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Accumulation of secondary compounds in barley and wheat roots in response to inoculation with an arbuscular mycorrhizal fungus and co-inoculation with rhizosphere bacteria

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Abstract Colonization of *Hordeum vulgare* L. cv. Salome (barley) and *Triticum aestivum* L. cv. Caprimus (wheat) roots by the arbuscular mycorrhizal fungus *Glomus intraradices* Schenck & Smith leads to de novo synthesis of isoprenoid cyclohexenone derivatives with blumenin [9-O-(2′-O-β-glucuronosyl)-β-glucopyranoside of 6-(3-hydroxybutyl)-1,1,5-trimethyl-4-cyclohexen-3-one] as the major constituent and to transient accumulation of hydroxycinnamate amides (4-coumaroylagmatine and -putrescine). Accumulation of these compounds in mycorrhizal wheat roots started 2 weeks after sowing together with the onset of arbuscule formation and proceeded with mycorrhizal progression. Highest levels were reached in 3- to 4-week-old secondary roots (root branches of first and higher order) characterized by the formation of vesicles. In the final developmental stages, the fungus produced massive amounts of spores, enclosing the stele of older root parts (older than 5 weeks) characterized by cortical death. In these root parts, the secondary compounds were detected in trace amounts only, indicating that they were located in the cortical tissues. Some rhizosphere bacteria tested, i.e. *Agrobacterium rhizogenes*, *Pseudomonas fluorescens*, and *Rhizobium leguminosarum*, markedly stimulated both fungal root colonization and blumenin accumulation, thus, acting as mycorrhiza-helper bacteria (MHB). Application of blumenin itself strongly inhibited fungal colonization and arbuscule formation at early stages of mycorrhiza development. This was associated with a markedly reduced accumulation of the hydroxycinnamate amides 4-coumaroylputrescine and -agmatine. The results suggest that both the isoprenoid and the phenylpropanoid metabolism are closely linked to the developmental stage and the extent of fungal colonization. Their possible involvement in the regulation of mycorrhiza development is discussed.

Key words Arbuscular mycorrhiza · *Hordeum vulgare* · *Triticum aestivum* · *Glomus intraradices* · Mycorrhiza-helper bacteria · Secondary compounds

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Introduction

We recently described arbuscular mycorrhizal (AM) fungus-induced accumulation of isoprenoid cyclohexenone derivatives with blumenin [9-O-(2′-O-β-glucuronosyl)-β-glucopyranoside of 6-(3-hydroxybutyl)-1,1,5-trimethyl-4-cyclohexen-3-one] as the predominant constituent in roots of various members of the Poaceae (Maier et al. 1995, 1997), occurring predominantly in the tribes Poeae, Triticeae and Aveneae. In *Hordeum vulgare* (barley) roots, AM fungus-induced transient accumulation of hydroxycinnamate amides has also been observed (Peipp et al. 1997). Stimulation of isoprenoid metabolism seems to be a typical phenomenon of the Poaceae. It is most likely that the AM fungus-induced accumulation of cyclohexenone derivatives results from stimulation of the non-mevalonate glyceraldehyde 3-phosphate (GAP)/pyruvate pathway. It has recently been shown, using ^{13}C-labelled glucose, that the biosynthesis of blumenin in mycorrhizal barley roots proceeds via the GAP/pyruvate pathway (Maier et al. 1998). Other studies showed AM fungus-induced accumulation of abscisic acid (Danneberg et al. 1993) and of a yellow "C_{14} carotenoid" ("mycorradicin") in maize (Klingner et al. 1995a) and some other graminaceous plants (Klingner et al. 1995b). Nemec and Lund (1990) described the induced accumulation of significant amounts of leaf sesquiterpenoids in mycorrhizal *Citrus jambhiri*. The role of these isoprenoids in mycorrhizal interactions of graminaceous plants is still obscure. The same holds true for the stimulation of phenylpropanoid metabolism, the accumulation of hydroxycinnamate amides in barley roots (Peipp et al. 1997)

or, in non-graminaceous plants, AM fungus-induced elevated levels of phenylalanine ammonia-lyase (EC 4.3.1.5) and chalcone synthase (EC 2.3.1.74) transcripts in arbuscule-containing cortical root cells of *Medicago truncatula* (Harrison and Dixon 1994) and *Phaseolus vulgaris* (Blee and Anderson 1996). The first example of fungus-stimulated tissue-specific and development-dependent accumulation of phenylpropanoids has been reported in larch ectomycorrhizas (Weiss et al. 1997). However, this area of research is still in its infancy, albeit numerous papers have been published within the last two decades on secondary metabolism in mycorrhizas (Duchesne et al. 1987; Morandi 1996).

