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Roots of nutrient-deprived *Brachiaria* species accumulate 1,3-di-*O*-*trans*-feruloylquinic acid

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Abstract

A novel di-hydroxycinnamoylquinic acid ester, 1,3-di-*O*-*trans*-feruloylquinic acid (DFQA), was isolated from roots of nutrient-deprived *Brachiaria* species — the most widely sown tropical forage grasses in South America. In contrast to other so far characterized quinic-acid esters, DFQA exists in a chair conformation with the carboxylic group in the axial orientation. It accumulates in older parts of the root system, but not in root apices or shoots. Higher levels were found in *B. ruziziensis*, which is poorly adapted to infertile acid soils, than in well adapted *B. decumbens*. DFQA was also found in the soil, most likely as a result of root decay, because it was not detected in root exudates of plants cultivated in solution culture. Nitrogen and phosphorus deficiency — but not aluminum toxicity or deprivation of other nutrients — stimulated its synthesis in roots. Its accumulation was correlated with a shift in biomass partitioning toward the root system.

Keywords: *Brachiaria ruziziensis*; *Brachiaria decumbens*; Poaceae; Hydroxycinnamoylquinic acid esters; Diferuloylquinic acid; Nitrogen and phosphorus deficiency; Biomass partitioning

1. Introduction

The genus *Brachiaria* (Poaceae) comprises about 100 species. It has its center of diversity in the savanna ecosystems of eastern Africa and is distributed pantropically (Renvoize et al., 1996). Given the lack of grazing-resistant native forage grasses, *Brachiaria* cultivars have been widely adopted for livestock production in South American savannas — with 40–70 million hectares in Brazil alone (Parsons, 1972; Boddey et al., 1996; Fisher and Kerridge, 1996). As part of an investigation of physiological attributes contributing to acid-soil adaptation, we investigated aromatic metabolites synthesized in roots of *B. decumbens* (well adapted to acid soils), and *B. ruziziensis* (poorly adapted to acid soils). The structure of the dominant compound was elucidated, and factors stimulating its synthesis were identified.

2. Results and discussion

2.1. Accumulation of aromatic metabolites in roots of plants grown in soil

Aromatic metabolites were extracted from roots of *B. ruziziensis* and *B. decumbens*, grown in a sandy-loam Oxisol in the glasshouse. Reverse-phase HPLC analysis showed two dominant, closely eluting, compounds: **1** (15.0 min) and **2** (15.3 min). The third major peak at 13.7 min was, at least in part, a product of heat-induced degradation or isomerization of **1** and **2** (Fig. 1a). Concentrations of **1** and **2** were higher in roots of plants grown under low-fertility conditions (Fig. 1b).

Roots of diploid *B. ruziziensis* accumulated larger amounts than those of tetraploid *B. decumbens*. Artificially induced tetraploids of *B. ruziziensis* contain more

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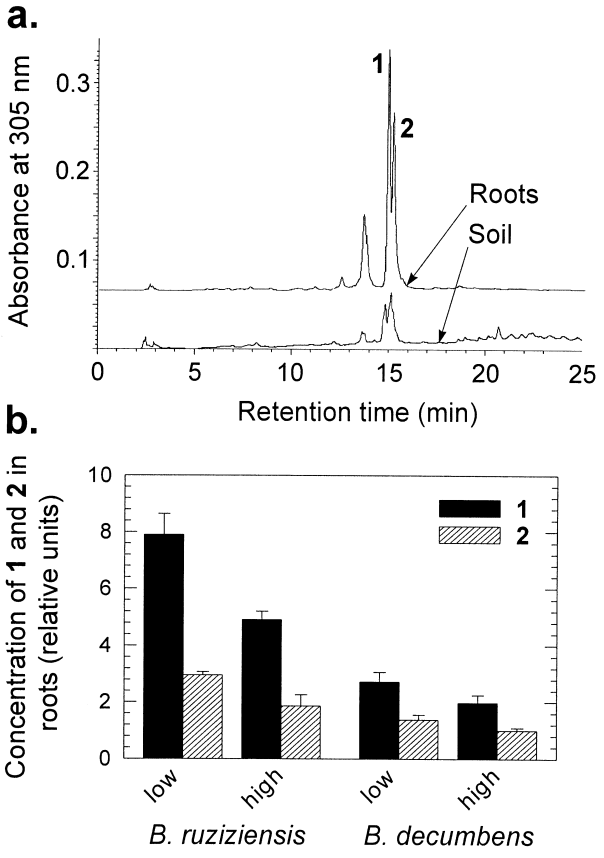

