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Identification and characterization of flavonoids in the root exudate of *Robinia pseudoacacia*

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Abstract Eight compounds exuded from young roots of black locust (*Robinia pseudoacacia*) were separated by two-dimensional HPTLC, by HPLC and GC, and were identified by spectroscopic methods (ultraviolet/visible spectroscopy and mass spectrometry) as 4′,7-dihydroxyflavone, apigenin, naringenin, chrysoeriol and isoliquiritigenin. Structural assignments were confirmed by comparison with authentic standards. The capacity to induce β-galactosidase activity in *Rhizobium* sp. NGR234 containing a *nod* box::*lac*Z fusion on plasmid pA27 identified these flavonoids and the chalcone as *nod* gene inducers. This indicates the important role of these compounds in nodulation of this legume tree.

Key words *Robinia pseudoacacia* · *Rhizobium* sp. NGR234 · Root exudate · Flavonoids · *nod* Gene induction

Introduction

Flavonoid compounds have been reported to be widely distributed throughout the plant kingdom (Harborne 1967) and are ubiquitous in roots, leaves and flowers of higher plants. Root flavonoids may play various functions in protecting the plants against pests and diseases, by regulating root growth and exerting allelopathic effects.

Flavonoids also play a significant role in the symbiotic legume-*Rhizobium* interaction by (1) enhancing the growth rate of bacterial cells, (2) promoting bacterial movement toward the plant, and (3) inducing transcription of rhizobial nodulation (*nod*) genes (Phillips and Tsai 1992). This sequence of events governs the early processes involved in nodulating a host plant (Mulligan and Long 1985; Spaink et al. 1989). Induction of the nodulation genes is dependent on NodD, a protein in the inner bacterial membrane (Mulligan and Long 1985). Flavonoid compounds that induce nodulation genes in concert with NodD have been isolated from exudates of seeds and roots of a wide variety of herbaceous legumes. Compatibility is first determined by preinfection events involving an exchange of molecular signals between the plant and the bacterium, a process that mediates their mutual differentiation (Fisher and Long 1992). The chemical structures of these compounds were found to be host-symbiont specific (Rossen et al. 1987).

The purpose of our study was to isolate and identify flavonoid compounds present in the root exudate of the black locust tree (*Robinia pseudoacacia*) and to characterize their biological activity in the *nod* gene induction assay. *R. pseudoacacia*, first introduced from North America to France and England in 1701, has become increasingly important throughout Europe and parts of Asia (Keresztesi 1988). It is one of the most useful trees for controlling erosion and rebuilding depleted soils. The presence of black locust may favour the development of other vegetation, probably through amelioration of the micro-climate and through nitrogen fixation. Growth of black locust trees on poor, nitrogen deficient soils can be enhanced by establishing the environmental conditions promoting the development and maintenance of symbiosis with rhizobia. In contrast to many other legume macrosymbionts, *R. pseudoacacia* is nodulated by very diverse *Rhizobium* strains (McCray-Batzli et al. 1992; Röhm and Werner 1992; Schäfers and Werner 1993). This raises an interesting question: is this nonspecific interaction also reflected by a rather nonspecific pattern of *nod* gene inducing flavonoid metabolites released by this woody legume? A large number of authentic plant derived phenolic compounds were assayed to determine the characterstics of black locust root exudate compounds capable of inducing *nod* gene transcription of the microsymbionts, and the structural features of the inducing compounds were analyzed.

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Material and methods

Chemicals

4',7-Dihydroxyflavone and chrysoeriol were purchased from Apin (Abingdon, UK). Naringenin and apigenin were purchased from Roth (Karlsruhe, Germany). Isoliquiritigenin was synthesized in our laboratory following the procedure of Kape et al. (1992).

