Levels of a Terpenoid Glycoside (Blumenin) and Cell Wall-Bound Phenolics in Some Cereal Mycorrhizas

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Four cereals, Hordeum vulgare (barley), Triticum aestivum (wheat), Secale cereale (rye), and Avena sativa (oat), were grown in a defined nutritional medium with and without the arbuscular mycorrhizal fungus Glomus intraradices. Levels of soluble and cell wall-bound secondary metabolites in the roots of mycorrhizal and nonmycorrhizal plants were determined by high-performance liquid chromatography during the first 6 to 8 weeks of plant development. Whereas there was no difference in the levels of the cell wall-bound hydroxycinnamic acids, 4-coumaric and ferulic acids, there was a fungus-induced change of the soluble secondary root metabolites. The most obvious effect observed in all four cereals was the induced accumulation of a terpenoid glycoside. This compound was isolated and identified by spectroscopic methods (nuclear magnetic resonance, mass spectrometry) to be a cyclohexenone derivative, i.e. blumenol C 9-0-(2'-0-glucuronosyl)-beta-glucoside. The level of this compound was found to be directly correlated with the degree of root colonization.

Arbuscular mycorrhizas are universally found symbiotic associations between plant roots and certain fungi, and there is ample evidence that these symbioses are of significant benefit for plants (reviewed by Powell and Bagyaraj, 1984; Gianinazzi and Schüepp, 1994). These benefits include enhanced nutrient supply (e.g. phosphate) and increased resistance to pathogen attack (e.g. root pathogenic fungi) or stress (e.g. drought). Despite increasing efforts in research on arbuscular mycorrhiza at the cellular and molecular levels within the last years (Harrison and Dixon, 1993, 1994; Dumas-Gaudot et al., 1994), very little is known about the basic biochemical interactions between the symbionts leading to formation of arbuscular mycorrhiza and maintenance of an active symbiotic relationship. This is in sharp contrast to our knowledge about pathogenic plant-fungus interactions (Dixon et al., 1994).

There is increasing evidence that secondary compounds in particular play a significant role in the interactions occurring between plants and their natural environment (Harborne, 1988). In this respect, secondary metabolites of roots might play an important role in mycorrhizal symbiosis. It has been documented, for example, that flavonoids promote spore germination of arbuscular mycorrhizal fungi (Gianinazzi-Pearson et al., 1989; Tsai and Phillips, 1991). Colonization of plants with arbuscular mycorrhizal fungi may lead to marked systemic reactions in terpenoid metabolism. Thus, Glomus intraradices induces accumulation of significant amounts of leaf sesquiterpenoids in Citrus jambhiri (Nemec and Lund, 1990). The sesquiterpenoid ABA reaches considerably higher levels in Glomus-colonized maize than in control plants (Danneberg et al., 1993).

As part of our studies of arbuscular mycorrhizal fungus-induced changes in secondary metabolism of cereals, we report here quantitative changes of root secondary products during formation of mycorrhizas of barley (Hordeum vulgare L.), wheat (Triticum aestivum L.), rye (Secale cereale L.), and oat (Avena sativa L.) with G. intraradices.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Four cereals, barley (Hordeum vulgare L. cv Salome), wheat (Triticum aestivum L. cv Hatri), rye (Secale cereale L. cv Petka) (spring cereals), and oat (Avena sativa L. cv Salvator) (Poaceae), as well as Tagetes erecta L. cv Discovery F1 Orange (Asteraceae), were grown from seeds (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; Leca-Deutschland, Halstenbek, Germany). Light intensity was approximately 230 μmol m⁻² s⁻¹ with sodium vapor lights for a 16-h daily light period. Constant temperature was approximately 23°C and RH was approximately 60%. The plants were supplied twice weekly with Long Ashton solution (Hewitt, 1966) (5 mL per pot), however, with a reduced phosphate level (30% Na₂HPO₄ of normal supply) to enhance compatibility of the plants to the arbuscular fungus (Schwab et al., 1991; Franken and Gnädinger, 1994).