Fig. 1. Aromatic metabolites in roots of *Brachiaria* species. (a) Reverse-phase HPLC chromatograms of root and soil extracts from *B. ruziziensis* grown in an Oxisol under low-fertility conditions. Two dominant metabolites, **1** (15.0 min) and **2** (15.3 min), accumulated in both roots and soil. The peak at 13.7 min was, at least partially, a product of heat-induced degradation or isomerization of **1** and **2**, because it augmented at their expense when the extract was heated. (b) Relative levels of **1** and **2** in roots of *B. ruziziensis* and *B. decumbens* grown in an Oxisol under low- and high-fertility conditions. Values are means±SE ($n=4$). Subsequent quantification, using purified compounds **1** and **2** as standards, indicated that a relative level of one corresponded to 0.18 μmol g fresh weight^{-1}.

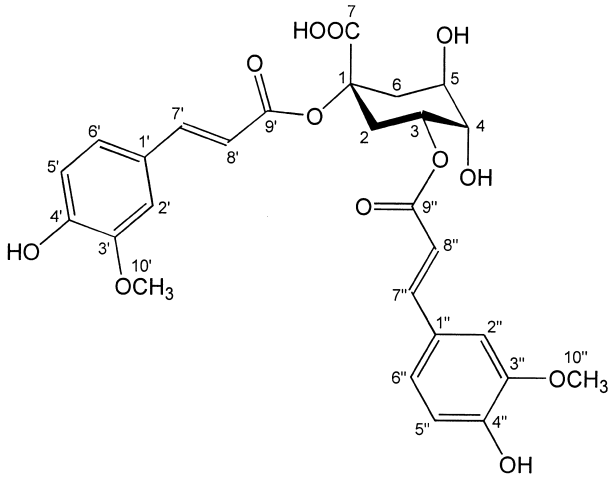

Fig. 2. Structure of compound **1**, 1,3-di-*O*-*trans*-feruloylquinic acid (DFQA), existing in a chair conformation with the carboxylic group in axial orientation. Compound **2** lacks one of the two methoxy groups of compound **1** (data not shown).

lignin (do Valle et al., 1988), which indicates an intensified phenylpropanoid metabolism of the tetraploid genotype. The ploidy level does therefore not account for the interspecific differences in accumulation of these compounds. Differential sensitivity to nutrient deficiency may have been the reason, because nutrient-limited growth conditions stimulated accumulation, and *B. ruziziensis* is poorly adapted to infertile soils (Rao et al., 1996). Both compounds were purified, and the structure of **1** was characterized using spectral methods.

2.2. Structural elucidation

The ^{1}H NMR spectrum of **1** displayed two sets of olefinic protons, at 6.36, 6.39 and 7.37, 7.52 ppm, with coupling constants of 15.9 Hz each, indicating that both pairs of proton were *trans* to each other. Two methoxy groups, at 3.75 and 3.78 ppm, and a pair of almost identical, tri-substituted, aromatic moieties, indicated by two pairs of three ABX protons at 6.73 to 7.24 ppm, were other characteristic signals of the ^{1}H NMR spectrum (Table 1). The UV absorption at 330 nm was consistent with an unsaturated carbonyl chromophore, conjugated with an aromatic residue. These data therefore suggested that **1** was a di-*trans*-hydroxycinnamoyl ester. Because both aromatic rings were tri-substituted and contained a total of two methoxy groups, **1** had either two *trans*-feruloyl moieties, or one *trans*-caffeoyl and one *trans*-dimethylcaffeoyl moiety. There was no signal at 7.02 ppm, the typical value of the H-2 in caffeic acid. Instead, two protons exhibited an identical chemical shift of 7.24 ppm. This confirmed that **1** contained two *trans*-ferulic acid moieties (Iwahashi et al., 1985). The fragment ion at m/z 177, observed by positive-ion FAB–MS, provided additional evidence for the ferulic acid functionalities.

