thorei Desm.	60	82 ± 22	–	–
Avena sativa L. cv. Salvator	80	125 ± 10	–	44 ± 18
A. s. L. var. *mutica* Alef.	50	78 ± 6	–	–
Calamagrostis arundinacea (L.) Roth	50	–	–	–
C. epigeios (L.) Roth	20	–	–	–
Corynephorus canescens (L.) P. Beauv.	20	78 ± 0.6	–	–
Danthonia alpina Vest	90	–	75 ± 23	–
D. decumbens (L.) Lam. et DC.	80	469[f] ± 29	–	82 ± 16
Deschampsia caespitosa (L.) P. Beauv.	70	21 ± 1	98 ± 4	–
Gaudinia fragilis (L.) P. Beauv.	80	89 ± 4	202 ± 1	–
Holcus lanatus L.	30	34 ± 7	–	–
Koeleria vallesiana (Honck.) Gaudin	25	–	–	–
Trisetum flavescens (L.) P. Beauv.	50	–	–	–
Chlorideae				
Cynodon dactylon (L.) Pers.	20	–	–	–
Coleantheae				
Eragrostis pilosa (L.) P. Beauv.	40	–	22 ± 7	–
Maydeae				
Zea mays L. cv. Garant	80	–	–	–
Nardeae				
Nardus stricta L.	40	–	–	–
Oryzeae				
Oryza sativa L. cv. Koshihikari	30	–	–	–
Paniceae				
Panicum miliaceum L.	80	–	27 ± 3	–
Setaria italica (L.) P. Beauv.	80	–	82 ± 4	–
Phalarideae				
Alopecurus pratensis L.	30	–	–	–
A. ventricosus Pers.	60	–	–	–
Anthoxanthum odoratum L.	10	110 ± 21	–	–
Phalaris arundinacea (L.) Motyka	50	–	–	–
Phleum phleoides (L.) Karsten	5	–	–	–
Spartina pectinata Link	70	–	75 ± 2	–
Poeae				
Briza media L.	10	–	–	–
Bromus arvensis L.	30	215 ± 9	93 ± 26	–
B. erectus Huds. var. *erectus*	40	–	83 ± 10	–
B. tectorum L.	15	72 ± 7	95 ± 22	–
Brachypodium silvaticum (Huds.) P. Beauv.	15	–	–	–
Dactylis glomerata L.	10	–	–	–
D. polygama Horvat.	10	–	–	–
Festuca gigantea (L.) Vill.	25	67 ± 12	–	–
F. ovina L. cv. Bornito	50	–	–	34 ± 3
F. pratensis Huds.	25	–	–	–
F. rubra L.	50	116 ± 11	–	38 ± 1
F. valesiaca Schleich ex Gaud.	30	–	–	–
Lolium multiflorum Lamk.	10	89 ± 14	–	–
L. perenne L.	20	88 ± 25	–	–
L. temulentum L.	10	101 ± 13	–	–
Poa badensis Haenke	5	–	–	–
P. compressa L.	40	56 ± 14	–	–
P. nemoralis L.	50	158 ± 18	51 ± 20	–
P. trivialis L.	10	–	–	–
Sesleria coerulea (L.) Ard.	60	–	–	–

Table 1. (*Contd.*)

Taxon[b]	Fungal colonization (%)[c]	Terpenoids (nmol)[a]		
		1	**2**	**3**
Triticeae				
Agropyron caninum (L.) P. Beauv.	60	–	–	–
A. elongatum (Host) P. Beauv.	70	–	176 ± 26	–
Elymus angustus Trin.	50	–	–	–
E. sibiricus L.	50	46 ± 23	64 ± 17	–
Hordelymus europaeus (L.) Jessen ex Harz	50	–	–	–
Hordeum vulgare L. cv. Salome	70	225[g] ± 18	88 ± 10	33 ± 2
Secale cereale L. cv. Petka	60	61 ± 11	81 ± 9	–
Triticum aestivum L. cv. Hatri	80	317 ± 34	97 ± 19	–
T. spelta L.	90	536 ± 69	105 ± 9	–

[a]Amount per gram fresh weight of mycorrhizal root
[b]Listed according to the tribes of the Poaceae
[c]Approx. percent values estimated from 30 root pieces (2–3 cm)
[d]Not detected by the applied HPLC method (less than 1 nmol per gram fresh weight)
[e]SD of the means of three independent experiments with 10 plants each
[f]161 ± 73 nmol compound **1** in nonmycorrhizal control plants
[g]Occasional occurrence of compound **1** in nonmycorrhizal control plants (in the range of 17 to 22 nmol in approx. half of 20 sets of cultivations)

1996). The latter two strains had been isolated from the roots of healthy barley. Reinfection of these isolates into barley plants showed that *Ggt* and *Drechslera*, the latter also considered to be a pathogenic genus (Brandenburger 1985), led to pronounced disease symptoms and death of the plants, whereas a reinfection with *Fusarium* caused no visible disease symptoms. Although some species of *Fusarium* are known to cause disease, others are apparently avirulent (Fischer and Petrini 1990; Zhang et al. 1996). The latter may reflect evolutionary adaptation of these species to their hosts, a theory that has recently been supported by a study on proteases suspected to be important in fungal pathogenicity (Reddy et al. 1996).

The results of fungal infection and application of abiotic stress are shown in Table 2. Whereas mycorrhiza formation induced blumenin (compound **1**) accumulation, neither the infection with the pathogens nor the endophyte, nor the abiotic stressors applied showed any effect on the cyclohexenone metabolism. Thus, we as yet have no explanation for the occasionally observed blumenin accumulation in the nonmycorrhizal cereals.