Inoculum Production and Arbuscular Mycorrhizal Fungus Application

The arbuscular mycorrhizal fungus Glomus intraradices Schenck & Smith (isolate 49) was supplied as propagules in expanded clay by H. von Alten (Hannover, Germany). This

Abbreviations: Blumenin, blumenol C 9-O-(2'-O-beta-glucuronosyl)-beta-glucoside; 1D, one dimensional; 2D, two dimensional; ε, extinction coefficient; λmax, maximum wavelength.
was used to further propagate the clay inoculum on *T. erecta* as described by Dehne and Backhaus (1986). Mycorrhization of the cereals was achieved by growing the plants in expanded clay mixed with 10% (v/v) arbucular mycorrhizal fungus inoculum.

The mycorrhizas were stained with trypan blue in lactophenol according to a procedure described by Phillips and Hayman (1970). The infection ratios were estimated microscopically with stained mycorrhizas by counting the frequency of colonization in 2- to 3-cm root pieces.

**Analytical Extraction of Soluble and Cell Wall-Bound Compounds**

Freshly harvested whole roots from 10 plants were washed with water and cut into small pieces, and 1 g fresh weight was transferred into 5 mL of 80% methanol in water, treated twice for approximately 1 min with an Ultra Turrax (Janke & Kunkel, Staufen, Germany) homogenizer, allowed to stand for 30 min, and centrifuged. The supernatants containing the soluble compounds were used for HPLC analysis (20-μL aliquots). Before cell wall-bound compounds were prepared, the pellets were consecutively treated with the following solvents, 25 mL each for 15 to 30 min with stirring followed by filtration on sintered glass discs: (a) 3× methanol, (b) 2× water, (c) 2× acetone, and (d) 2× diethylether. To make sure that there were no major cytosolic contaminants, we included in preliminary experiments treatments with 1 m NaCl and 0.5% SDS.

The crude cell wall preparation (cell debris of the roots) was dried in an exsiccatior. The white powder (30-mg dry weight aliquots) was suspended in 2 mL of 1 N sodium acetate solutions treatments with 1 n HCl and kept for 90 min at 95°C. The supernatants were used for HPLC analysis (20-μL aliquots). Before cell wall-bound compounds were prepared, the pellets were consecutively treated with the following solvents, 25 mL each for 15 to 30 min with stirring followed by filtration on sintered glass discs: (a) 3× methanol, (b) 2× water, (c) 2× acetone, and (d) 2× diethylether. To make sure that there were no major cytosolic contaminants, we included in preliminary experiments treatments with 1 m NaCl and 0.5% SDS.

The crude cell wall preparation (cell debris of the roots) was dried in an exsiccatior. The white powder (30-mg dry weight aliquots) was suspended in 2 mL of 1 N sodium methylate in 80% aqueous methanol, kept for 2 h at 80°C, and allowed to stand for 1 h at room temperature with continuous stirring. The hydrolysates were centrifuged, 1 mL of the supernatant was acidified by adding 100 μL of 85% aqueous ortho-phosphoric acid and centrifuged, and 20 μL of the supernatants were taken for HPLC.

**Analytical HPLC**

The liquid chromatograph (Waters 600-MS system controller) 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 solvent A (1.5% ortho-phosphoric acid in water) to solvent B (80% acetonitrile in water). Injections of 20 μL were carried out by an automatic sampler (Waters 717 autosampler). Compounds were photometrically detected (maxplot between 230 and 400 nm) by a Waters 996 photodiode array detector. Quantitative values were calculated from external standardization with ABA (Fluka) for blumenin and authentic hydroxycinnamic acids (Sigma) for 4-coumaric and fericulic acids from cell wall hydrolysates using the Millennium software 2010 (Millipore). Each value is the mean ± sd from three replicates of 10 plants each. Exact quantitative data of blumenin were obtained by multiplying the ABA data by 2.2. This conversion factor was calculated from the ε of ABA, determined to be log ε = 4.32 for λ_{max} (methanol) = 240 nm and the respective ε of blumenol C glucoside log ε = 3.98 for λ_{max} (methanol) = 240 nm, taken from Miyase et al. (1988).