The protonated molecular ion at m/z 545 suggested the molecular formula $C_{27}H_{28}O_{12}$ ($M_r=544$), which is in agreement with that of diferuloylquinic acid. The ^{13}C NMR spectrum of **1** displayed signals for two *trans*-feruloyl moieties, one carboxylic acid functionality (173.7 ppm), and five additional carbons with chemical shifts similar to quinic-acid resonances in hydroxycinnamoylquinic acid esters (Rumbero-Sánchez and Vázquez, 1991; Merfort, 1992; Agata et al., 1993; Scholz et al., 1994; Maruta et al., 1995). The C-6 signal of the quinic acid moiety was covered by the solvent peak (Table 1). Therefore, **1** was concluded to be a di-*trans*-feruloylquinic acid ester.

Acylation of hydroxyl groups of quinic acid causes paramagnetic chemical shifts of geminal protons — a feature that can be used to distinguish positional isomers of hydroxycinnamoylquinic acid esters (Morishita et al.,

Table 1
^{1}H NMR (300 MHz) and ^{13}C NMR (125 MHz) data of compound **1** in DMSO-d_6. Off-diagonal peaks detected by COSY (500 MHz) are given as well. Chemical shifts δ_H and δ_C are in ppm, and coupling constants J in Hz[a–i]

Position	δ_H (Number of protons; multiplicity J)	δ_H (COSY)	δ_C
Quinic acid moiety			
1	–	–	82.6
2	2.28 (1 H_{ax}; *t* 13, 13)	2.00, 5.18	33.3
	2.00 (1 H_{eq}; *dd* 12, 3)	2.28, 5.18	
3	5.18 (1 H_{ax}; *dt* 10, 4, 4)	2.00, 2.28, 3.64	69.8[a]
4	3.64 (1 H_{eq}; *m*)	3.69, 5.18	71.1
5	3.69 (1 H_{eq}; *m*)	1.85, 3.64	68.5[a]
6	(H_{ax} covered by DMSO)	1.85	(covered by
	1.85 (1 H_{eq}; *d* 12)	2.49, 3.69	DMSO)
7 (carboxyl)	–	–	173.7
Ferulic acid moieties			
1′	–	–	125.7[b]
2′	7.24 (1 H; *s*)	7.03	111.0[c]
3′	–	–	148.9[d]
4′	–	–	147.9
5′	6.73 (1 H; *d* 7.8)[a]	7.03	115.5
6′	7.03 (1 H; *d* 8.1)	6.73, 7.24	122.7[e]
7′ (β olefinic)	7.37 (1 H; *d* 15.9)[b]	6.36	143.5[f]
8′ (α olefinic)	6.36 (1 H; *d* 15.9)[c]	7.37	115.1[g]
9′ (ester carbonyl)	–	–	165.2[h]
10′ (-OMe)	3.78 (3 H; *s*)[d]	–	55.6[i]
1″	–	–	126.0[b]
2″	7.24 (1 H; *s*)	7.03	111.1[c]
3″	–	–	149.2[d]
4″	–	–	147.9
5″	6.75 (1 H; *d* 7.8)[a]	7.03	115.5
6″	7.03 (1 H; *d* 8.1)	6.75, 7.24	122.9[e]
7″ (β olefinic)	7.52 (1 H; *d* 15.9)[b]	6.39	144.7[f]
8″ (α olefinic)	6.39 (1 H; *d* 15.9)[c]	7.52	116.7[g]
9″ (ester carbonyl)	–	–	166.0[h]
10″ (-OMe)	3.75 (3 H; *s*)[d]	–	55.7[i]

[a–i] Identically labeled resonances within each column are interchangeable.

1984). Because there was only a single deshielded proton signal (5.18 ppm; Table 1), one of the *trans*-feruloyl residues must have been attached to the C-1 of quinic acid, which has no proton. The paramagnetic shift of the ^{13}C signal of C-1, compared to hydroxycinnamoylquinic acid esters substituted at other positions, confirmed substitution at this position (Agata et al., 1993; Scholz et al., 1994).