Plant material and growth conditions

Seeds of *R. pseudoacacia* (supplied by B. Keresztesi, Hungary) were washed with 0.1% Tween 20 (polyoxyethene sorbitan monolaurate; Serva, Heidelberg, Germany) for 3 min, rinsed several times with sterile tap water, and surface-sterilized for 10 min in 30% hydrogen peroxide. Both procedures were carried out in an ultrasonic bath (35 kHz, Sonorex RK 510S, Bandelin Electronic, Germany). After sterilization, seeds were washed ten times with sterile tap water and allowed to germinate on NB-agar (nutrient broth 8 g/l, agar 15 g/l). The preparation of the black locust root exudate was performed according to Kape et al. (1992): 2 days after germination 100 seedlings were individually transferred onto a stainless steel mesh, which was placed in a glass petri dish (diameter, 22 cm; height, 7 cm) supplied with a cellulose acetate filter (SM111, Sartorius, Göttingen). The roots of the seedlings grew through the holes of the mesh and then along the surface of the cellulose acetate filter. This filter material most effectively bound the *nod* gene-inducing compounds as compared with several other filters (Recourt et al. 1991). Plants were grown in a controlled environmental chamber under a 16/8 light/dark cycle, 25/20 °C, 70% relative humidity and a photosynthetically active radiation of 124 μE m^{-2} s^{-1} provided by fluorescent tubes (Sylvania, cool white, F195 W/CW/VHO, USA).

Preparation of root exudate

After 7 days the cellulose acetate filters were first rinsed with distilled water to remove all water soluble compounds and the cell debris, and were then rinsed with hexane to remove lipids. The washed filters were extracted three times with methanol. The methanolic extracts were pooled, filtered through a glass fiber filter (Whatman GF/C, Maidstone, England) concentrated under vacuum at 60 °C to a volume of 2 ml and further concentrated to dryness using a Speedvac concentrator (Savant Instruments, Farmingdale, N.Y.). The residues were stored at −20 °C in the dark for later analysis.

Purification and separation of flavonoids was done by *two dimensional high performance thin-layer chromatography* (2D-HPTLC) on silica gel plates (nano-Sil 20 UV254, Machery & Nagel, Germany). The above residues were dissolved in 50 μl methanol, and 3 μl of the solution was spotted on to nano-Sil layers. Separation of the flavonoids was performed with chloroform-methanol-formic acid (93:6:1, v:v:v) in the first dimension and toluol-ethyl acetate-methanol-acetic acid (75:25:4:1, v:v:v:v) in the second dimension. All chromatographic steps were carried out at 28 °C in a saturated chamber. The migration distance chosen was 8 cm. HPTLC plates were evaluated with a Desaga CD60 densitometer (Desaga, Heidelberg, Germany), which allowed us to record UV-visible absorption spectra of single spots without prior elution from the HPTLC plate.

Crude extracts dissolved in 50 μl methanol were purified prior to HPLC by column chromatography (column length, 70 mm; diameter, 5 mm). The column packing was Sephadex LH 20 (Pharmacia, Uppsala, Sweden). Flavonoids were eluted with an increasing methanol-water gradient (20 to 100% methanol). Fractions of 1 ml were collected and their identities as flavonoids confirmed by HPTLC. Flavonoid containing fractions were dried in a Speedvac concentrator and dissolved in 50 μl methanol before injection to HPLC.

For *high performance liquid chromatography* (HPLC) 20 μl aliquots of the extracts were injected into a LKB system equipped with a reversed phase C 18 column (ODS-Hypersil, 250×4 mm, 5 μm, Hewlett Packard, Böblingen, Germany) and separated using an acetontrile-water gradient elution protocol: 18–55% acetonitrile-H_2O, pH 3 in 25 min, flow rate 1 ml/min. Water was acidified with acetic acid. The absorption spectra of the eluting compounds were analyzed with a diode array detector (Spectra Focus, Spectra-Physics, San Jose, Calif., USA).