The present study was aimed at gaining a closer insight into the relation between changes in the levels of secondary compounds and mycorrhizal development in two members of the Poaceae, barley and wheat (*Triticum aestivum*). Investigations focused on tissue-specific and development-dependent responses, including effects of co-inoculation of the AM fungus and so-called mycorrhiza-helper bacteria (MHB), as well as on the possible roles of the secondary compounds in root-AM fungus interactions.

Material and methods

Plant material and AM fungus inoculation

Barley (*Hordeum vulgare* L. cv. Salome) and wheat (*Triticum aestivum* L. cv. Caprimus) were grown from grains (Walz, Stuttgart, Germany) in growth chambers in 1-l plastic pots (10 plants per pot) filled with expanded clay (Lecaton, 2- to 5-mm particle size; Fibo Exclay Deutschland, Pinneberg, Germany). The plants were 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). This isolate was routinely propagated in our laboratory by inoculating *Tagetes erecta* L. roots for 4 months in expanded clay. Mycorrhizal colonization was achieved by growing the plants in expanded clay mixed with 10% (v/v) of the AM fungal inoculum. Details of plant growth conditions are described elsewhere (Maier et al. 1995). Approximate values of colonization frequency % were estimated by counting the proportion of 30 root pieces (2 cm) with mycorrhizal structures (arbuscules, vesicles, spores) after staining with trypan blue in lactophenol according to Phillips and Hayman (1970).

Inoculation with mycorrhiza-helper bacteria

The following bacterial strains were provided by Gisela Höflich (Müncheberg, Germany): *Agrobacterium rhizogenes* strain A1A4 (isolated from *Brassica napus* roots), *Pseudomonas fluorescens* strain PsIA12 (isolated from wheat roots), *Rhizobium leguminosarum* bv *trifolii* strain R39 (isolated from clover nodules). Bacteria were inoculated by incubating the barley grains for 4 h in the respective bacterial suspension prior to inoculation with the AM fungus.

Extraction of secondary compounds and application of blumenin

Barley and wheat roots were either extracted immediately after harvest or were frozen in liquid nitrogen and stored at –20 °C prior to extraction. Whole barley roots from 10 plants were cut into small pieces, and aliquots (1 g fresh weight) were treated twice for 1 min with an Ultra Turrax homogenizer (Janke & Kunkel, Staufen, Germany) in 5 ml of 80% aqueous methanol.

Various parts of wheat roots were examined to follow the different stages of mycorrhization (Fig. 1) and some root parts were dissected for extraction of secondary compounds (Fig. 2). These root parts were homogenized in a mortar in the presence of liquid nitrogen followed by extraction with 80% aqueous methanol. The homogenates were centrifuged and the supernatants (20 µl-aliquots) were used for HPLC analysis. Preparative isolation (HPLC) of blumenin from mycorrhizal barley roots was as described previously (Maier et al. 1998). Blumenol C was obtained by preparative HPLC from acidic hydrolyses of blumenin with 1 N HCl for 1 h at 95 °C. Blumenin (30 or 150 nmol in 30 ml water) was added to mycorrhizal barley plants twice a week to the pots with 10 plants each over a period of 4, 6 and 8 weeks.

High-performance liquid chromatography

The liquid chromatograph (Waters 600-MS) was equipped with a 5-µm Nucleosil C_{18} column (250 × 4 mm i.d.; Macherey-Nagel, Düren, Germany) and a linear gradient elution system was applied at a flow rate of 1 ml min^{-1} within 30 min from 5 to 80% solvent B (acetonitrile) in solvent A (1.5% ortho-phosphoric acid in water). Compounds were photometrically detected (maxplot between 230 and 400 nm) by Waters 996 photodiode array detection. The chromatograph, identification of the compounds and their quantification by external standardization have been described elsewhere (Maier et al. 1995; Peipp et al. 1997). All data shown are the means (± SD) of three independent experiments with 10 plants each.