If the second *trans*-feruloyl moiety had been attached to the C-4 of quinic acid, the proton at 5.18 ppm would have shown only two vicinal couplings, with C-3 and C-5 protons. However, it appeared as a doublet of triplets. Therefore, only the C-3 and C-5 carbons remained as possible substitution sites. Only protons in *trans*-diaxial orientation have large vicinal coupling constants in cyclohexanes ($J \sim 10$ Hz; Karplus, 1963). From this, it followed that the proton at 5.18 ppm ($J = 10$, 4 and 4 Hz; Table 1) must have been in axial orientation, and that one vicinal proton was in axial, and the other two in equatorial, orientation. For the naturally occurring stereoisomer of quinic acid, (−)-quinic acid, this is only possible if the second *trans*-feruloyl residue is attached to C-3. The resulting 1,3-substitution pattern was corroborated by non-overlapping signals of the two C-2 protons. This has previously been observed only for quinic acid simultaneously acylated at C-1 and C-3 (Pauli et al., 1998). In addition, the carbon signal of the C-2 of quinic acid was shifted upfield to a similar extent as in other 1,3-diacylquinic acids (Agata et al., 1993; Scholz et al., 1994; Maruta et al., 1995).

To confirm the substitution pattern inferred from the ^{1}H and ^{13}C NMR spectra, H–H connectivities were established with a 2D-homonuclear-correlated proton spectrum (COSY). All predicted cross peaks of quinic acid protons were detected — with exception of the H_{6ax}–H_{5eq} peak, presumably because the H_{6ax} signal was very broad and covered by the solvent peak (Table 1). Therefore, compound **1** is 1,3-di-*O*-*trans*-feruloylquinic acid (DFQA), a novel di-hydroxycinnamoyl ester of quinic acid (Fig. 2). 1-*O*-acylquinic acid esters are particularly prone to trans-esterification stimulated by heat (Herrmann, 1978). This may explain the instability of DFQA at high temperatures, observed during the initial investigation of aromatic metabolites in roots (Fig. 1a).

Quinic-acid proton connectivities and multiplicities — particularly that of H_{3ax} — demonstrated that the cyclohexane ring existed in a chair conformation that places the carboxylic group in axial, and both *trans*-feruloyl residues in equatorial, orientation (Fig. 2). In contrast, free quinic acid, and all other so far characterized quinic-acid esters, exist in the alternative chair conformation, with the carboxylic group in equatorial orientation (Flores-Parra et al., 1989; Pauli et al., 1998). The latter is also true for cynarin, 1,3-di-*O*-*trans*-caffeoylquinic acid, whose substitution pattern is identical to that of DFQA (Horman et al., 1984).

Molecular modeling has suggested a charge-transfer interaction between the two aromatic rings in 3,5-di-*O*-*trans*-caffeoylquinic acid (Pauli et al., 1998). Accordingly, the asynchronous signals of aromatic protons in the two *trans*-caffeoyl moieties of cynarin (Pauli et al., 1998) may reflect anisotropic ring-current effects, caused by an interaction between the two axially oriented substituents, which are spatially close to each other (Horman et al., 1984). The two additional methyl groups in DFQA, compared to cynarin, may disrupt the interaction between the two aromatic rings to such an extent that the alternative chair conformation, with both hydroxycinnamoyl residues in equatorial orientation, becomes the lowest-energy conformer of the molecule. The co-purified Na^+ and K^+ ions may also have contributed to a conformeric rearrangement (see FAB–MS data in the Section 3).

The ^1H NMR spectrum of **2** demonstrated that this compound was closely related to **1**. It showed that one of the two ferulic-acid residues of **1** was replaced by *p*-coumaric acid in **2** — a conclusion that was confirmed by FAB–MS (data not shown). Therefore, **2** is a hitherto unreported 1,3-substituted quinic-acid ester of *trans*-ferulic and *p*-coumaric acid. It existed in the same chair conformation as **1**. A comparison of the chemical shifts of olefinic protons of compound **2** with those of DFQA, and other quinic-acid esters described in the literature, suggested that the *trans*-feruloyl residue was attached to the C-1 of quinic acid, and the *p*-coumaroyl residue to the C-3. Logistic reasons prevented us from carrying out additional spectroscopic analyses to definitively decide between the two possible positional isomers.