**Isolation of Blumenin and Preparation of Blumenol C**

The methanolic extracts left from analytical work on barley mycorrhizas (corresponding to more than 100 g fresh weight) were combined and reduced at 30°C (in vacuo) to approximately 10 mL and fractionated on a Sep-Pak cartridge (C_{18}, Waters) with water-methanol mixtures (water, 5 and 30% methanol in water, and 100% methanol). The fraction containing most of the blumenin (30% methanol) was repeatedly chromatographed on a preparative HPLC column (two linked Prep Nova-Pak cartridges C_{18}, 100 × 25 mm i.d., 6 μm, Waters; HPLC system as in analytical chromatography) with linear gradient elution within 30 min from 30% solvent B (80% aqueous methanol) to 70% B in solvent A (1% formic acid in water) at a flow rate of 8 mL min⁻¹. Blumenin was obtained in a yield of approximately 10 mg with more than 95% purity.

To prepare blumenol C, 1 mg of blumenin was dissolved in 2 mL of 1 n HCl and kept for 90 min at 95°C. The liberated aglycone (approximately 50%), blumenol C, was extracted three times with chloroform. The combined organic extracts were taken to dryness.

**Spectroscopic Methods**

Positive and negative electrospray mass spectroscopy was performed on a Finnigan (Bremen, Germany) TSQ 7000 (electrospray voltage 4.5 kV; nitrogen as sheath gas) using a syringe pump (Harvard Apparatus, South Natick, MA) operating at a flow rate of 3 μL min⁻¹. Electron impact mass spectra were recorded on a double-focusing mass spectrometer (AMD 402) at 70 eV and with an ion source temperature of 200 to 250°C.

¹H and ¹³C NMR spectra were recorded at ambient temperature on Bruker (Rheinstetten, Germany) AM 600 (¹H, 600 MHz; ¹³C, 150 MHz) and WM 400 (¹H, 400 MHz; ¹³C, 100 MHz) NMR spectrometers locked to the major ²H resonance of the solvent, CD₃OD. All 1D and 2D (correlation spectroscopy, ¹H-detected direct and long range ¹³C-¹H correlations) spectra were recorded using the standard Bruker software package. Chemical shifts are given in parts per million relative to tetramethylsilane and coupling constants in Hertz. Abbreviation used in the ¹H NMR data correspond to signal multiplicities and are: d = doublet, dd = doublet doublets, ddd = doublet of doublets of doublets, m = multiplets, and s = singlet.

**Carbohydrate Methylation Analysis**

Additional chemical proof of the 1,2-bond of the glucuronosylglucose moiety was performed as described previously (Heuer et al., 1994). In short, blumenin was methylated, hydrolyzed, reduced, peracetylated, and analyzed by GC-MS. The partially methylated alditol acetates were identified from their characteristic fragmentation patterns and by comparison with standard compounds.
RESULTS

Four cereals, barley, wheat, rye, and oat, were grown in defined nutritional media with low phosphate content in expanded clay with and without G. intraradices inoculum. The time courses of root colonizations were followed. First signs of mycorrhization were observed after 2 weeks of plant development. After 4 to 5 weeks, colonization and signs of mycorrhization were observed after 2 weeks of barley roots, and 60 to 70% of the roots of wheat and rye. There was an evenly distributed mycorrhization of the roots.

HPLC traces of methanolic extracts from control roots and arbuscular mycorrhizas from 6-week-old barley plants showed fungus-induced changes in the accumulation pattern of several UV light-absorbing peaks (λ_max below 320 nm) (Fig. 1). One of the components, which showed the most dramatic increase, was isolated and identified by spectroscopic methods (NMR, MS) as 9-O-blumenol C 9-0-(2′-O-β-glucuronosyl)-β-glucoside (Fig. 2) for which we propose the name blumenin. It has been found thus far exclusively in the roots and mycorrhizas of the four cereals. No trace of this compound was detected in the aerial parts of these plants.