Bioassay

Blumenin and its aglycone, blumenol C, were tested in a bioassay for possible fungitoxic properties according to Gottstein et al. (1984). Blumenin up to 200 nmol (110 µg) and blumenol C up to 500 nmol (105 µg) were applied to silica gel thin-layer plates which were sprayed with a spore suspension of *Cladosporium cucumerinum* Ell. et Arh.. The plates were incubated for 48 h at 25 °C in the dark in a closed container. In this assay, fungitoxic activity is recognized as white areas of silica gel where the dark grey fungus fails to grow.

Results and discussion

AM fungal colonization of wheat roots

Wheat was chosen for the present studies because, under the growth conditions used, it showed intense root proliferation with pronounced morphological differences between the main roots and root branches (root branches of first and higher orders) and reproducible patterns of mycorrhiza development. A schematic representation of the different stages of AM fungal colonization of the wheat root system is given in Fig. 1. Plants develop homorhizal root systems where all the roots emanating from the shoot base follow the same pattern of development. During the first 2 weeks of a given root, longitudinal growth is much greater than lateral branching. Thereafter branching increases, especially in older parts of the root. Finally, after 5 weeks, cortical tissue in the old root parts is lost, leaving the vascular cylinder. This phenomenon is due to root cortical sen-

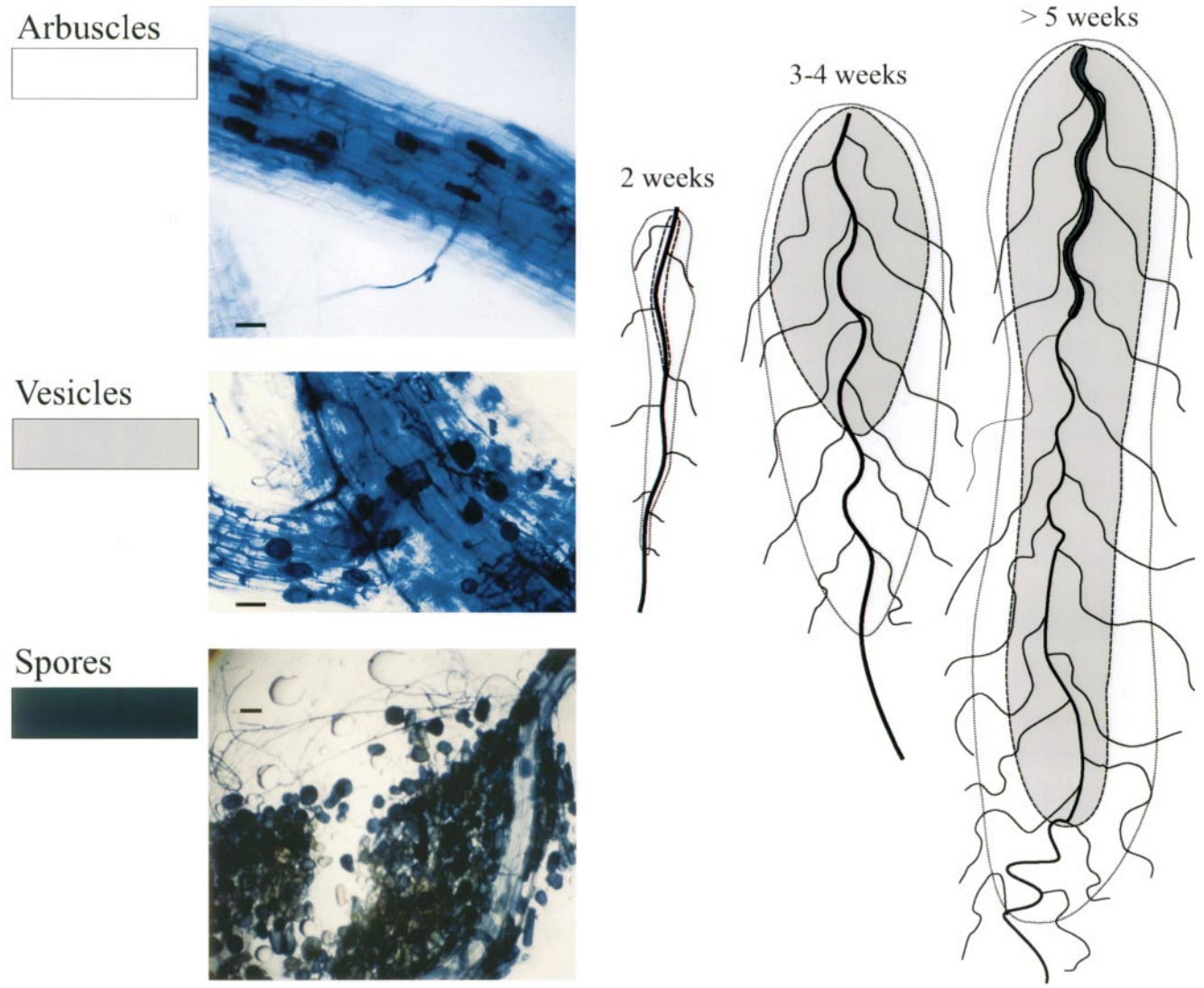

Fig. 1 Schematic representation of the different stages of *Glomus intraradices* colonization of wheat roots marked by different shadings as indicated. Many of the main roots drawn here with first branches emanate from the shoot base, forming a homorhizal root system. The photomicrographs (left; *bars* 200 μm) show mycorrhizal root segments at stages of the predominance of arbuscules, vesicles and spores. The 'spore stage' is characterized by the loss of cortical tissue due to root cortical senescence