2.3. Spatial pattern of accumulation

Both the hydroxycinnamoyl and the quinic acid moieties of DFQA and compound **2** are derived from metabolites of the shikimate pathway. The latter appears to be particularly active in root plastids, as suggested by the high expression levels of the involved enzymes (Weaver and Hermann, 1997). Accordingly, roots but not shoots were found to accumulate DFQA and compound **2**. This contrasts with limpograss (*Hemarthria altissima*), another African forage grass, which accumulates higher concentrations of chlorogenic acid (5-*O*-*trans*-caffeoylquinic acid) in shoots than in roots (Cherney et al., 1990). Other, so far uncharacterized, aromatic metabolites accumulated under nutrient deficiency in *Brachiaria* shoots, however (data not shown).

When cultivated in an unfertilized Oxisol, concentrations of DFQA and compound **2** in roots of *B. ruziziensis* were as high as 4.3±0.7 and 3.5±0.6 μmol g^{-1} fresh weight (means±SD) — that is, about 3.3 and 2.6% of the dry root biomass (data not shown). Neither of the two compounds could be detected in root apices. In addition, primary roots contained higher concentrations than seminal and adventitious roots. This pattern suggests that synthesis of DFQA and compound **2** starts beyond the apical elongation zone and continues during the root's lifespan, thus resulting in a progressive accumulation in older parts of the root system.

DFQA and compound **2** were also found in methanolic extracts of the soil, in which *B. ruziziensis* had been cultivated (Fig. 1a). Yet, we were unable to detect them in root exudates of plants cultivated in nutrient solutions, either in the glasshouse or under sterile conditions in a growth chamber (data not shown). When accumulation of shikimate pathway intermediates was artificially stimulated by treating plants with inhibitors of shikimate-pathway enzymes, they were sequestered in vacuoles (Schmid and Amrhein, 1995). Another hydroxycinnamoyl ester has been shown to accumulate in vacuoles (Sharma and Strack, 1985). These findings, and the absence of DFQA and compound **2** in root exudates, suggest that these compounds are compartmentalized into vacuoles, and only released into the soil upon root decay.

2.4. Factors stimulating accumulation

Shikimate kinase, an enzyme of the pre-chorismate portion of the shikimate pathway, is subject to control by energy charge (Schmidt et al., 1990). Under energetically unfavorable conditions, a larger proportion of shikimate molecules may be diverted from the main path — leading to the synthesis of aromatic amino acids — and converted into quinic acid and its derivatives (Herrmann, 1995). The latter, and the transcriptional regulation of genes coding for enzymes of the shikimate pathway by nutrient deficiency (Weaver and Herrmann, 1997), may account for the accumulation of DFQA and compound **2** under nutrient-deficient growth conditions (Fig. 1b).

To investigate which nutrient deficiencies stimulate the synthesis of DFQA and compound **2**, plants were cultivated in low-ionic-strength nutrient solutions, designed to simulate nutrient concentrations in soil solutions of Oxisols in neotropical savannas (Wenzl et al., in preparation). Deprivation of either nitrogen or phosphorus induced accumulation of both compounds in

roots of *B. ruziziensis*, while depletion of other nutrients, such as potassium, sulfur, calcium and magnesium, or aluminum toxicity, had no effect (Fig. 3a). Caffeoylquinic-acid esters have been found to accumulate under nitrogen and phosphorus deprivation — but also under sulfur and potassium deficiency — in other plant species such as tobacco and sunflower (Gershenzon, 1983). As in soil-grown plants, DFQA and compound **2** accumulated to higher concentrations in roots of hydroponically cultivated *B. ruziziensis* than in those of *B. decumbens* (Fig. 3b). Their accumulation may therefore reflect the susceptibility of the species to nitrogen and phosphorus deficiency.