The molecular weight of blumenin is indicated by peaks at m/z 571 ([M+Na]+, 51% relative intensity) and m/z 549 ([M+H]+, 5%) in the positive electrospray mass spectrum as well as m/z 547 ([M-H]-, 100%) in the negative one. A peak at m/z 373 ([M+H-C6H4O6]+ (7%) results from the loss of a glucuronic acid moiety. The subsequent loss of a Glc unit results in a peak at m/z 211 ([M+H-C12H10O11]+ (100%), which corresponds to the protonated ion peak of the aglycone, blumenol C. Additional peaks were [M+Na-C6H4O6]+ at m/z 395 (16%) and [M+H-C12H10O11H2O]+ at m/z 193 (13%). The electron impact mass spectrum of the aglycone derived from acid hydrolysis supports these results. The ion at m/z 210 and the fragmentation pattern of the aglycone (m/z [percentage of relative intensity]: 210 [52], 192 [14], 150 [56], 138 [33], 136 [24], 135 [100], 126 [13], 123 [38], 108 [50], 93 [36], 84 [15]) correspond with literature data from blumenol C (Miyase et al., 1988).

The NMR data corroborate the MS analyses. 1H NMR: δ 5.58 [d, H-4, J = 0.8], 4.56 [d, H-1″, J(1″-2″) = 7.8], 4.54 [d, H-1′, J(1′-2′) = 7.6], 3.95 [m, H-9, J = 6.0], 3.88 [dd, H-6′/A, J(5′-6′A) = 2.1], 3.75 [d, H-5′, J(5′-6′) = 3.68] [dd, H-6′/B, J(6′A-6′B) = 11.7], 3.59 [dd, H-3′, J(3′-4′) = 8.9], 3.54 [dd, H-4′, J(4′-5′) = 9.7], 3.44 [dd, H-5′, J(3′-4′) = 8.9], 3.39 [dd, H-2′, J(2′-3′) = 8.9], 3.36 [dd, H-4′, J(4′-5′) = 9.1], 3.28 [dd, H-5′, J(5′-6′B) = 5.7], 2.51 [d, H-2B, J(2A-2B) = 17.2], 2.09 [d, 13-CH3, J = 1.1], 2.01 [d, H-2A, J = 17.5], 2.51 [d, H-2B, J(2A-2B) = 17.2], 2.03 [m, H-6, J not measurable, this proton is the X part of an AA′BB′..X system], 1.5–1.7 [m, H-7A, H-7B, H-8A, H-8B, J not measurable], 1.21 [d, 9-CH3, J = 6.11], 1.14 [s, 12-CH3], 1.05 [s, 11-CH3]. 13C NMR: δ 202.83 (C-3), 175.49 (C-6′), 170.28 (C-5), 125.36 (C-4), 104.93 (C-1″), 100.69 (C-1′), 83.75 (C-2″), 77.62, 77.47, 77.39, 77.11, 75.59 (C-3″, C-5″), 75.81 (C-9), 73.55 (C-4″), 71.69 (C-4′), 62.81 (C-6″), 52.39 (C-6), 48.16 (C-2), 37.84 (C-8), 36.15 (C-11), 29.04 (C-10). The nature of the aglycone, blumenol C, was established form homo- and heteronuclear 2D NMR spectra. Cross-peaks in the 2D 1H correlation spectroscopy spectrum allowed the fragment -CH=C(CH3)CH=CH2 to be identified, and a further two methyl groups and the characteristic AB signals of an isolated methylene group were apparent in the 1D 1H spectrum. Protonated carbons were assigned from the 2D 1H-detected direct (one-bond) 13C-1H correlation. Cross-peaks in the 2D 1H-detected long-range 13C-1H correlation, which showed the presence of a quaternary carbon and the ring-keto group and also provided sufficient data to unambiguously establish the exact positions of these in the aglycone. The β-configuration of the Glc and the glucuronosyl moieties were determined from the large 1H coupling con-
The level of blumenin was directly correlated with the degree of root colonization by the mycorrhizal fungus, i.e. high degrees of colonization correlate with high levels of blumenin. Figure 3 shows the accumulation pattern of blumenin in control roots was demonstrated by its preparative isolation from 50 plants (not documented). The three other cereals studied, wheat, rye, and oat, showed the same phenomenon as barley (Table 1). As an additional control in these experiments, another group of barley plants was grown in heat-treated inoculum (200°C for 30 min). These plants gave the same results (data not shown) as those grown in noninoculated expanded clay.