Conclusion

It is not possible to draw any definite conclusions from the present results on the distribution of the AM-fungus-

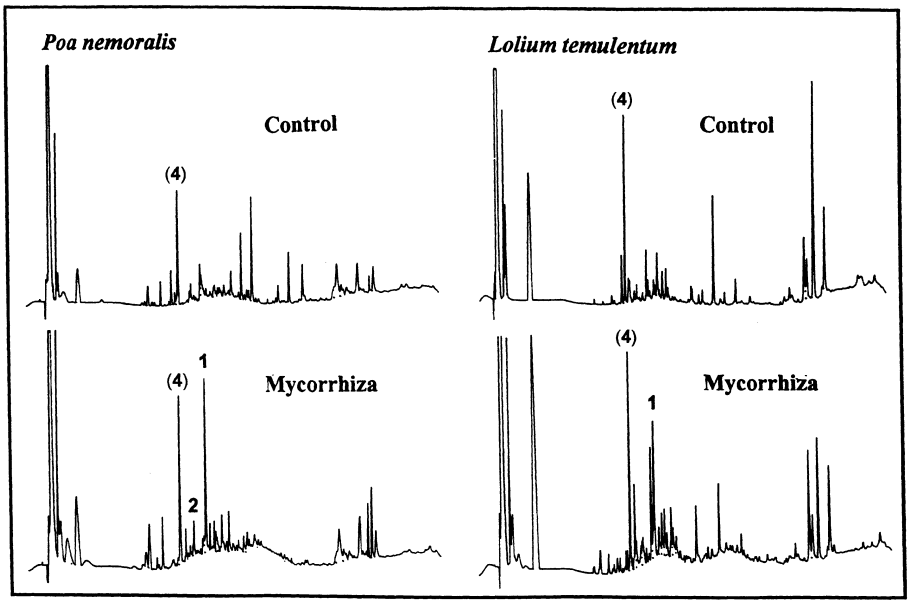

Fig. 2. HPLC traces (40 min) of methanolic extracts from six-week-old noncolonized plant roots (*Control*) and the respective roots colonized with *Glomus intraradices* (*Mycorrhiza*). For each 5 ml of methanolic extraction volume from 1 g fresh weight of root material, 20 µl was injected. Components were traced by maxplot detection between 210 and 400 nm (0.1 and 0.05 full-scale absorbance in traces from *P. nemoralis* and *L. temulentum*, respectively). For peak identification (**1** and **2**), see structures in Fig. 1; (**4**) marks the peak corresponding to tryptophan. Note also some dramatic changes (increase or decrease) of as yet unknown compounds

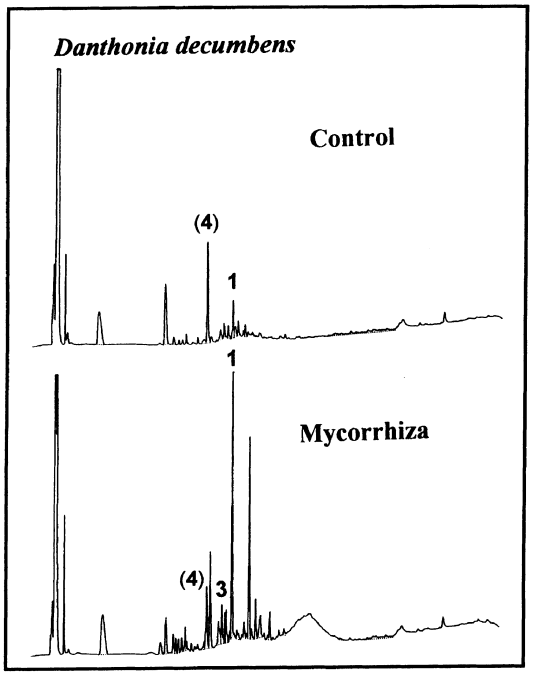

Fig. 3. HPLC traces as described in Fig. 2 from *Danthonia decumbens* extracts (0.26 full-scale absorbance). For peak identification of the cyclohexenone derivatives **1** and **3** see structures in Fig. 1; Peak (**4**) corresponds to tryptophan. Note also the dramatic changes (increase or decrease) of as yet unknown compounds

induced accumulation of the cyclohexenone derivatives in gramineous mycorrhizas. There are definitely multiple factors underlying the formation of AMs. We do not yet know whether these terpenoids are important factors in the physiological functioning of AMs, characteristic for those grass species that respond positively as listed in Table 1. Compared to the phytoalexin concept, it might well be that there are some parallel phenomena to mycorrhization that the accumulation of sesquiterpenoid cyclohexenone derivatives is part of the "metabolic perturbation" (Kuc 1995) caused by the penetration of AM fungi. Formation of AMs might initiate a cascade of metabolic events, among which the accumulation of the cyclohexenone derivatives could be part of some stress-induced side reactions. Nevertheless, the metabolism of the described sesquiterpenoid cyclohexenone derivatives is part of a response pattern of many gramineous roots, involved in as yet unknown areas of AM metabolism. It is important to note that we never observed AM formation with the "cyclohexenone-positive plants" without the induction of the cyclohexenone derivatives.

Table 2. Effect of inoculation of barley roots with some nonmycorrhizal fungi and application of various abiotic stressors to barley plants in comparison with AM fungus (*Glomus intraradices*) inoculation on the accumulation of blumenin

Treatment	Blumenin (**1**)
Glomus colonization	428 nmol ± 43
Ggt infection[a]	–[b]
Fusarium infection[c]	–
Drechslera infection[d]	–
Heat	–
Cold	–
Light	–
Drought	–
Heavy metal (Cu^{2+})	–

[a]Typical symptoms of the take-all disease
[b]Not detected by the applied HPLC method (less than 1 nmol per gram fresh weight)
[c]No disease symptoms
[d]Disease symptoms

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