Concentrations of DFQA and compound **2** in roots of nitrogen or phosphorus-deprived plants were correlated with a shift in biomass partitioning toward the root system, at the expense of the shoot system. The latter was quantified using the "root-weight ratio", that is, the root fresh weight per unit of plant fresh weight (Fig. 3b). This correlation suggests that preferential biomass allocation to roots, and accumulation of these quinic-acid esters, are either causally linked, or regulated by the same events. Seedling bioassays with DFQA indicated a statistically significant, though marginal, stimulatory activity on root elongation (about 10% at 20 μM DFQA; data not shown).

Experiments using transgenic plants with down-regulated phenylpropanoid metabolism have indicated that phenylpropanoids, such as chlorogenic acid, may play an important role in preventing premature tissue senescence (Tamagnone et al., 1998), and act as a chemical barrier against fungi (Maher et al., 1994). The possibility that DFQA and compound **2** might act in a similar manner — thus increasing root lifespan under nutrient-limited growth conditions — is a hypothesis that deserves future research.

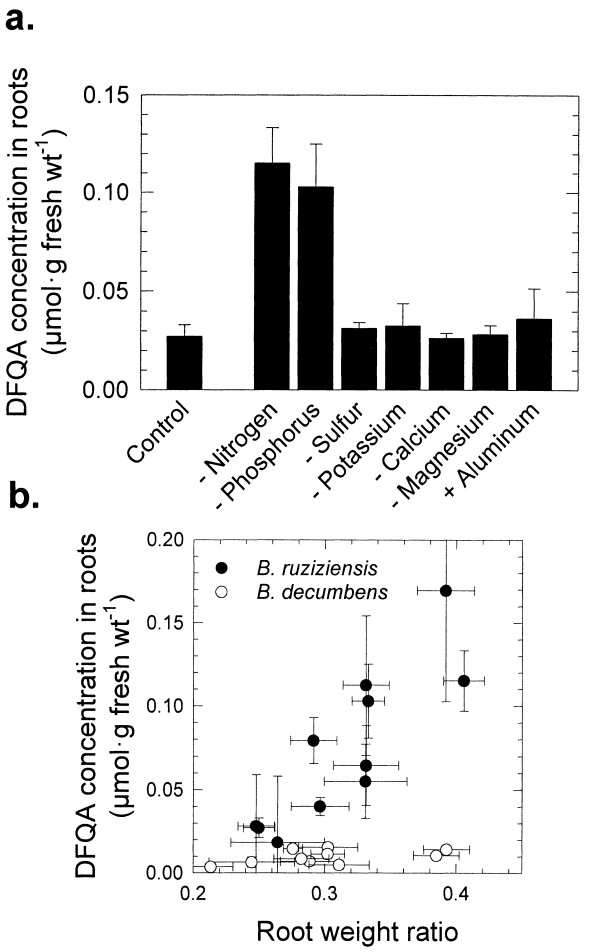

Fig. 3. Factors stimulating accumulation of DFQA in roots of *Brachiaria* species. (a) Concentration of DFQA in roots of *B. ruziziensis*, cultivated for 10 days in nutrient solutions lacking a single nutrient, or containing phytotoxic aluminum. Compound **2** accumulated in parallel with DFQA (data not shown). (b) Correlation between the preferential allocation of biomass to roots — quantified by the root-weight ratio (root fresh weight per unit of plant fresh weight) — and the accumulation of DFQA in roots of *B. ruziziensis* and *B. decumbens*. Plants were grown in nutrient solutions with a varying level of nitrogen or phosphate. All values are means±SE ($n=4$ for DFQA content, and $n=8$ for root-weight ratio). Compound **2** accumulated in parallel with DFQA (data not shown).