escence, which is much faster in wheat than in many other cereals (Liljeroth 1995).

As shown in Fig. 1, three essential stages of the AM fungal life cycle could be followed in different parts of the wheat roots. Stage one was characterized by appressoria formation and by predominance of arbuscules. This could be localized in all of 2-week-old root systems. Later on, this stage was mainly observed in young parts of secondary roots. Stage two was characterized by a predominance of vesicles, observed in older parts of the root branches and the main roots. In stage three, spore formation was predominant. The fungus produced massive amounts of spores, enclosing the vascular cylinder of root parts older than 5 weeks.

Mycorrhizal status and accumulation of secondary compounds in wheat roots

Figure 2 illustrates the levels of one prominent isoprenoid cyclohexenone derivative (blumenin) and one hydroxycinnamate amide (4-coumaroylputrescine) in mycorrhizal main roots and root branches of wheat. Accumulation of these compounds started 2 weeks after sowing in roots containing mainly arbuscules and increased with mycorrhizal progression. Highest levels of about 1.3 μmol blumenin and 1 μmol 4-coumaroylputrescine per g fresh weight were reached in 3- to 4-week-old secondary roots characterized by the formation of vesicles. The most rapid accumulation of blumenin seemed to coincide with this mycorrhizal stage. The rate of blumenin accumulation in vesicle-producing secondary wheat roots was estimated to be about 400 nmol day^{-1} (g fresh $weight)^{-1}$. A markedly lower rate of increase in blumenin concentration of about 10 nmol day^{-1} (g fresh $weight)^{-1}$ of the whole root was calculated in previous work with mycorrhizal barley (Maier et al. 1995).