For *gas chromatography-mass spectrometry* (GC-MS) analysis, dry flavonoid samples were derivatized by incubation with 100 μl bis (trimethylsilyl)trifluoroacetamide (BSTFA) contaning 1% trimethylchlorosilane (TMCS) (Sigma, Dorset, UK) in a sealed glass tube for 15 h at 60 °C to obtain the trimethylsilyl (TMS)- derivatives. GC-MS was performed according to Greenaway et al. (1989) with slight modifications: the derivatized samples were separated and analysed in a Finnigan ITD 800 automated GC-MS system; the GC system (Varian 3400) was fitted with a 30 m×0.25 mm ID J&W Scientific silica column with 0.25 μm DB-1, and a splitless injector with a flush 30 s after sample injection to remove residual gases. The outlet of the column was introduced directly into the mass spectrometer manifold. The system was operated under the following conditions: helium pressure 15 psi; injector temperature 280 °C, GC-temperature 70–300 °C at 5 °C min^{-1}. The mass spectrometer was set to scan 200–650 a.m.u. per nominal second with an ionizing voltage of 7 eV or 70 eV when using a Taylor disk.

Identification of compounds

Peaks were identified by computer search of user-generated reference libraries, either based on GC retention times and mass spectra, or on HPLC retention times and UV-spectra. After tentative identifications, commercial standards were compared by spectroscopic analysis and co-chromatography to confirm R_f-values, retention times and spectra.

Preparation of flavonoid stock solutions and *nod* gene induction assays

Quantification of substances on HPTLC plates was done by comparison of UV-absorption with flavonoid spots of known concentrations. Silica gel corresponding to the spots was scratched from the plates, eluted with methanol and aliquots were transferred into 2-ml glass vials with Teflon-lined screw caps (Renner, Darmstadt, Germany). After vaporizing of the methanol in a Speedvac concentrator, flavonoids were diluted with RMM medium (Broughton et al. 1986) to concentrations from 0.1 to 100 μM.

The *nod* gene induction assay was done according to Miller (1972). The following changes were introduced. *Rhizobium* sp. NGR234 (pA27) containing a *nod* box::*lac* Z construct and resistant to tetracycline was used to monitor *nod* gene induction (Lewin et al. 1990). The bacteria were grown in TY medium (tryptone 5 g/l, yeast extract 3 g/l, $CaCl_2$ 0.4 g/l, agar 15 g/l) containing 10 mg/l tetracycline. Cultures were incubated at 28 °C on a rotary shaker (100 rpm) for 16 h in the presence of varying concentrations of the test compounds as given above.

Nod gene induction was measured as β-galactosidase activity and reported as Miller units, which indicate enzyme activity standardized for bacterial cell number (Miller 1972).

Background activity was determined with extracts of silica gel from portions of the plates that did not contain spots. Constitutive expression of β-galactosidase was tested as a control with *Rhizobium* sp. NGR234 (pMP220) containing a promotorless *lac*Z gene. The orgin of the strains have been published by Lewin et al. (1990).

Results

Detection of flavonoids in root exudate

Cultivation of black locust seedlings in the presence of cellulose acetate filters allowed the rapid preparation of a flavonoid containing root exudate fraction from tree seed-

Table 1 Thin layer chromatography data of compounds from root exudates of *Robinia pseudoacacia*, and of authentic standards. R_f values (R_{f1} = 1st dimension, R_{f2} = 2nd dimension). Spot colours were observed upon irradiation at 366 nm in the dark

Unknown compound standard	R_{f1}	R_{f2}	Spot colour (366 nm)
Compound 1	0.20	0.18	blue
4′,7-dihydroxyflavone	0.22	0.17	blue
Compound 2	0.29	0.18	light blue
Compound 3	0.26	0.29	yellow
Compound 4	0.30	0.30	dark purple
Apigenin	0.28	0.30	dark purple
Compound 5a	0.32	0.41	fluor.extinct
Naringenin	0.31	0.41	fluor.extinct
Compound 5b	0.31	0.43	dark purple
Isoliquiritigenin	0.32	0.42	dark purple
Compound 6	0.33	0.32	dark purple
Chrysoeriol	0.33	0.32	dark purple
Compound 7	0.34	0.28	yellow

lings. Separation of the metabolite fraction by 2D-HPTLC resulted in a total of 34 spots. Ten of these spots were selected for further characterisation. Spot no. 5 contained two compounds named compounds 5a and 5b.