3. Experimental

3.1. Plant varieties and growth conditions

Seeds of *Brachiaria ruziziensis* cv. Common (sexual, diploid) were obtained from FundeAgro (Pucallpa, Peru). Seeds of the commercial cultivar *Brachiaria decumbens* cv. Basilisk (apomictic, tetraploid) were purchased from Agrosemillas (Medellín, Colombia). Plants were cultivated in pots containing a sandy-loam Oxisol — supplemented with fertilizer mixtures to establish two fertility levels — in the glasshouse at CIAT (Cali, Colombia). The low-fertility mixture contained triple superphosphate, KCl, dolomitic lime and elemental sulfur. The high-fertility mixture also contained urea, $ZnCl_2$, $CuCl_2$, H_3BO_4 and Na_2MoO_4 (Rao et al., 1992). For each combination of species and fertility level, ten seeds were sown in each of three pots containing 4 kg of soil. Two weeks later, all but the three largest plants were removed. After 50 days of growth, roots of each pot were gently spread out so that soil particles would fall down upon shaking. Subsamples (100 g) of soil particles were taken from *B. ruziziensis* pots of the low-fertility treatment, after passing them through a sieve to remove residual root fragments. Roots were repeatedly washed with deionized water, ground in liquid nitrogen and stored at −80°C.

3.2. Characterizing aromatic metabolites in roots and soil

Soil samples were extracted twice with 200 ml MeOH each. After filtration, the extracts were dried under reduced pressure at 40°C, dissolved in 2×750 μl MeOH,

dried in a SpeedVac centrifuge at 40°C, and dissolved in 200 µl MeOH. The resulting samples were analyzed by reverse-phase HPLC (C-18 column; 250×4 mm), using a gradient of 0–55% acetonitrile in H_2O (pH adjusted to 3.0 with HOAc) in 25 min at 1.5 ml min^{-1} (Graham, 1991).

Aliquots of root powder (100 mg) were homogenized in 400 µl MeOH for 1 min, using a hand-held Ultraturrax homogenizer. Extraction with MeOH yielded identical results as extraction with 80% EtOH, indicating that esterification of **1** and **2** with MeOH, and migration of hydroxycinnamoyl residues along the quinic-acid ring via trans-esterification (Herrmann, 1978), were insignificant under the condition used for extraction. Heat, however, degraded **1** and **2** and was therefore avoided. After centrifugation at 14000***g*** during 3 min, the supernatant was removed, passed through a 0.45-µm filter, and analyzed by HPLC as above. Re-extraction of the pellet resulted in negligible increases in yield. UV detection at 250, 280 and 305 nm showed two dominant peaks (**1** and **2**). Concentrations of compound **1** in roots were quantified, using purified DFQA as an external standard.

3.3. Purification of compound *1*

Plants were cultivated in soil under low-fertility conditions and harvested as described above (ten pots). The root powder was lyophilized at −40°C (9.2 g), and extracted with 3×50 ml MeOH at 50°C/1000 psi, using an Accelerated Solvent Extractor (Dionex). The extracts were combined, evaporated to dryness under reduced pressure at 40°C, and the resulting residue (1.0 g) was dissolved in 4 ml MeOH. Insoluble material was removed by centrifugation at 8000 ***g*** during 10 min, the pellet was re-extracted with 2 ml MeOH, and the combined supernatants were loaded on an MPLC silica-gel column, equilibrated with $CHCl_3$–MeOH–H_2O (1:1:0.01). The column was first eluted with $CHCl_3$–MeOH–H_2O (1:1:0.01; Fraction I), next with $CHCl_3$–MeOH–H_2O (1:1:0.1; Fraction II), and finally with MeOH (Fraction III). Sub-fractions of II were analyzed by TLC (silica gel; $CHCl_3$–MeOH–H_2O, 1:1:0.01). Two UV-absorbing spots showed strong fluorescence under 366 nm, after spraying with 10% H_2SO_4 and heating at 100°C. Preparative TLC (bands eluted with MeOH) and HPLC confirmed that these spots corresponded to compounds **1** and **2**.

Sub-fractions containing a mixture of compounds **1** and **2** were pooled, evaporated under reduced pressure at 40°C, and dissolved in 3 ml MeOH. Insoluble material was removed by centrifugation at 14000 ***g*** for 3 min, the supernatant (47 mg) was passed through a 0.45-µm filter, and aliquots of about 10 mg were separated by preparative HPLC, using two Shodex GS-310 columns (20×500 mm), connected in series, and isocratic elution with 30% MeOH in H_2O at 25°C. The final yield for **1** was 7.8 mg.