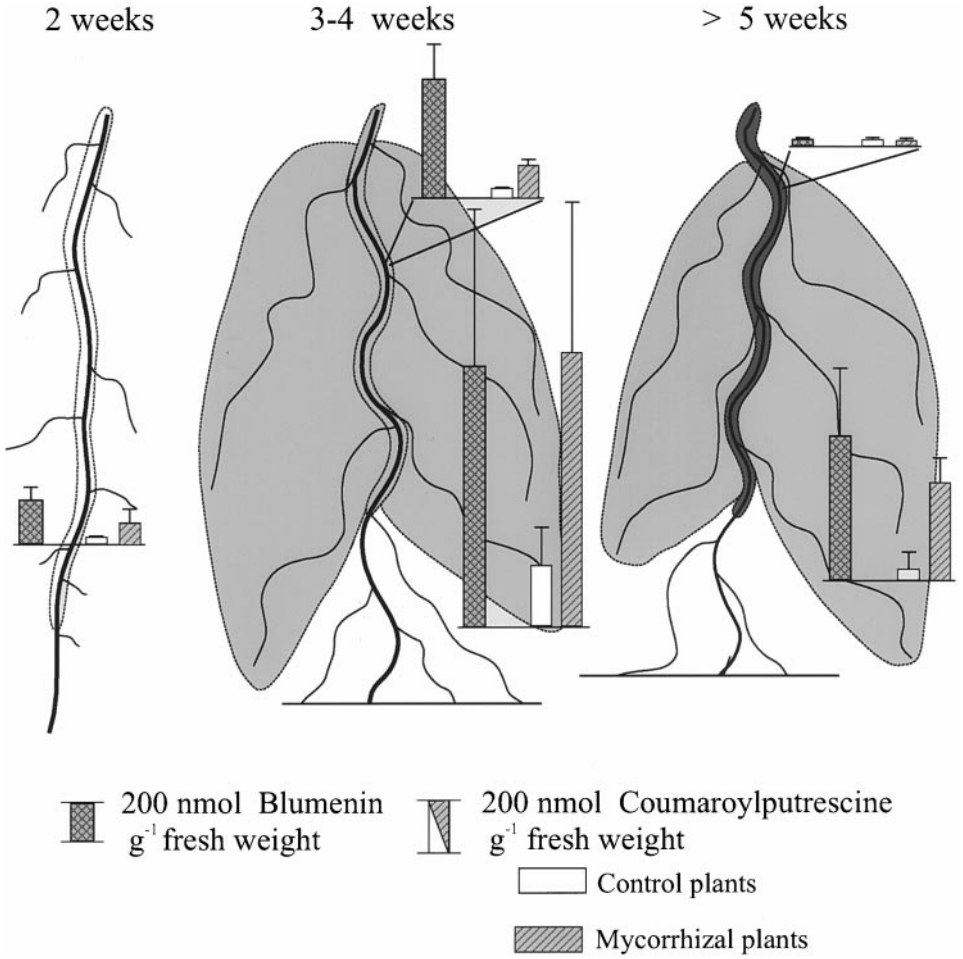

Fig. 2 Accumulation of blumenin and 4-coumaroylputrescine in mycorrhizal main roots and root branches of wheat as indicated by the different shadings (see Fig. 1). Levels of blumenin in control plants were below the detection limit of HPLC in all cases

Blumenin could not be detected either in control plants or in very young root parts of mycorrhizal plants. Although fungal colonization occurred very close to the root tip (<5 mm), blumenin could not be detected by HPLC in segments up to a distance of 1.5 cm from the root tip. 4-Coumaroylputrescine accumulated also in control plants but its accumulation was markedly stimulated by fungal colonization of wheat plants, as shown previously in barley (Peipp et al. 1997). In old parts of the main roots, characterized by spore production, both blumenin and 4-coumaroylputrescine were detected in trace amounts. Since these root parts are devoid of cortical tissue, this result indicates that the fungus-induced blumenin accumulation and the increase of 4-coumaroylputrescine concentration take place within the root cortex.

Effect of MHB on AM fungal colonization and blumenin accumulation in barley roots

The rhizosphere of plants in natural stands involves far more complex interactions than those between a single plant and a single mycorrhizal fungus. There are increasing indications that bacteria, which are most abundant in soils, play an especially important role in root-AM fungus interactions. These so-called mycorrhiza-helper bacteria (MHB) enhance the extent of fungal colonization (Meyer and Linderman 1986). The mechanisms underlying these effects are virtually unknown (Garbaye 1994), although there is some evidence that MHB produce cell wall-degrading enzymes that might soften root cell walls and thus make it easier for the AM fungi to penetrate the root (Mosse 1962).

It is well known that AM fungal root colonization is enhanced in the presence of rhizosphere bacteria, which promote plant growth and nutrient uptake (Höflich et al. 1994). The three species of potential MHB tested in the present study for their effects on barley root mycorrhiza increased mycorrhiza levels two- to threefold (Fig. 3). The frequency of mycorrhiza colonization achieved with *G. intraradices* alone was about 20%. Co-inoculation with *P. fluorescens*, *R. leguminosarum* or *A. rhizogenes* enhanced fungal colonization up to 40, 50 and 60%, respectively. If blumenin accumulation is an integral part of a response pattern of graminaceous roots, as discussed in previous work (Maier et al. 1995, 1997), the bacteria-stimulated mycorrhiza development should lead to a significant increase in blumenin levels. Figure 3 shows that this is