Identification of compounds

The R_f values and spot colours on HPTLC-plates of the 8 spots with flavonoid specific UV-spectra are given in Table 1. The overlay graphs of the UV spectra revealed good correspondence (Fig. 1) between putative flavonoids and the authentic standards.

HPLC analysis confirmed these results. Comparable retention times were obtained for 4',7-dihydroxyflavone and compound 1 (15:70 min vs 15:82 min), naringenin and compound 5a (19:43 min vs 19:65 min), isoliquiritigenin and compound 6 (21:75 min vs 21:84 min), and the identity of the paired compounds was supported by on-line diode array spectroscopy.

The flavonoid metabolites of the root exudate were further identified as their TMS-derivatives by comparison of their GC and MS characteristics with those of known reference standards (Table 2). The identification of 4',7-dihydroxyflavone, naringenin and the chalcone isoliquirtigenin was confirmed, while compounds 4 and 6 were additionally identified as apigenin and chrysoeriol, respectively. Three samples were analysed and in each sample these five flavonoids could be identified.

Nod gene-inducing activities of compounds

Eleven distinctive spots, separated on HPTLC-plates, were analysed for their *nod* gene inducing activity. Eight root exudate compounds, namely 1, 2, 3, 4, 5a, 5b, 6, and 7,

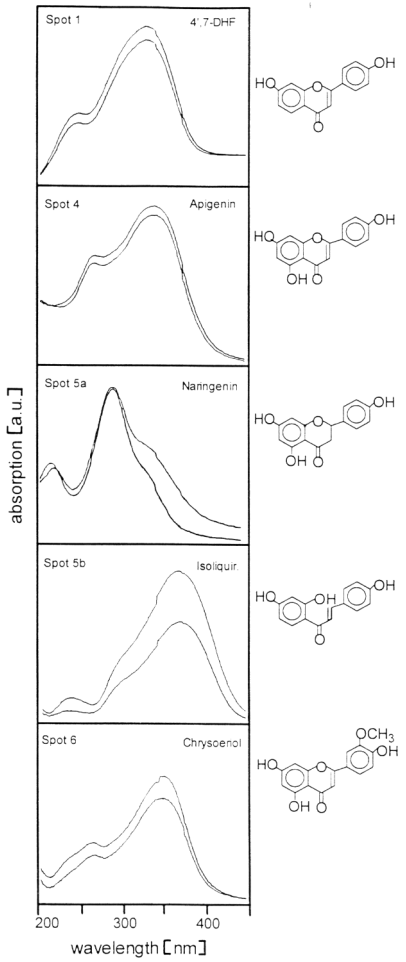

Fig. 1 UV spectra of putative flavonoids and authentic standards obtained by direct densitometry of spots HPTLC plates. Upper spectra represent the natural metabolite except for spot 5b. The chemical structures are also given

Table 2 GC/MS data of compounds in root exudates of *Robinia pseudoacacia*, and of authentic standards. Retention time, calculated molecular masses of the trimethylsilylderivatives and the m/z values observed are given. The reverse fit (*Rfit*) value quantifies the degree to which the unknown spectrum is included in the library spectrum. A Rfit of more than 700 implies a close resemblance between the components

Unknown compound standard	Retention time (min : sec)	Mol mass calculated (Da)	Mol mass observed (m/z)	Rfit
Compound 1	20:12	399	384	728
4′7-dihydroxyflavone	20:06	399	384	
Compound 4	21:10	487	472	741
Apigenin	21:06	487	472	
Compound 5a	18:52	489	474	712
Naringenin	18:54	489	474	
Compound 5b	19:06	473	458	786
Isoliquiritigenin	19:02	473	458	
Compound 6	22:00	516	502	729
Chrysoeriol	22:00	516	502	