HPLC analysis of the products from alkaline treatments of cell wall preparations showed the presence of 4-coumaric and ferulic acids as the only phenolics liberated. There were no significant differences in the levels of these hydroxycinnamic acids in 6-week-old cereal mycorrhizas compared with their control roots (Table II). The changes of the levels of these phenolics in root and mycorrhiza cell walls were followed during development of barley plants (Fig. 4). Both the control roots and the mycorrhizas show the same pattern. Young roots and mycorrhizas contain about 50 μmol g⁻¹ dry weight hydrolyzable cell wall-bound 4-coumaric and ferulic acids that decreases to about 20 to 30 μmol in older plants.

**DISCUSSION**

In this paper we describe a new natural compound, blumenol C, 9-O-(2'-O-β-glucuronosyl)-β-glucoside (blumenin), that occurs in roots of barley, wheat, rye, and oat in trace amounts but accumulates to high concentrations during mycorrhization with *G. intraradices*. The spectroscopic data of the disaccharide moiety and those of the aglycone, compared with literature data (Galbraith and Horn, 1972; Miyase et al., 1988), unambiguously identified blumenin as a new natural blumenol C glycoside. With regard to the elucidation of the structure and its occurrence, there is no systematic distribution of blumenol C and its conjugates in plants. The aglycone of blumenin, blumenol C, was found for the first time, together with its possible derivatives blumenol A and B, in leaves of *Podocarpus blumei* (Podocarpaceae) (Galbraith and Horn, 1972). A blumenol-related glycoside, blumenol C glucoside, has been isolated from leaves of *Nicotiana rustica* (Solanaceae) (Kodama et al., 1984) and *Isatis tinctoria* (Brassicaceae) (Hartleb and Seifert, 1994) and the aerial parts of *Epimedium grandiflorum* (Berberidaceae) (Miyase et al., 1988).

The most obvious effect observed in changes of second-order products of all four cereal roots was the fungus-induced accumulation of blumenin. Although there is nothing known about biological activities of blumenol C and its conjugates, it seems that the accumulation of blumenin is directly correlated with mycorrhiza formation. The microscopically estimated increase in the degree of mycorrhiza-

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**Table I.** Blumenin (nmol g⁻¹ fresh weight of roots) in 6-week-old noncolonized cereal roots (control) and roots colonized with *G. intraradices* (mycorrhiza)

<table>
<thead>
<tr>
<th>Plant</th>
<th>Control</th>
<th>Mycorrhiza</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley</td>
<td>228 ± 27</td>
<td>317 ± 34</td>
</tr>
<tr>
<td>Wheat</td>
<td>61 ± 11</td>
<td>35 ± 8</td>
</tr>
</tbody>
</table>

| a Trace, Less than 2 nmol g⁻¹ fresh weight. |

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**Table II.** Alkaline-labile cell wall-bound hydroxycinnamic acids (μmol g⁻¹ dry weight of cell debris of the roots) in 6-week-old noncolonized cereal roots (control) and roots colonized with *G. intraradices* (mycorrhiza)

<table>
<thead>
<tr>
<th>Plant</th>
<th>4-Coumaric Acid</th>
<th>Ferulic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Mycorrhiza</td>
</tr>
<tr>
<td>Barley</td>
<td>26.5 ± 2.8</td>
<td>26.7 ± 4.2</td>
</tr>
<tr>
<td>Wheat</td>
<td>43.8 ± 1.8</td>
<td>46.0 ± 1.5</td>
</tr>
<tr>
<td>Rye</td>
<td>56.3 ± 4.7</td>
<td>57.6 ± 5.6</td>
</tr>
<tr>
<td>Oat</td>
<td>5.6 ± 0.8</td>
<td>6.8 ± 0.5</td>
</tr>
</tbody>
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tion during plant development parallels the induced blumenin accumulation.