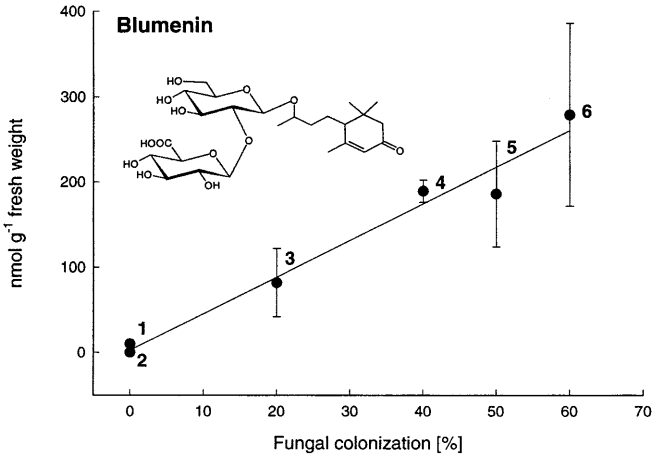

Fig. 3 Co-inoculation efficacy of the arbuscular fungus *G. intraradices* and some rhizosphere bacteria on mycorrhizal development and blumenin accumulation in mycorrhizal barley roots. *1* control, *2* plus *Pseudomonas fluorescens* or *Rhizobium leguminosarum* or *Agrobacterium rhizogenes*, *3* plus *Glomus intraradices*, *4* plus *Glomus* and *P. fluorescens*, *5* plus *Glomus* and *R. leguminosarum*, *6* plus *Glomus* and *A. rhizogenes*

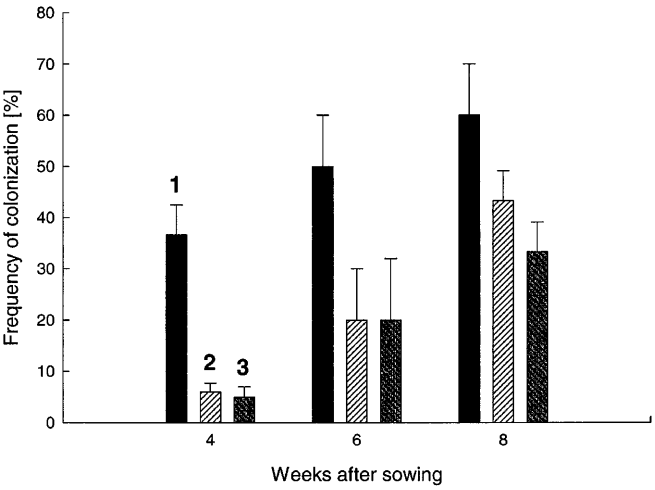

Fig. 4 Mycorrhization of barley roots in the presence of exogenous blumenin added twice a week. Bars are grouped at 4, 6 and 8 weeks and identified as control (*1*), addition of 30 nmol blumenin (*2*), and addition of 150 nmol blumenin (*3*)

indeed the case. The strongest effect was observed with *A. rhizogenes*. Co-inoculation with this bacterium led to a threefold higher mycorrhiza frequency associated with a threefold higher blumenin level. Inoculating barley roots with bacterial isolates alone did not induce blumenin accumulation.

Effect of exogenous blumenin on AM fungal colonization and accumulation of secondary compounds in barley roots

In a first attempt to elucidate the role of blumenin in the root-AM fungus association, standard blumenin