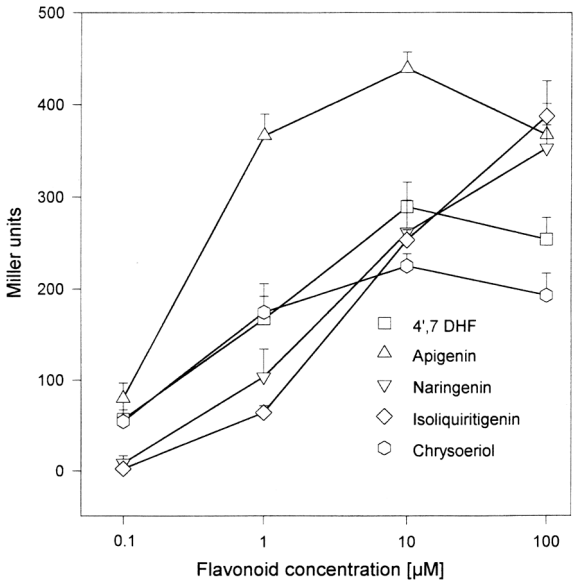

Fig. 2 *Rhizobium nod* gene induction by 4', 7-dihydroxyflavone (4', 7-DHF), apigenin, naringenin, isoliquiritigenin and chrysoeriol. *Rhizobium* sp. NGR234 (pA27) containing a *nod* Box:lacZ fusion was used as a test organism, and induced β-galactosidase was measured. The background level of β-galactosidase was subtracted. *Errors bars* show standard deviations

induced β-galactosidase activity in *Rhizobium* sp. NGR234 (pA27) at least two times higher than the background activity (data not shown).

After successful identification of spots 1, 4, 5a, 5b and 6, quantitative tests were performed with the respective commercial standards. Results are shown in Fig. 2 (mean values of three independent experiments). Apigenin was the substance with the highest I_{max} and the lowest I_{50} value (0.3 µM). It can therefore be regarded as the most active *nod* gene inducer of *Rhizobium* sp. NGR234 (pA27) in this test system. At a concentration of 100 µM, apigenin showed a significant inhibition of β-galactosidase activity, as did the same concentrations of 4′,7 dihydroxyflavone and chrysoeriol. The background activity determined was 102 Miller units. The control strain *Rhizobium* sp. NGR234 (pMP220) showed the same level of β-galactosidase activity (data not shown).

Comparison of the biological activity of commercial standards with isolated compounds (10 µM) from the root exudate revealed corresponding *nod* gene inducing activity within a variation coefficent of less than 10% (data not shown).

Unfractionated root exudate, reflecting the original flavonoid concentration in the cultivation system, resulted in a *nod* gene inducing activity of 452 Miller units (mean value of two independent experiments).

Discussion

R. pseudoacica has been found to be primarily associated with fast-growing *Rhizobium* strains, but it may also form nodules with slow-growing *Bradyrhizobium* strains (McCray-Batzli et al. 1992). Rhizobial diversity may be favoured by the fact that black locust nodules are perennial (Allen and Allen 1981), maintaining distinct rhizobia in nodules from year to year without repeated competition for reinfection sites with other strains in the soil. It is also possible for more than one strain of *Rhizobium* to occupy one and the same black locust nodule (McCray-Batzli et al. 1992). Differences in strain preferences may be influenced by the fact that different soil microsite conditions, such as aeration, nutrient availability, moisture content, temperature, and competition, may favour different serotypes (Postgate 1982). In root systems of woody and perennial plants the complexity and spatial variability of microsites may be more pronounced than in annual herbs. In *R. pseudoacacia* this variability is possibly reflected by a very complex pattern of exuded flavonoids.

Recent analysis of extracts of black locust organs identified robinetin, dihydrorobinetin, dihydrofisetin, fisetin, robtin, butin, robtein and robinin in the heartwood, and acacetin, quercetin and apigenin in the leaves of the tree (Smith et al. 1989a, b), but there was so far no information on flavonoids in the root exudate of *R. pseudoacacia*. Since it is impossible to collect exudate from an adult tree, our special cultivation system was designed for *R. pseudoacacia* seedlings. The cultivation period of 7 days allowed the preparation of metabolites at amounts sufficient for structural elucidation in a short time.