The phenomenon of fungus-induced blumenin accumulation in gramineous arbuscular mycorrhizas seems to be of restricted occurrence within the Poaceae. So far, we detected blumenin accumulation in mycorrhizas of members of the tribes Triticeae (barley, wheat, rye) and Aveneae (oat) of the subfamily Pooidae, but we were unable to detect blumenin in mycorrhizas of some species of Festuca, Pea, Lolium (tribe Poaeae, subfamily Pooidae), Phileum (tribe Phalarideae, subfamily Pooidae), Panicum, Setaria, Sorghum (subfamily Panicoideae), and Zea (subfamily Andropogoneae). Results of an extensive taxonomic study of the occurrence of blumenin accumulation in mycorrhizas of members of the Poaceae will be published elsewhere.

Studies of the effects of blumenol C and blumenin on fungal development are in progress. In this context it will be crucial to localize blumenin biosynthesis and accumulation within the cereal mycorrhizas. It will also be of great interest to study the fungus-induced increases in the levels of the enzymes involved in blumenin biosynthesis, e.g., the glucosyl- and the glucuronosyltransferases, and eventually the dioxygenase involved in the biosynthesis of the aglycone blumenol C. Dioxygenase activity is believed to catalyze the rate-limiting cleavage of a carotenoid as a potential precursor in the biosynthetic route of the structurally related phytohormone ABA (Parry and Horgan, 1991). The present results and related studies of induction of the accumulation of ABA (Danneberg et al., 1992) and a C₁₄ carotenoid in maize (Klingner et al., 1995a) and some other gramineous plants (Klingner et al., 1995b) suggest that mycorrhiza formation in members of the grass family (Poaceae) is somehow correlated with the terpenoid metabolism of the roots. Future studies will localize the transcripts encoding the enzymes catalyzing blumenin formation, in analogy to a study by Harrison and Dixon (1994). They showed a cell type-specific differential expression of genes of phenylpropanoid biosynthesis in Medicago truncatula roots colonized with the arbuscular fungus Glomus versiforme.

The fact that there was no difference in the levels of cell wall-bound phenolics between colonized and noncolonized plant roots nevertheless needs attention. Obviously neither appressorium formation nor hyphal penetration into root cells induces changes in the pattern of cell wall-bound phenolics. This is in agreement with the work of Codignola et al. (1989), who showed that there was no difference in cell wall-bound phenolics in endomycorrhizas of Allium porrum and Ginkgo biloba with G. versiforme. The plant seems to recognize the symbiotic fungus and does not reject hyphal penetration by wall reinforcement. The mycorrhizal fungus might also be able to suppress cell wall defense reactions of the plant. This is in contrast to pathogenic plant-fungus interactions, in which fungus-induced increased phenolic levels of the cell wall may constitute a barrier against pathogen invasion (Graham and Graham, 1991; Beimen et al., 1992; Kauss et al., 1993) by decreasing the digestibility of the cell wall (Fry, 1986). On the other hand, it has been observed that the levels of cell wall-bound ferulic and 4-coumaric acids as well as a ferulic acid tyramine amide increase during mycorrhization of Allium cepa with G. intraradices and G. versiforme (Grandmaison et al., 1993). This may lead to an increased resistance of mycorrhizas to pathogenic fungi. With regard to the cereals studied in the present work, we cannot exclude fungus-induced changes in cell wall composition, involving accumulation of nonhydrolyzable polymeric phenolics and increased lignification (Dehne and Schönbeck, 1979).

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LITERATURE CITED