3.4. Structural elucidation

Compound **1** was dissolved in DMSO-d_6, and ^{1}H NMR (300 MHz) and ^{13}C NMR (125 MHz) spectra were recorded. To resolve the multiplicity of the proton signal at 5.18 ppm, a second ^{1}H NMR spectrum was taken at 500 MHz. A 2D-homonuclear-correlated proton spectrum (COSY) of **1** was recorded as well (500 MHz). The protonated fraction of DMSO-d_6 was used as internal standard. Positive-ion FAB mass spectra were used to determine M_r.

3.4.1. Compound *1* (1,3-di-O-trans-feruloylquinic acid; DFQA)

Pale yellowish amorphous powder. ^{1}H NMR and ^{13}C NMR: see Table 1. Positive-ion FAB–MS (NBA) *m*/*z* (rel. int.): 177 $[feruloyl]^+$ (100), 545 $[M+H]^+$ (5), 567 $[M+Na]^+$ (37), 583 $[M+K]^+$ (4), 589 $[M-H+2Na]^+$ (19), 605 $[M-H+Na+K]^+$ (4); positive-ion FAB–MS (glycerol) *m*/*z* (rel. int.): 177 $[feruloyl]^+$ (100), 545 $[M+H]^+$ (14), 567 $[M+Na]^+$ (23), 583 $[M+K]^+$ (5). UV $\lambda_{max}^{H_2O}$ nm (log ϵ): 295sh, 330 (4.26), 388sh.

3.5. Studying the pattern of accumulation and exudation

To investigate the distribution and exudation of DFQA, plants were cultivated in low-ionic-strength nutrient solutions in the glasshouse. Seeds of both species were surface-sterilized with 70% EtOH during 1 min, and 2% NaOCl, 0.1% Triton X-100 during 15 min. They were then thoroughly rinsed with deionized water, and germinated on mosquito nets glued to Styrofoam(®) frames, floating on the surface of 10 l of a basal nutrient solution (nutrient concentrations in µM: 500 NO_3^-, 50 NH_4^+, 5 $H_2PO_4^-$, 300 K^+, 300 Ca^{2+}, 150 Mg^{2+}, 5 Fe^{3+}, 1 Mn^{2+}, 1 Zn^{2+}, 0.2 Cu^{2+}, 160 Na^+, 6 H_3BO_3, 0.001 MoO_4^{2-}, 5 $EDTA^{4-}$, 5 SiO_3^{2-}, 286 SO_4^{2-}, 332.4 Cl^-, pH adjusted to 4.2 with HCl; Wenzl et al., in preparation). After 7 days, groups of four seedlings were transferred to pots, wrapped in polyethylene bags, and filled with 1.4 l of the same nutrient solution, which was aerated constantly.

Three days later, plants were transferred to nutrient solutions containing decreasing nitrogen or phosphorus levels, or nutrient solutions lacking another macronutrient. Cultivation was continued for a further 10 days, during which the solutions were replaced daily. Treatments with decreasing nitrogen levels contained 550, 246, 110, 49, 10 and 0 µM of mineral N, with a constant NO_3^- to NH_4^+ ratio of 10:1, and a correspondingly increasing Cl^- concentration. Concentrations of all other nutrients and the pH were kept constant. In a similar manner, treatments with decreasing phosphorus levels (5, 2.2, 1, 0.4 and 0 µM of phosphate) contained increasing Cl^- concentrations. *B. ruziziensis* was also transferred to nutrient solutions lacking either SO_4^{2-}, K^+,

Ca^{2+} or Mg^{2+}, and to a nutrient solution containing 113 μM Al^{3+}. In these solutions, Cl^- or Na^+ ions were used to achieve electroneutrality.

At harvest, roots and shoots were separated, roots were blotted dry, and the root-weight ratio (root fresh weight per unit plant fresh weight) was determined for each individual plant. Roots and shoots of individual plants (or groups of two plants from the treatments containing 0 or 10 μM nitrogen, or 0 μM phosphorus) were ground in liquid nitrogen and stored at −80°C until HPLC analyses. In some cases, root apices of seminal and adventitious roots were collected and stored separately, or the primary and secondary root system of single plants were ground separately.

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