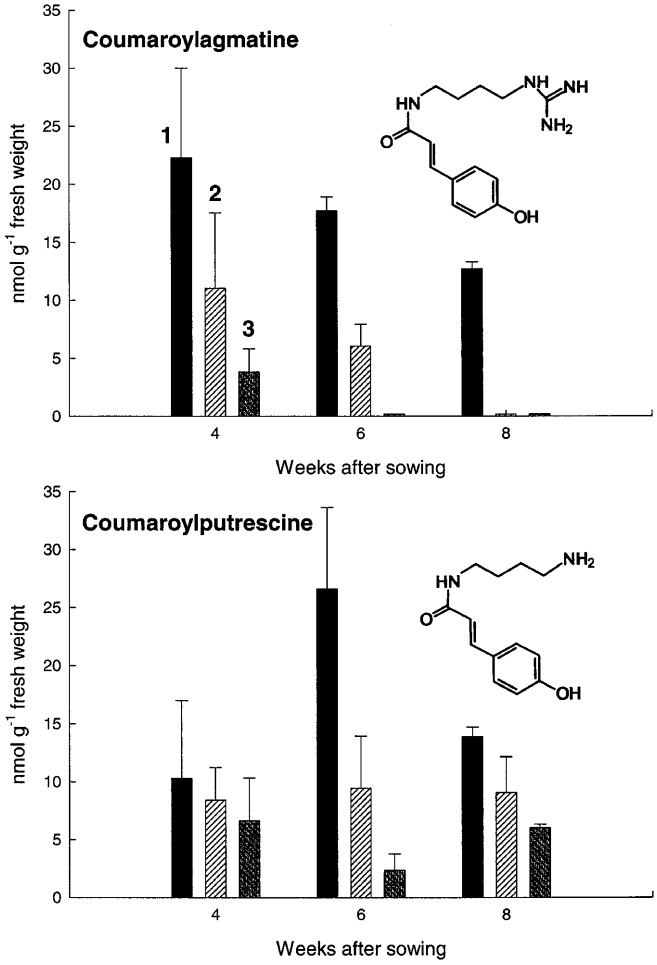

Fig. 5 Accumulation of 4-coumaroylagmatine and -putrescine in mycorrhizal barley roots in the presence of exogenous blumenin added twice a week. Bars are grouped at 4, 6 and 8 weeks and identified as control (*1*), addition of 30 nmol blumenin (*2*), and addition of 150 nmol blumenin (*3*)

was applied to mycorrhizal roots. As can clearly be seen in Fig. 4, blumenin dramatically reduced mycorrhiza development. Both 30 and 150 nmol blumenin, supplied twice a week, led to a reduction of mycorrhiza colonization to 15% of the control in 4-week-old plants. In the following weeks, mycorrhiza development recovered to 40 and 60% of the control at 6 and 8 weeks after sowing, respectively. An additional important effect was the lack of arbuscules in blumenin-treated plants up to 6 weeks, whereas untreated mycorrhizal plants showed an abundant number of arbuscules already after 4 weeks (not shown). This effect was no longer observed in 8-week-old plants. Thus, blumenin affected barley mycorrhiza in two ways: by a strong inhibition of root colonization and virtually complete inhibition of arbuscule formation up to 6 weeks.

Although 2.4 μmol blumenin was added to 10 plants (1.5–2.0 g total root weight), this amount was not recovered from either the endogenous blumenin pool or from the growth containers. There were no significant

differences between extractable blumenin from the untreated mycorrhizal plants and those supplied with blumenin. After 4, 6 and 8 weeks, there was 50, 110 and 220 nmol blumenin g^{-1} root fresh weight, respectively, in the roots of all mycorrhizal plants.

Addition of blumenin to the nutrition medium strongly inhibited the AM fungus-induced accumulation of 4-coumaroylagmatine and -putrescine (Fig. 5). This was most dramatic for 4-coumaroylagmatine. It supports the previous suggestion (Peipp et al. 1997) that accumulation of these amides in mycorrhizal barley plants reflects the initiation of a transient defense response. We have observed in several experiments that the extent of this reaction depends on the rate of fungal colonization (not shown). It seems that this part of root metabolism could control the speed of colonization, i.e. a rapid fungal root colonization relates to a rapid accumulation of these amides. Thus, inhibition of fungal root colonization and arbuscule formation by an exogenous application of blumenin leads to a lower rate of endogenous amide accumulation.

In order to explain the described effects of blumenin and to obtain an initial insight into its possible biological activity, a well-established bioassay was used testing fungitoxic activity with *C. cucumerinum*, a plant pathogenic fungus with known sensitivity to various chemicals (Gottstein et al. 1984). Although we applied high concentrations (more than 100 μg) of blumenin and its aglycone, blumenol C, the test fungus remained unaffected. However, this does not mean that it is not toxic to the mycorrhizal fungus and this should be tested by bioassays on mycorrhizal spore germination and hyphal growth.

In summary, there is no doubt that the cyclohexenone derivatives with its predominant constituent, blumenin, play an important role in root-AM fungus interactions. However, their function in mycorrhiza development of barley, wheat and other members of the Poaceae remains to be elucidated.

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