Table 3 Collected data on flavonoids present in root exudates of various legumes, and presence or absence of *nod* gene inducing activity in the appropriate microsymbionts (+ = flavonoid produced/*nod* gene induction; – = no flavonoid produced/no *nod* gene induction; ? = no data available)

Flavonoid	Exuded by macrosymbiont/effective in microsymbiont					
	T. repens/ R. leg. trifolii	*V. sativa supsp.nigra/ R. leg. viciae*	*P. vulgaris/ R. leg. phaseoli*	*M. sativa/ R. meliloti*	*G. max/ B. japonicum*	*R. pseudoacacia/ R. NGR 234*
4′,7-DHF	+/+[b]	?/?	?/?	+/+[c, d]	?/?	+/+[a]
Apigenin	+/+[d, e]	?/+[d, e]	?/+[d, e]	?/+[d, e]	?/?	+/+[a]
Naringenin	?/+[e]	+/+[e–g]	+/+[e–g]	?/+[e]	?/?	+/+[a]
Isoliquiritigenin	?/+[g]	+/+[g]	?/?	?/?	+/+[h]	+/+[a]
Chrysoeriol	?/?	?/?	?/?	+/+[i]	?/?	+/+[a]

[a] this work; [b] Djordjevic et al. 1987; [c] Redmond et al. 1986; [d] Sadowsky et al. 1988; [e] Rolfe 1988; [f] Hungria et al. 1991; [g] Recourt et al. 1991; [h] Kape et al. 1992; [i] Hartwig et al. 1991

We used three different methods to analyse the UV-active part of the root exudate to identify the flavonoids 4',7-dihydroxyflavone, naringenin, chrysoeriol, apigenin and the chalcone isoliquiritigenin. High similarity in UV-spectra comparing the putative flavonoids with authentic standards, and the mass-spectrometric analysis verified the identifications. Differences of 15 m/z in calculated molecular weights of flavonoid-trimethylsilylderivates and detected base peaks are known (Kape et al. 1992) and are probably due to the loss of one CH3-group during ionization.

The induction studies with all isolated compounds clearly reflect the broad host range of *Rhizobium* sp. NGR234. Interestingly, all identified flavonoids are prominent *nod* gene inducers of diverse fast growing *Rhizobium* strains (Djordjevic et al. 1987; Recourt et al. 1991; Sadowsky et al. 1988; Hartwig et al. 1990) and are released by a wide variety of legumes, e.g. alfalfa, white clover, vetch, and common bean (Table 3). Isoliquiritigenin, on the other hand, has been identified as a strong *nod* gene inducer of a *Bradyrhizobium* strain (Kape et al. 1992) that nodulates soybean, and has so far only been isolated from *Glycine max* and *Vicia sativa* ssp. *nigra* root exudate (Recourt et al. 1991).

All identified compounds, in addition to sharing somewhat similar flavonoid ring structures, have free hydroxyl groups at the C-4' and C-7 positions. The hydroxylation of C-7 is necessary for the *nod* gene induction of NGR234 (Rolfe 1988). The C-4'-hydroxylation of chalcones is structurally analogous to the C-7-hydroxylation of flavonoids. The presence of so many structurally different *nod* gene inducing flavonoids in the root exudate differs markedly from exudates of other legumes. With common bean as the only exception (Hungria et al. 1991), all other legumes roots investigated exuded not more than four compounds with *nod* gene inducing activity.

Nod gene induction is dependent on flavonoid concentration (Fig. 2). Unfractionated root exudate reflecting the original flavonoid concentration in the cultivation system seems to contain flavonoids in optimal concentrations for *nod* gene induction. The activity of 452 Miller units corresponds to the most active *nod* gene inducer apigenin in our test system. The quantity of exuded flavonoids is approximatly 950 pmol per plant of 7-day-old seedlings in the test system, but can be highly variable possibly due to changing light intensities. Preliminary tests in our laboratory have shown that the quantity of exuded flavonoids was considerably lower at lower light intensities (data not shown). Although direct extrapolation from our petri dish experiments to the soil environment is not possible, results reported here suggest that legume trees have developed their own strategy to ensure efficient nodulation in their extensive root system by exudation of a great variety of *nod* gene inducing flavonoids.

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